Q5A(R2) Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin

Guidance for Industry

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologic Evaluation and Research (CBER)

January 2024
ICH - Quality
Revision 2

Q5A(R2) Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin

Guidance for Industry

Additional copies are available from:

Office of Communications, Division of Drug Information
Center for Drug Evaluation and Research
Food and Drug Administration
10001 New Hampshire Ave., Hillandale Bldg., 4th Floor
Silver Spring, MD 20993-0002
Phone: 855-543-3784 or 301-796-3400; Fax: 301-431-6353; Email: druginfo@fda.hhs.gov
https://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances-drugs

and/or

Office of Communication, Outreach, and Development
Center for Biologics Evaluation and Research
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 71, Room 3128
Silver Spring, MD 20993-0002
Phone: 800-835-4709 or 240-402-8010; Email: ocod@fda.hhs.gov
https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics/biologics-guidances

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologic Evaluation and Research (CBER)

January 2024
ICH - Quality
Revision 2

FOREWORD

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has the mission of achieving greater regulatory harmonization worldwide to ensure that safe, effective, and high-quality medicines are developed, registered, and maintained in the most resource-efficient manner. By harmonizing the regulatory expectations in regions around the world, ICH guidelines have substantially reduced duplicative clinical studies, prevented unnecessary animal studies, standardized safety reporting and marketing application submissions, and contributed to many other improvements in the quality of global drug development and manufacturing and the products available to patients.

ICH is a consensus-driven process that involves technical experts from regulatory authorities and industry parties in detailed technical and science-based harmonization work that results in the development of ICH guidelines. The commitment to consistent adoption of these consensus-based guidelines by regulators around the globe is critical to realizing the benefits of safe, effective, and high-quality medicines for patients as well as for industry. As a Founding Regulatory Member of ICH, the Food and Drug Administration (FDA) plays a major role in the development of each of the ICH guidelines, which FDA then adopts and issues as guidance to industry.

TABLE OF CONTENTS

Q5A(R2) Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin Guidance for Industry[1]

I. INTRODUCTION (1)[2]

This guidance describes the evaluation of the viral safety of biotechnology products including viral clearance and testing, and it outlines what data should be submitted in marketing applications for those products. Biotechnology products include biotherapeutics and certain biological products derived from cell lines of human or animal origin (e.g., mammalian, avian, insect). In this guidance, the term *virus* excludes nonconventional transmissible agents like those associated with mammalian prions (e.g., bovine spongiform encephalopathy, scrapie). Applicants are encouraged to discuss transmissible spongiform encephalopathy associated issues with the appropriate regulatory authorities because they are not in scope of this guidance.

This guidance includes products such as cytokines, monoclonal antibodies (mAbs), and subunit vaccines produced from in vitro cell culture using recombinant DNA technologies. This guidance also includes certain genetically engineered viral vectors and viral vector-derived products (e.g., viral vaccines, gene therapy products), provided they are amenable to viral clearance, without a negative effect on the product. These products can include viral vectors, for example, adeno-associated virus (AAV), produced using transient or stable transfected cell lines, or by infection using a recombinant virus. It also includes viral vector-derived products, for example, baculovirus-expressed virus-like particles (VLPs), protein subunits, and nanoparticle-based protein vaccines and therapeutics. AAV gene therapy vectors include those that depend on helper viruses such as herpes simplex virus or adenovirus for their production. Specific guidance on genetically engineered viral vectors and viral vector-derived products is provided in Appendix F (Annex 6).

[1] This guidance was developed within the Expert Working Group (Quality) of the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Assembly at *Step 4* of the ICH process, November 2023. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the ICH regions.

[2] The numbers in parentheses reflect the organizational breakdown of the document endorsed by the ICH Assembly at Step 4 of the ICH process, November 2023.

Inactivated viral vaccines and live attenuated viral vaccines containing self-replicating agents are excluded from the scope of this guidance. Cell therapies are out of the scope of this guidance; however, the principles can be used as applicable (e.g., for biological starting or raw materials).

It is no longer encouraged that materials be manufactured from hybridoma cells grown in vivo as ascites due to the contamination risk as well as to the ongoing global initiative to replace, reduce, and refine the use of animals. Where this situation exists, the principles of this guidance should be followed, including replacement of the in vivo assay by next generation sequencing (NGS).

The risk of viral contamination is a concern for all biotechnology products derived from cell lines and needs to be reduced because such contamination could have serious clinical consequences. This risk can arise from the contamination of the source cell lines themselves (cell substrates) or from exogenous introduction of adventitious virus during production. However, biotechnology products derived from cell lines have not been historically implicated in the transmission of viruses. The viral safety of these products has been reasonably assured by applying a comprehensive virus testing program and assessing virus removal and inactivation achieved by the manufacturing process, as outlined below. Three principal, complementary approaches are applied to control the potential viral contamination of biotechnology products:

- Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable infectious viruses

- Assessing the capacity of the production processes to clear adventitious and endogenous viruses

- Testing the product at appropriate steps of production for demonstrating the absence of contaminating infectious viruses

Some viral clearance steps used during production of genetically engineered viral vectors and viral vector-derived products may not be as effective as those used for recombinant proteins. Therefore, considerations for further risk reduction (e.g., treatment of raw materials, extensive testing for broad virus detection) should be applied (see Appendix F (Annex 6)).

Viral tests have inherent limitations. For example, the ability to detect low virus concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the viral safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification process is capable of removing and/or inactivating (clearing) viruses.

The type and extent of viral tests and viral clearance studies performed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be considered include the origin of the cell line, the extent of cell bank characterization and testing; the nature of any viruses detected, culture medium constituents, culture methods; facility and equipment design; the results of viral tests after cell culture; the ability of the process to clear viruses; and the type of product and its intended clinical use.

The recommendations presented here should be adjusted to the specific product and its production process. Moreover, the approach used to assure viral safety should be explained and justified. In addition to the detailed data, an overall summary of the viral safety assessment should be provided. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination.

In general, FDA's guidance documents do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. POTENTIAL SOURCES OF VIRUS CONTAMINATION (2)

Virus contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during generation of a production cell line and/or cell banking and during the production process. Use of well-characterized cell banks and virus seeds can reduce the risk of virus contamination. Introduction and control of potential adventitious viruses from a master virus seed or working virus seed (WVS) are discussed in Appendix F (Annex 6). Viruses used for production of the products (production viruses) described in Appendix F (Annex 6) are considered to be process-related impurities.

A. Viruses That Could Occur in the Master Cell Bank (2.1)

Adventitious virus contaminants can be introduced in the master cell bank (MCB) by several routes such as (1) derivation of the cell line from an infected animal; (2) use of a virus to establish the cell line; (3) use of contaminated biological reagents (e.g., antibodies for selection) or raw materials for cell culturing (e.g., animal or human serum and porcine trypsin); or (4) contamination during cell handling and cell banking processes.

Cells have endogenous retroviruses that are maintained from one cell generation to the next. The endogenous viral sequences may be constitutively expressed or may become activated to produce infectious or defective viral particles. Cells may also contain latent or persistent viruses (e.g., herpesvirus).

B. Adventitious Viruses That Could Be Introduced During Production (2.2)

Adventitious viruses may contaminate the production process by several routes including, but not limited to: (1) the use of contaminated biological raw materials or reagents such as animal serum components during cell culture; (2) the use of a contaminated virus seed for production (see Appendix F (Annex 6)); (3) the use of a contaminated raw material or reagent used during downstream purification, such as a mammalian derived antibody-coupled affinity resin for product selection or purification; (4) the use of a contaminated inactive ingredient during formulation; and (5) contamination from the environment, including storage of nonbiological raw materials or during cell culture and medium handling by personnel.

Monitoring cell culture parameters (e.g., cell growth and viability) may also be helpful in the early detection of potential adventitious viral contamination. Manufacturers should avoid

using human- and animal-derived raw materials (e.g., human serum, bovine serum, porcine trypsin) in their manufacturing processes when possible. When this is not possible, the use of human- and animal-derived raw materials should be supported by the relevant documentation or qualification of the material, commensurate with risk. Information such as the country of origin, tissue of origin, virus inactivation, or removal steps applied during the manufacturing process of the material, and the types of virus testing that have been performed on the raw material should be provided. This testing, if needed according to risk assessment, should be performed before inactivation, when possible.

When possible, biological materials with a high risk of introducing adventitious viruses such as animal or human serum or porcine trypsin should be subjected to viral inactivation such as ionizing irradiation. Additional virus risk-mitigation strategies can include, for example, cell culture media or media supplement treatments, for example, virus filtration, high temperature short-time processing, or ultraviolet C irradiation, where appropriate.

III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES (3)

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of viruses.

A. Virus Tests for Master Cell Bank, Working Cell Bank, and Cells at the Limit of In Vitro Cell Age Used for Production (3.1)

Table 1 shows the virus tests recommended to be performed once on the MCB, working cell bank (WCB) and cells at the limit of in vitro cell age (LIVCA) that are used for production.

1. MCB (3.1.1)

Extensive screening for both endogenous viruses and adventitious viral contamination should be performed on the MCB. For hetero-hybrid cell lines, for example, in which one or more partners are human or nonhuman primate in origin, tests should be performed to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular risk. Testing for adventitious viruses should include both broad and specific virus detection assays as described in Table 1. Introduction of new methodologies such as NGS for detecting a broad range of adventitious viruses is encouraged. To ensure detection of contaminating viruses, the testing approach should be based on a risk assessment, considering the origin and history of the parental cell line and the potential exposure to materials of human or animal origin during the generation of production cells and banking of the MCB. An alternative approach in which complete testing is carried out on the WCB rather than on the MCB could also be acceptable.

2. WCB (3.1.2)

Each WCB should be tested for adventitious viruses as described in Table 1. When appropriate, if adventitious virus tests have been performed on the MCB, and cells cultured up to or beyond the LIVCA have been derived from the WCB and tested for the presence of adventitious viruses, then similar tests can be omitted from this WCB.

3. Cells at the LIVCA Used for Production (3.1.3)

The established LIVCA for production should be based on data derived from production cells expanded at small scale using a representative scale-down model and/or under pilot plant-scale or commercial-scale conditions to the proposed in vitro cell age or beyond. LIVCA cells are obtained by expansion of the WCB or the MCB. Cells at the LIVCA should be evaluated once for viruses that may have been undetected in the MCB and/or WCB. The performance of suitable tests (as outlined in Table 1) once on cells at the LIVCA used for production will provide further assurance that the production process does not lead to induction of endogenous viruses, reactivation of latent viruses, or amplification of low-level or slow-growing viruses. If any adventitious viruses are detected at this stage, the process, including the MCB and WCB should be checked carefully to determine the source of the contamination. Cells at the LIVCA can also be referred to as end of production cells.

B. Recommended Virus Detection and Identification Assays (3.2)

A number of assays are used to detect endogenous and adventitious viruses. Table 2 lists examples of such assays. These assays are recommended, but the list is not all-inclusive or definitive. The most appropriate techniques may change with scientific progress; proposals for alternative techniques should be accompanied by adequate supporting data. A comprehensive testing strategy should be developed following a thorough viral risk assessment that includes consideration of the cell line origin; the passage history; and the raw materials and reagents used for cell line generation, cell bank preparation, and production (e.g., steps that can inactivate or remove viruses). The strategy should include additional assays as appropriate based on risk assessment. For example, if there is a relatively high possibility of the presence of a particular virus, specific tests or other approaches for detection of that virus should be included unless otherwise justified. Appropriate controls should be included to demonstrate adequate assay sensitivity and specificity. Furthermore, potential matrix interference, where applicable, should be considered.

The following is a brief description of a general framework that the manufacturer should use to develop a comprehensive virus testing scheme that is specific (or appropriate) to the product and manufacturing process. The testing strategy should be accompanied with appropriate justification for the approach.

1. Tests for Retroviruses (3.2.1)

Cell lines should be characterized for the presence of retroviruses. Tests for retroviruses should be performed on the MCB and cells cultured up to or beyond the LIVCA used for production. These tests can include infectivity assays by direct inoculation of the cell supernatant or cocultivation of the cells, assays for reverse transcriptase (RT) activity using cell-free supernatant, and evaluation of the cells for particles by transmission electron microscopy (TEM). TEM is also able to detect other agents and, therefore, is generally recommended for characterization of cell banks.

If a cell line is producing retroviral particles (as occurs in some cell lines derived from rodent, insect, and avian species), RT activity is expected and therefore a polymerase chain reaction (PCR)-based RT assay (e.g., product-enhanced reverse transcriptase (PERT) assay) may not be needed. TEM should be performed to examine the type of retroviral particles (e.g., type-A, type-C) present. To determine whether the endogenous retroviral particles are

infectious, infectivity assays should be performed using relevant permissive cells (e.g., Mus dunni for general murine retrovirus detection, SC-1 cells for ecotropic murine retrovirus detection) with sensitive readout assays for retrovirus detection (e.g., PERT assay, a sarcoma-positive, leukemia-negative (S+L-) assay, or an XC plaque assay).

If it is not known whether the cell line produces retroviral particles, TEM should be performed on cells, and an RT assay (e.g., PERT assay) should be carried out on clarified supernatant. The PCR-based RT assay is particularly useful because it can detect retrovirus RT activity with high sensitivity; however, the RT activity can be associated with an infectious or noninfectious retrovirus. Because some cellular DNA polymerases can lead to a positive RT result, confirmation of the RT activity (because of a retrovirus contamination) or a positive TEM result should be followed by testing to detect infectious retroviruses in permissible cells, including human cell lines and a sensitive readout assay for retrovirus detection.

Retroviral testing results should be interpreted considering all available data. Cell lines expressing endogenous retrovirus particles are not precluded from use in manufacturing based on risk evaluation as discussed in sections III.C (3.3) and V (5).

Chemical induction studies need not be performed for cell lines that have been well characterized for endogenous retroviruses (e.g., Chinese hamster ovary (CHO), NS0, Sp2/0, Vero, other cell lines based on prior knowledge). However, such studies may help to evaluate a new cell substrate for the presence of an unknown inducible endogenous retrovirus. Furthermore, induction studies for latent DNA viruses (e.g., herpesvirus in human cells) and latent RNA viruses (e.g., nodavirus in insect cells) may also be appropriate based on risk assessment. These studies may help inform the virus testing and clearance strategy for products derived from a new cell substrate.

2. In Vitro Cell Culture Infectivity Assays (3.2.2)

In vitro assays are carried out by inoculating a test article (see Table 2) on to various indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The panel of indicator cell lines should include a cell line of the species of origin, human diploid cells (e.g., MRC-5), and a monkey kidney cell line (e.g., Vero). Additional cell lines can be included. The choice of cells used in the test should be based on a risk assessment considering the species of origin of the cell substrate to be tested.

The nature of the infectivity assay and the sample to be tested are governed by the type of virus that may be present based on the origin or handling of the cells. The indicator cell cultures should be monitored for cytopathic viruses, hemadsorbing and hemagglutinating viruses, consistent with existing regional regulations and guidance. For qualification of the MCB, WCB, and LIVCA, 28-day testing on permissive cells should be performed with at least one subpassage at 2 weeks.

NGS or other molecular methods can be used to supplement or replace the assay. This could address general limitations of the in vitro cell culture infectivity assay (e.g., susceptibility of cell lines to infection) and specific limitations of the production system (e.g., test article-mediated interference or toxicity).

3. In Vivo Assays (3.2.3)

In vivo testing can be performed based on risk assessment considering the history and manufacturing of the cell bank and the testing strategy. However, in vivo testing is not necessary for extensively used, well-characterized cell lines such as CHO, NS0, and SP2/0, based on prior knowledge. Risk-based considerations include prior in vivo virus testing or NGS testing of the parental untransfected cell line and control of the derivation of the MCB from the parental cell bank. Prior knowledge of viral safety testing of other MCB derived from the same parental cell bank including the method used to establish the MCB also should be considered. The test is generally not necessary for the WCB if it is prepared under approved controlled conditions. For cells at the LIVCA, the test may not be necessary based on prior knowledge and other risk-based considerations.

If residual risk remains, retention of the test or replacement with nontargeted NGS can be considered to detect viruses that may have been introduced during establishment of the MCB or during culture of the cells to the LIVCA stage.

The use of the in vivo assay can include inoculation of the test article (see Table 2) into suckling mice, adult mice, and embryonated hens' eggs. The health of the animals should be monitored, and any abnormality should be investigated to establish the cause.

Nontargeted NGS is encouraged as a replacement for in vivo assays due to its breadth and sensitivity of virus detection and the limitations of the in vivo assays. Furthermore, this promotes the global initiative to replace, reduce, and refine the use of animal testing.

4. Tests for Specific Viruses (3.2.4)

The list of specific viruses to be tested for is defined based on a viral contamination risk assessment and considers (but is not limited to) the origin of the cells and the potential sources of viral contamination (e.g., biological raw materials, especially those of human or animal origin). For cell lines with exposure to human- or animal-derived raw materials, (e.g., bovine serum, porcine trypsin), tests for human, bovine, and porcine viruses should be performed. For cell lines of rodent origin or exposure to rodent materials, nucleic acid amplification techniques (NATs) or antibody production tests in mice, rats, or hamsters are used to detect species-specific viruses. These tests should be performed when the potential exists for exposure to viruses of a specific animal species. For example, the presence of rodent viruses in cell lines of rodent origin, or cell lines generated by passage through rodents and the use of reagents that may have been derived from rodent materials, can be detected by inoculation of the test article (see Table 2) into specific pathogen-free animals, such as mice, rats, and hamsters, that are subsequently tested for antibodies to specific agents.

Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in Table 3.

NATs such as PCR assays or targeted or nontargeted NGS or other molecular methods can be used for replacing the animal assays described in Table 3, without head-to-head comparison.

5. Molecular Methods (3.2.5)

Molecular methods such as NAT and NGS are used for virus detection. NGS may be appropriate for broad (nontargeted) detection of known and novel viruses. NGS can also be used for targeted detection of viruses by the sequencing strategy or by bioinformatic analysis. Nontargeted NGS can be used to replace the in vivo assays and supplement or replace the in vitro cell culture assays, without a head-to-head comparison as long as the method is demonstrated to be suitable for its intended purpose. A head-to-head comparison is not recommended due to the different endpoints of the assay systems and limitations of the breadth of virus detection by the in vitro and in vivo methods compared to the enhanced capability of NGS for broad virus detection of known and unknown viruses. The results of the in vitro and in vivo assays rely on virus replication and biological properties for detection in the specific target system, which limits the breadth of detection. Replacement of in vivo assays by NGS also meets the intent of the global objective to replace, reduce, and refine the use of animals for testing.

NATs such as PCR-based methods can be used for virus-specific detection. Targeted or nontargeted NGS can be used to replace the large number of PCR assays for virus-specific detection without a head-to-head comparison. This can also help overcome the limitation of detection of virus variants. Positive results should be investigated to determine whether detected nucleic acids are associated with an infectious virus.

a. NATs (3.2.5.1)

NATs such as PCR-based methods are typically used singly or in a multiplex format to detect viral sequences from known viruses or known closely related virus families. These molecular methods can be used to supplement cell culture assays when there are limitations because of assay interference, and they are effective tools for specific virus detection when such viruses cannot be readily grown in cell culture for detection by infectivity assays. NATs also have the capacity to be adapted for broader range virus detection (e.g., degenerate PCR), but specificity may be reduced. Because of the assay specificity, multiple virus-specific PCR assays can be used to detect a broader range of viruses that would be detected by a single more general biological assay. NATs should be appropriately validated for their intended uses. This includes the method validation and matrix-specific verification, as applicable.

b. NGS (3.2.5.2)

NGS (also known as high-throughput sequencing) is available with demonstrated capabilities for broad virus detection. NGS can provide defined sensitivity and breadth of virus detection and can reduce animal use and testing time. Nontargeted NGS can replace the in vivo tests with broad virus detection for unknown or unexpected virus species without a head-to-head comparison.

Nontargeted NGS can also be used without a head-to-head comparison to supplement or replace the in vitro cell culture assays for detection of known and unknown or unexpected viruses. This could address general limitations of the in vitro cell culture infectivity assay (e.g., susceptibility of cell lines to infection) and specific limitations of the production system (e.g., test article-mediated interference or toxicity).

NGS can be used for targeted detection of known viruses based upon availability of their sequence information. Alternatively, the NGS bioinformatic analysis can be targeted for detection of specific viruses.

NGS (targeted or nontargeted) can replace virus-specific PCR assays and rodent antibody production tests (see section III.B.4 (3.2.4)) without a head-to-head comparison. Replacement of in vivo assays by NGS also meets the intent of the global objective to replace, reduce, and refine the use of animals for testing.

Use of NGS can be considered for characterization of the cell line or testing of the cell bank, virus seed, or unprocessed bulk harvest. This can be particularly useful in case of assay interference as a result of lack of effective neutralization of the viral vector (see Appendix F (Annex 6)) or toxicity caused by the product or media components. In such applications, NGS can be applied to analyze all genomic viral nucleic acids (genomics), viral mRNAs (transcriptomics), or encapsidated viral genomes (viromics). When analyzing cell culture-derived materials, nucleic acids prepared from cells are used for genomics and transcriptomics, and cell culture supernatants or cell-free virus preparations are used for the viromics. The rationale for selecting these different strategies should be provided.

When applying NGS for sensitive and broad detection of known and novel viruses, there are several critical steps in the NGS workflow for consideration: (1) sample pretreatment (when performed) and virus enrichment steps that may be needed to maximize virus detection based on the type of sample material; (2) efficiency of viral nucleic acid extraction (from enveloped and nonenveloped particles) and library preparation (DNA and RNA viruses); (3) selection of a suitable sequencing platform for sensitive virus detection; and (4) bioinformatics analysis against a database with diverse representation of viral sequences of different viral families using strategies for broad virus detection. A follow-up strategy may be needed to confirm the detection of a virus-specific signal to distinguish from nonviral sequences that could be present in the database.

Suitable reference materials should be used for method qualification and validation to evaluate performance of the different steps involved in the workflow and to demonstrate sensitivity, specificity, and breadth of virus detection. This can include using currently available reference virus reagents/panels, which contain viruses of distinct physical (size, enveloped and nonenveloped), chemical (low, medium, and high resistance), and genomic (DNA, RNA, double- and single-stranded, linear, circular) characteristics to evaluate the performance of the entire NGS workflow or specific steps. A comprehensive viral database should be used with diverse viral sequences for broad virus detection. Other types of reference materials can be used to evaluate the specific technical and bioinformatic steps.

For any NGS method used, validation/qualification should be provided to support its intended use for the application. This includes method validation and matrix-specific verification when used as a replacement method. When used as a supplementary method, this includes method qualification and matrix-specific verification. Method validation requires predefined performance criteria while method qualification only evaluates the performance characteristics of the method. NGS is used as a limit test and, as such, the performance characteristics (specificity/breadth of detection and sensitivity) for method

validation/qualification should consider the principles of the ICH draft guidance for industry *Q2(R2) Validation of Analytical Procedures* (August 2022).[3]

C. Acceptability of Cell Lines (3.3)

Some cell lines used to manufacture a product will contain endogenous retroviruses, other viruses, or viral sequences that may become reactivated as infectious viruses. In such circumstances, the action plan recommended for manufacture is described in section V (5). The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the appropriate regulatory authorities, considering a benefit-risk analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

IV. TESTING FOR VIRUSES IN UNPROCESSED BULK (4)

It is recommended that manufacturers develop programs to routinely test for adventitious viruses in production batches. The extent of virus testing on the unprocessed bulk should consider several points including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, the raw material and reagent sources, and the results of viral clearance studies. See Appendix F (Annex 6) for testing of unprocessed bulk for genetically engineered viral vectors and viral vector-derived products.

For batch processing, the unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. For processes that involve a continuous product harvest from cell culture, there may be pooled intermediate or unprocessed bulk sample(s) collected from a flow stream. A representative sample of the unprocessed bulk, removed from the production reactor before further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Therefore, appropriate testing for viruses should be performed on the unprocessed bulk. The potential impact of cell separation technology on detecting cell-associated virus in the bioreactor should be considered. If unprocessed bulk is toxic or causes interference with virus detection in cell culture assays, minimal sample dilution or alternative testing (e.g., NGS) can be considered (see section III.B (3.2)). To detect cell-associated virus, a mixture of both intact cells and cell lysates (disrupted cells and cell culture supernatants) that are removed from the production reactor should be tested. For perfusion or continuous manufacturing (CM) processes, cells may not be readily accessible (e.g., because of the use of hollow fiber or similar microfiltration systems). In such cases, cell-free product harvest from cell culture can be used. For processes that involve continuous product harvest from cell culture, the sampling strategy (including periodicity and composition of the samples) should be justified because the level of adventitious viruses and endogenous virus particles may vary along the cell culture duration (see section VII (7)).

[3] When final, this guidance will represent the FDA's current thinking on this topic. For the most recent version of a guidance, check the FDA guidance web page at https://www.fda.gov/regulatory-information/search-fda-guidance-documents.

Adventitious virus testing should be routinely applied to each unprocessed bulk. This testing can include in vitro assays using several indicator cell lines or nontargeted NGS (see section III.B (3.2)). The indicator cell cultures should be observed for 28 days including at least one subpassage at 2 weeks. Assay duration can be reduced with justification to 14 days for cell lines based on a risk assessment (considering the cell substrate, cultivation period for production, use of animal-derived raw materials or reagents, and level of viral clearance of the process). Additionally, methods, such as NAT or targeted NGS, may also be appropriate for detection of specific viruses (e.g., minute virus of mice) or virus families, based on risk assessment for potential introduction of contaminants. Such rapid test methods can facilitate real-time decision-making.

If any adventitious viruses are detected at the unprocessed bulk stage, the product harvest from cell culture should not be used for product manufacture unless justified. (See section V (5) for guidance on the use of material in which an adventitious virus has been detected in the harvest material.) The process should be carefully checked to determine the root cause and extent of the contamination, and appropriate actions should be taken. For CM processes, final release of a batch should include documented absence of viral contamination for the period during which cultivation fluids were harvested for manufacture of that batch. If an adventitious virus is detected, a procedure to segregate potentially contaminated material should be established to mitigate a wider production effect.

V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK (5)

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, and to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance play a critical role in this scheme. The goal should be to obtain assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between evaluating processes for their ability to clear viruses that are known to be present and estimating the robustness of the process by characterizing the clearance of nonspecific *model* viruses (described later). Definitions of *relevant*, specific, and nonspecific model viruses are given in the glossary. Viral risk evaluation should include knowledge of how much virus may be present in the process, such as in the unprocessed bulk, and how much can be cleared, to assess product safety. Knowledge of the time dependence for inactivation procedures is necessary in ensuring the effectiveness of the inactivation process. When evaluating viral clearance, in-depth time-dependent inactivation studies, demonstration of reproducibility of inactivation or removal, and evaluation of process parameters should be performed as appropriate. When a manufacturing process is characterized for robustness of clearance using nonspecific model viruses, particular attention should be paid to nonenveloped viruses in the study design (see section VI.A.1 (6.1.1) for explanation of model viruses). The extent of viral clearance studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described below (see section VI (6)).

Table 4 presents an example of an action plan used in response to the results of virus tests on cells or unprocessed bulk. The plan includes the process evaluation and the characterization of viral clearance and virus tests on purified bulk. Various cases are presented in the table and

are described below. In all cases, characterization of clearance using nonspecific model viruses should be performed. The most common situations are Cases A, B, and F. Production systems contaminated with a virus other than a rodent retrovirus normally are not used. When there are well-justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the appropriate regulatory authorities. In Cases C, D, and E, it is important to have validated and effective steps to inactivate and/or remove the virus in question from the manufacturing process.

Case A: When no virus, no VLP, or no retrovirus-like particle (RVLP) other than the drug substance (e.g., viral vector particles) has been demonstrated in the cells or the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific model viruses, as previously stated. If no RVLPs are detected and if the PERT assay is negative, no estimation of retroviral particles per dose is required.

Case B: In rodent cell lines, if only a rodent retrovirus (such as rodent A-, C-, and R-type particle) is present, the process evaluation using a specific model virus (such as a murine leukemia virus (MLV)) should be performed in addition to nonspecific viral clearance evaluation as in Case A. For marketing authorization, typically RVLP quantification data from three lots or more of purified bulk at pilot plant scale or commercial scale should be provided. Well-characterized cell lines such as CHO, C127, BHK, and murine hybridoma cell lines (e.g., NS0 or SP2/0 cells) have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is usually not recommended to test for the presence of the noninfectious particles in the purified bulk or drug substance. This also applies to other well-characterized cell lines (e.g., Sf9 insect cell lines) that produce endogenous retroviral-like particles that have been extensively characterized.

Case C: When the cells or unprocessed bulk are known to contain a virus (other than a rodent retrovirus) for which there is no evidence of infectivity to humans (e.g., Sf9 rhabdovirus), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, relevant or specific model viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or relevant or specific model) viruses at the critical inactivation steps should be obtained as part of the process evaluation for these viruses. Purified bulk should be tested using suitable methods with high specificity and sensitivity for detecting the virus in question. For the purpose of marketing authorization, typically data from three batches or more of purified bulk manufactured at pilot plant scale or commercial scale should be provided.

Case D: If a virus infectious to humans (such as those viruses indicated in Table 3, footnote a) is identified, the product should be considered acceptable only under exceptional circumstances. In such instances, the identified virus should be used for virus removal and inactivation evaluation studies and specific methods with high specificity, and sensitivity for the detection of the virus in question should be used. If it is not possible to use the identified virus, relevant and/or specific model viruses (described later) should be used. The process should be shown to remove and inactivate the selected viruses during downstream processing. Time-dependent inactivation data for the critical inactivation steps should be obtained as part of the process evaluation. Purified bulk should be tested using suitable methods with high specificity and sensitivity for the detection of the virus in question. For

marketing authorization, typically data from three batches or more of purified bulk manufactured at pilot plant scale or commercial scale should be provided.

Case E: When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable because the virus may be pathogenic. In the rare case in which there are well-justified reasons for drug production using such a cell line, this should be discussed with the appropriate regulatory authorities before proceeding further.

Case F: When a production virus (helper virus or viral vector for expression of recombinant protein or VLP) is used in product manufacturing, clearance of the virus should be demonstrated using the production virus or a specific model virus (e.g., baculovirus, adenovirus, herpesvirus). Testing for the absence of the production virus should be performed for each purified bulk, unless justified by robust excess clearance (see section VI.C (6.3)). In this case, absence of residual production virus should be confirmed by testing of at least three batches of purified bulk.

VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES (6)

Evaluation and characterization of virus removal or inactivation procedures are important for establishing the safety of biotechnology products. Past instances of contamination have occurred with agents whose presence was not known or even suspected. Though this happened due to various source materials other than fully characterized cell lines, it reinforces that assessment of viral clearance provides a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a well-documented and controlled manner.

Process characterization/evaluation of viral clearance is performed to demonstrate the clearance of a virus known to be present endogenously in the cell substrate, or as a result of using production viruses as well as to ensure that adventitious viruses that could not be detected, or that might enter the production process, would be cleared. The objectives of viral clearance studies are (1) to assess process steps that effectively inactivate or remove viruses and (2) to estimate quantitatively the overall level of virus reduction obtained by the process. These should be achieved by the deliberate addition (i.e., *spiking*) of significant amounts of virus to the unprocessed bulk or to process intermediates obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by using fewer steps. It should be considered that other steps in the process may have an indirect effect on the viral inactivation or removal achieved. Manufacturers should explain and justify the approach used in studies to evaluate viral clearance. When appropriate, to determine the amount of virus particles that enter the purification process, quantification should be performed on three cell culture lots/batches. These data should be submitted as part of the marketing application.

The reduction of virus infectivity can be achieved by removing virus particles or by inactivating viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it results from inactivation

or removal. For inactivation steps, the study should be planned so that samples are taken at different times and an inactivation curve is constructed (see section VI.B.5 (6.2.5)).

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove or inactivate other viruses should be conducted. The purpose of studies using viruses with diverse biochemical and biophysical properties is to characterize the robustness of the procedure to clear viruses that are not known or not expected to be present rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C (6.3)). Such studies are not performed to evaluate a specific safety risk; therefore, achieving a specific clearance value is not needed.

A. Choice of Viruses for Evaluation and Characterization of Viral Clearance (6.1)

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses that may contaminate the product and to represent a wide range of physicochemical properties to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses according to the aims of the evaluation and characterization study provided in this guidance.

1. Relevant Viruses and Model Viruses (6.1.1)

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: (1) relevant viruses, (2) specific model viruses, and (3) nonspecific model viruses. Relevant viruses are used in the viral clearance studies. Relevant viruses are the identified viruses or of the same species as the viruses that are identified or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The downstream process should demonstrate the capability to remove and/or inactivate such viruses. When a relevant virus is not available or when it is not well adapted to the process evaluation of viral clearance studies (e.g., it cannot be grown in vitro to sufficiently high titers), a specific model virus should be used as a substitute. An appropriate specific model virus can be a virus that is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus. Cell lines derived from rodents usually contain endogenous retroviruses or RVLPs, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This can be accomplished by using an MLV — a specific model virus in the case of cells of rodent origin.

For CHO cell-derived products, CHO-derived endogenous virus particles can also be used for viral clearance experiments. There is no infectivity assay for these particles, and the detection assay (e.g., molecular, biochemical) should be qualified for its intended use. When human cell lines have been obtained by the immortalization of B lymphocytes by Epstein-Barr virus, the ability of the manufacturing process to remove and/or inactivate a herpesvirus should be determined. Pseudorabies virus can also be used as a specific model virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general (i.e., to characterize the robustness of the clearance

process), viral clearance characterization studies should be performed with nonspecific model viruses with differing properties. Data obtained from studies with relevant and/or specific model viruses can also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used should be influenced by the quality and characterization of the cell lines and the production process. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

Appendix 1 (Annex 1) and Table A-1 provide examples of useful model viruses representing a range of physicochemical structures and examples of viruses that have been used in viral clearance studies.

2. Other Considerations (6.1.2)

Additional points to be considered include the following:

- Viruses that can be grown to high titer are desirable, although this may not always be possible.
- There should be an efficient and reliable assay for the detection of each virus used for each stage of manufacturing that is evaluated.
- The health hazard that certain viruses may pose to the personnel performing the clearance studies should be considered.

B. Design and Implications of Viral Clearance Evaluation and Characterization Studies (6.2)

1. Facility and Staff (6.2.1)

It is inappropriate to introduce any unintended virus into a production facility because of good manufacturing practice constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in collaboration with production personnel with experience in the specific process involved in designing and preparing a scale-down model of the purification process.

2. Scale-Down Model of the Production Process (6.2.2)

The representativeness of the scale-down model should be demonstrated and should represent the production process as closely as possible. The scale-down model should also consider the robustness of viral clearance (i.e., ability of a process or process step to tolerate variability of materials and changes of the process without negative impact). In this context, it can be advisable to perform viral clearance studies under worst-case conditions for relevant parameters. It can also be acceptable that conditions may be outside of an operating range to demonstrate the robustness of viral clearance and to be prepared for potential manufacturing changes. The performance of the scale-down model should be demonstrated to represent the production process (e.g., comparable yield and purity). For chromatographic steps, for

example, column bed-height, linear flow rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and resin types, pH, temperature, conductivity, and concentration of product should be shown to be representative of commercial-scale manufacturing. A similar chromatogram should result. For other procedures, similar considerations apply. Unavoidable deviations should be discussed with regard to their influence on the results.

3. Analysis of Stepwise Clearance of Virus (6.2.3)

When viral clearance studies are performed, assessment of the contribution of more than one production step to virus elimination should be considered. Steps that are likely to clear virus should be individually assessed for their ability to remove or inactivate virus, and the exact definition of an individual step should be considered. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to process intermediate at each step to be tested. In some cases, adding high titer virus to an early process intermediate and testing its concentration between subsequent steps can be justified. When virus removal results from separation procedures, it is recommended that the distribution of the virus load in the different fractions be investigated, if appropriate and if possible. When buffers that inactivate viruses are used within the manufacturing process, alternative strategies such as parallel spiking in less virus-inactivating buffers (e.g., in pH adjusted buffer) can be carried out as part of the overall process assessment. Inactivation by the buffer itself might be tested in separate spiking experiments. Using quantitative assays not associated with infectivity may be applicable to determine partitioning of viral particles. The virus titer before and after each step being evaluated should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity can be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (see Appendix B (Annex 2)).

As a general principle, reproducible viral clearance should be confirmed in at least two independent experiments, unless a reduction of studies is justified by prior knowledge (see section VI.F (6.6)). Experiments can be performed at the same site, using the same experimental setting and with the same lot of in-process material although it is not expected that the two independent experiments are performed identically.

4. Determining Physical Removal Versus Inactivation (6.2.4)

Reduction of infectious virus can be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of the loss of viral infectivity should be described as related to inactivation and/or removal. It may be necessary to distinguish between removal and inactivation for a particular step. As an example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step (i.e., the contribution to inactivation by a buffer shared by several chromatographic steps), the clearance achieved by each of these chromatographic steps should be distinguished.

5. Inactivation Assessment (6.2.5)

For the assessment of viral inactivation, process intermediate should be spiked with infectious virus, and the reduction factor calculated. It should be recognized that virus inactivation is not a simple first order reaction and is usually more complex with an initial fast phase and a subsequent slow phase. Therefore, the study should be planned in such a way that samples are taken at different times and an inactivation curve be constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important if the virus is a relevant virus known to be a human pathogen, and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific model viruses are used or when specific model viruses are used as surrogates for virus particles such as the CHO RVLPs, reproducible clearance should be demonstrated in typically two independent studies. Whenever possible, the initial virus load should be determined from the virus, which can be detected in the spiked starting material. If this is not possible, the initial virus load can be calculated from the titer of the spiking virus preparation. When inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls (e.g., samples with lower concentration of the inactivating agent) should be performed to demonstrate that infectivity is indeed lost by inactivation.

6. Function and Regeneration of Columns (6.2.6)

Over time and after repeated use, the ability of chromatography resins and membranes used in the purification process to clear virus may vary. Chromatography media/resin lifetime use should be indicated, and parameters with potential impact on viral clearance should be defined. Viral clearance studies should be performed to support media/resin reuse.

For Protein A affinity capture chromatography, prior knowledge indicates that virus removal is not affected or slightly increases for used (e.g., end of life) chromatography media/resin. Therefore product-specific studies with used resin are not expected. Prior knowledge might also apply to other chromatography types involved in viral clearance (e.g., anion exchange, cation exchange). Accordingly, in cases where product-specific studies with end-of-lifetime resin are not considered necessary to support repeated resin use for other chromatography types, equivalent prior knowledge including in-house experience and a detailed justification should be provided (see section VI.F (6.6)).

Assurance should be provided so that any virus potentially retained by the production system would be adequately inactivated or removed before reusing the system. For example, evidence can be provided demonstrating that the cleaning and regeneration procedures inactivate or remove virus, for example, during viral clearance evaluation, and this can be supported by prior knowledge.

7. Specific Precautions (6.2.7)

The following specific precautions should be considered:

- Using highly pure and concentrated virus stocks for spiking can be helpful to minimize dilution of the product intermediate as well as to reduce introduction of nonrepresentative impurities that may impact process performance. However, care should be taken in preparing high titer virus to avoid aggregation, which may enhance

physical removal and decrease inactivation thus distorting the correlation with actual production.

- Consideration should be given to the minimum quantity of virus that can be reliably assayed.

- The study should include parallel control assays to assess the loss of infectivity of the virus because of such reasons as the dilution, concentration, filtration, or storage of samples before titration.

- The virus spike should be added to the product in a small volume so as not to dilute or change the characteristics of the product.

- Small differences in buffers, media, or reagents (for example) can affect viral clearance.

- Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.

- Buffers and product should be evaluated independently for toxicity and interference in assays used to determine the virus titer because these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH or dialysis of the buffer containing spiked virus might be necessary. If the product itself has antiviral activity, the clearance study may need to be performed without the product in a *mock* run, or prior knowledge may be applicable as described in section VI.F (6.6). However, omitting the product or substituting a similar protein that does not have antiviral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.

- In case molecular biology assays are used for quantification of viral genomes, care should be taken to consider the effect from nonencapsulated viral nucleic acids, which may be nonrepresentative of intact viral particles removal in certain process steps.

- Many purification processes use the same or similar buffers or columns in more than one purification step. When the overall virus reduction factor for a complete production process is based on the sum of the reduction factors of such purification processes, this approach should be justified; for example, the effectiveness of virus elimination by a particular process step may vary with the stage of manufacture at which it is used and the presence of accompanying proteins and other impurities clearly affecting the virus reduction capacity.

- Overall reduction factors may be overestimated or underestimated when production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated because of the inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies (6.3)

The objective of assessing virus inactivation/removal is to evaluate and characterize process steps considered effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For viruses, as in Cases B through F, it is important to show that not only is the virus removed or inactivated, but that there is excess capacity for viral clearance built into the downstream process to ensure an appropriate level of viral safety for the final product. The amount of virus removed or inactivated by the manufacturing process should be compared to the amount of virus that may be entering the downstream process.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as TEM or a comparable quantitative NAT. The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single dose equivalent of unprocessed bulk. See Appendix C (Annex 3) for calculation of virus reduction factors and Appendix D (Annex 4) for calculation of estimated particles per dose. Manufacturers should recognize that clearance mechanisms may differ among virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include the following:

- The appropriateness of the viruses evaluated
- The design of the clearance studies
- The virus reduction factor achieved
- The time dependence of virus inactivation
- The potential effects of variation in-process parameters on virus inactivation/removal
- The assay sensitivity
- The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses

It is recommended to design a downstream process that clears a wide range of potential viruses (potential adventitious virus contaminants, endogenous and/or production virus). In this context, whenever feasible and not adversely affecting the product, implementing two distinct viral clearance steps, one of which should provide effective clearance of nonenveloped virus, that complement each other in their mode of action is recommended. An effective viral clearance step generally gives reproducible reduction of virus load in the order of 4 $\log_{10}$ or more shown by at least two independent studies. However, it is recognized that steps giving a reproducible reduction of 1 to 3 $\log_{10}$ contribute toward viral safety and can be considered for assessment of overall virus reduction. Process steps dedicated to virus inactivation/removal such as solvent/detergent (SD) treatment, treatment with detergent alone, or incubation at low pH, have been very successful in clearing a wide range of enveloped viruses, and virus filtration removes viruses based on their sizes. Using virus filters designed for removal of small viruses is also an effective viral clearance step for the smaller viruses such as parvovirus or polyomaviruses. Finally, there is experience of efficient

inactivation of MLV and pseudorabies virus by incubation at low pH after the Protein A capture step for purification of mAbs.

Acceptable overall clearance can be achieved by any of the following steps: multiple complementary inactivation steps, multiple complementary removal (separation) steps, or combinations of inactivation and removal steps. Virus removal methods may be dependent on the extremely specific physicochemical properties of viruses, which influence their interaction with stationary phases for chromatography (e.g., resins or chromatography membranes) and their precipitation properties. Therefore, model viruses can be separated in a different manner than a relevant virus. Manufacturing parameters influencing removal should be properly defined and controlled. Despite these potential variables, effective clearance can be obtained by a combination of complementary removal steps or combinations of inactivation and removal steps. Therefore, well-designed removal steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps if they are performed under appropriately controlled conditions.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus load less than 1 log10 is considered negligible and should be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. The factors listed above will be considered in evaluating the results.

D. Limitations of Viral Clearance Studies (6.4)

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. A number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

- Virus preparations used in clearance studies for a production process are usually obtained from specific cell cultures. The behavior of such virus spike in a production step may be different from that of the native viral contaminant from a biological raw material in the cell culture medium or replicating in the manufacturing cells. For example, this might include if virus particles used for spiking and native virus from a respective production intermediate differ in purity or degree of aggregation.

- Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

- The ability of the overall process to remove or inactivate virus is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors from steps with nonsignificant reduction (e.g., below 1 $\log_{10}$), may overestimate the

true potential for virus elimination, and should not be performed. Addition of individual virus reduction factors resulting from similar inactivation mechanisms during the manufacturing process may also overestimate overall viral clearance. Furthermore, if reduction values achieved by repetition of identical or near identical procedures are included, they should be justified.

- The expression of reduction factors as logarithmic reductions in titer implies that, although residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 $\log_{10}$ infectious units per milliliter (mL) by a factor of 8 $\log_{10}$ leaves 0 $\log_{10}$ per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.

- Processing during validation studies for viral clearance performed at small scale may differ from processing at commercial scale despite care taken to design the scale-down process.

E. Statistics (6.5)

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (refer to Appendix B (Annex 2)).

F. Application of Prior Knowledge for Evaluation of Viral Clearance (6.6)

As a general principle, viral clearance is evaluated by experiments when the virus is added to the product-specific process intermediate of each step to be investigated. When a manufacturer is developing similar products by established and well-characterized processes (i.e., using the same platform technology), viral clearance data generated for other products might be applicable to the new product for the same processing step. However, to make use of data from such a step, the process step should be well understood. The representativeness of the prior knowledge for the specific process step should be clearly justified. The prior knowledge including external and in-house experience should cover the aspects outlined below:

- There should be an understanding of the mechanism underlying viral clearance.

- There should be comprehensive understanding of the process parameters that may affect viral clearance.

- It should be clear that interactions between virus and product do not affect viral clearance. If there is a potential risk that the virus-product interaction may affect viral clearance, applying prior knowledge from manufacturing of other products should be justified. If data for more than one product is available for the specific step, the effectiveness of virus reduction should be comparable in each case.

- The composition of a specific process intermediate may affect viral clearance. For some process steps, even small differences in buffers, media, reagents, and profile of impurities (for example) may affect viral clearance. Therefore, the representativeness of the composition of the process intermediate(s) from other products should be justified. Processing before the specific step for the new and the established product(s)

should follow a similar strategy unless prior knowledge indicates robustness of viral clearance with respect to composition of the process intermediate.

- The general limitations of viral clearance studies as outlined in section VI.D (6.4) should be considered when applying prior knowledge to a specific product.

External prior knowledge (including published data) can be supportive in indicating the potential of a step to inactivate/remove viruses and can provide insight to the mechanisms involved. Such data can also be used to define the process parameters critical for viral clearance and in setting worst-case limits for testing in a specific viral clearance step. Performing viral clearance studies at worst-case conditions can help reduce the number of product-specific experiments. However, the application of published reduction factors to a specific product should be supported by demonstration of similarity of the processes across manufacture of different products involved, similarity of the product intermediates, and an assurance that product-specific attributes do not affect virus reduction. Therefore, published data should be carefully assessed and supplemented with in-house experience (internal prior knowledge) for a given platform.

The decision on the acceptability of viral clearance data without product-specific experiments is made on a case-by-case basis while considering the whole viral safety strategy for a medicinal product, including the nature and characterization of the cell substrate and raw materials, and the overall viral clearance strategy. If the data package does not sufficiently support the use of prior knowledge, product-specific viral clearance studies should be performed.

When deriving a reduction factor claim using prior knowledge, the claim should be justified considering all reduction factors from the relevant platform data. A conservative reduction factor claim is advised to avoid the risk of overestimating the reduction capacity of the process step.

Appendix E (Annex 5) provides cases when, according to current understanding, prior knowledge including in-house experience with viral reduction data from other products could be used to claim a reduction factor for a new product from the same manufacturing platform.

G. Reevaluation of Viral Clearance (6.7)

Whenever significant changes in the upstream or downstream manufacturing processes are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system reevaluated, as needed. For example, changes in upstream manufacturing processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

Changes in the manufacturing process during life-cycle management that may affect viral clearance efficacy could be evaluated using internal knowledge and the platform concept. If the internal knowledge (in-house experience) with other products cannot be extrapolated to specific products and/or the platform concept can no longer be applied, product-specific viral clearance studies should be performed.

VII. POINTS TO CONSIDER FOR CM (7)

CM can involve the continuous feeding of input materials into a manufacturing process comprised of a series of linked unit operations that transform the feed and provide a continuous stream of an output material (i.e., product). CM can be applied to some or all unit operations of a manufacturing process. An understanding of the integrated process and its dynamics, in addition to each unit operation, is essential to identify and mitigate the risk to viral safety. A description of the modes of CM processes for the manufacture of therapeutic proteins can be found in the ICH guidance for industry *Q13 Continuous Manufacturing of Drug Substances and Drug Products* (March 2023), (at Annex III).

In terms of viral safety, technical aspects for CM may differ from those encountered in batch processes, including approaches of detection and removal of viruses, material traceability, process dynamics, and monitoring frequency start-up/shutdown.

However, basic principles and expectations (such as science- and risk-based approaches and their implementation to control virus contamination risk) that are based on process understanding are the same as for batch manufacturing. This also includes contamination prevention strategies (see section II.B (2.2)). For example, the physical and chemical conditions to inactivate or remove viruses derived from experience or prior knowledge of batch production may also be applicable to CM processes (see section VI.F (6.6)).

A. Viral Safety in CM (7.1)

The viral safety strategy in CM processes should be based on a risk assessment of potential sources of contamination (e.g., the starting and raw materials and extended cell culture duration), the ability of the process to remove viruses, and the testing capability to assure absence of viruses. Guidance on testing provided in sections III (3) and IV (4) is also considered applicable to CM. Based on this assessment, a strategy should be developed to include the type and frequency of adventitious virus testing undertaken to demonstrate that the23rocesss is free of contamination during manufacturing.

2. General Considerations for Viral Clearance in CM (7.2)

To design the manufacturing process and the viral clearance study, the following should be considered:

- The manufacturing process can be partially run in an integrated (connected) or continuous mode of operation, and it is possible to use the scientific understanding of viral clearance performance based on batch processes for the evaluation of unit operations if suitable (see section VII.C.2 (7.3.2)).

- The potential risk of each unit operation and the connection between equipment (e.g., use of a surge or mixing tank between unit operations to mitigate differences in mass flow rate or nonhomogenous input materials) should be assessed to address any effects on the virus reduction capabilities.

- There should be appropriate process monitoring in place to detect (1) process disturbance (e.g., pressure/flow interruption that impacts viral clearance) and (2) adventitious virus contamination. The final impact of process disturbance may differ based on the type of event and should be addressed on a case-by-case basis.

- If conducting real-time decision-making, this should include a procedure to determine the effects of (1) the process disturbance on viral clearance or (2) the contamination on the output material quality and product. Based on these considerations, the diversion of the potential nonconforming material from the product stream or the disposition of the material produced should be considered (see section IV (4)).

- The viral clearance study designs should consider potential effects of the following, if applicable:

  — Variability of input material attributes (e.g., concentration and homogeneity of protein or impurities, level of aggregation)

  — Residence time

  — Planned events (e.g., process start-up, shutdown, and pause) and unplanned transient events (e.g., disturbances)

  — Operational loading capacity

  — Alternative loading strategies (e.g., multicolumn cycling, serial loading)

  — Virus control strategy

2. Unique Considerations for Viral Clearance in CM (7.3)

CM also presents unique aspects to consider for viral safety as described below.

2. Potential Risk Related to Longer Periods in Production Culture (7.3.1)

Fluctuations in the levels of endogenous virus may occur over time in the production culture, so an assessment should be made of the appropriate sampling strategy so as not to affect the dose risk factor calculation for the drug product (see section IV (4) and considerations in section III (3) for cell line qualification).

2. Approach to Viral Clearance Study (7.3.2)

Although CM is expected to maintain a state of control, the manufacturing process can include periods when the process output can vary during start-up, termination, and process disturbance (e.g., potentially high virus load for a short period of time in case of a virus contamination). The risks for such periods are addressed elsewhere in this guidance (see section VI.B (6.2)).

Furthermore, simultaneous validation of two or more connected unit operations could be an option according to the equipment design and system integration.

Considerations specific to CM include the following:

- Chromatography

  — In the case of repeating cycles (e.g., multicolumn processing), a batch process could serve as a scale-down model with well-justified target process conditions (e.g., flow rate, resin load versus column overload, resin cleanability); see also guidance for scaling down in section VI.B.2 (6.2.2).

  — Validation of two or more connected chromatography steps can be considered (e.g., bind and elute mode of cation exchange chromatography and flow through mode of anion exchange chromatography). For connected unit operations, it is an option to evaluate viral clearance using a conventional batch scale-down model if the operating conditions during manufacturing are adequately reflected, with respect to the load material.

- Viral inactivation

  — Validation as a batch process could be appropriate with well-justified target process conditions.

  — For virus inactivation (e.g., pH, SD), the control of relevant dynamic process parameters should be ensured (e.g., pH, SD concentration, homogeneity and mixing, temperature, residence time distribution).

  — Care should be taken in evaluating/justifying the effects of scale on the process dynamics (e.g., residence time distribution) and control strategy when a scale-down model is applied for inactivation in a dynamic process.

- Virus filtration

  — Validation as a batch process could be appropriate if settings of parameters, which affect viral clearance, do not vary beyond ranges tested in the viral clearance study (e.g., worst-case set point).

  — Process controls should be defined to allow for filter changes and post use integrity testing while maintaining viral clearance capacity.

VIII. SUMMARY (8)

This guidance recommends approaches for evaluating the risk of viral contamination of the product, controlling potential sources of virus, and qualifying the manufacturing process for viral clearance, thus contributing to the production of safe biotechnology products derived from animal or human cell lines. These control and clearance approaches include the following:

- Thorough characterization/testing of cell substrate starting material to identify which, if any, virus contaminants are present
- Assessment of potential risk from virus contaminants by determination of the human cell tropism or knowledge of human infections
- Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk
- Establishment of a program for appropriate viral clearance
- Careful design and performance of viral clearance studies for different methods of virus inactivation or removal in the same production process.

GLOSSARY (9)

Adventitious virus

Unintentionally introduced contaminant viruses. See *virus*.

Cell substrate

Cells used to manufacture product.

Control cells

Cells cultured in parallel with the production of the virus or viral vector without inoculation of the virus/viral vector seed. Control cells are maintained in conditions that are essentially equivalent to those used for the production cell cultures, including use of the same batches of media and media changes.

End of production cells (EOPCs)

Cells harvested (under conditions comparable to those used in production) from the master cell bank (MCB) or working cell bank (WCB) cultured to a passage level or population doubling level comparable to or beyond the highest level reached in production. In certain situations, the chronological time in culture can be measured. The EOPCs are also referred to as extended cell banks (ECB), and these terms can be used interchangeably with limit of in vitro cell age (LIVCA) cells.

Endogenous virus

Retrovirus whose genome is part of the germ line of the species of origin of the cell line and is stably integrated into the genome of the host species from which the parental cell line was derived. In this guidance, this also includes intentionally introduced, nonintegrated viruses such as Epstein-Barr virus used to immortalize cell substrates or bovine papilloma virus.

Extended cell bank (ECB)

Cells cultured from the MCB or WCB and propagated to the proposed in vitro cell age used for production or beyond. May also be referred to as *EOPC*.

Inactivation

Reduction of virus infectivity caused by chemical or physical treatment.

In vitro cell age

A measure of the period between thawing the MCB vial(s) and harvesting the production vessel that is measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Limit of in vitro cell age (LIVCA) cells

LIVCA cells are derived from production cells at or beyond in vitro cell age by expansion of the MCB or WCB. LIVCA cells may be also referred to as EOPC or ECB, and these terms can be used interchangeably.

Master cell bank (MCB)

An aliquot of a single pool of cells, which generally has been prepared from the cell substrate or selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions.

Master virus seed (MVS)

An MVS (stock, lot, or bank) is a preparation of a vector virus, a viral vector or production virus (i.e., helper virus or viral protein expression vector), from which all future production will be derived, either directly or via a working virus seed (WVS). It is a live viral preparation of uniform composition (although not necessarily clonal) derived from a single culture process, aliquoted into appropriate storage containers, and stored under appropriate conditions.

Minimum exposure time

The shortest period for which a treatment step will be maintained.

Next generation sequencing (NGS)

Also referred to as high-throughput sequencing or massive parallel sequencing or deep sequencing, multistep nucleic acid-based technology with broad capabilities for nontargeted (agnostic) detection of known and unknown adventitious viruses. In some cases, NGS can be used for targeted detection of known viruses by the sequencing strategy or by bioinformatic analysis.

Platform manufacturing (see the International Council for Harmonisation guidance for industry *Q11 Development and Manufacture of Drug Substances* (November 2012))[1]

The approach of developing a production strategy for a new drug starting from manufacturing processes similar to those used by the same applicant to manufacture other drugs of the same type (e.g., as in the production of monoclonal antibodies (mAbs) using predefined host cell, cell culture, and purification processes for which considerable experience already exists).

[1] We update guidances periodically. To make sure you have the most recent version of a guidance, check the FDA guidance web page at https://www.fda.gov/regulatory-information/search-fda-guidance-documents.

Platform validation[2]

Throughout this guidance, this term exclusively refers to validation of the process platform regarding viral clearance. In this context, platform validation is defined as the use of prior knowledge including in-house experience with viral reduction data from other products, to claim a reduction factor for a new similar product, according to current understanding.

Prior knowledge

Prior knowledge refers to existing knowledge and includes internal knowledge (e.g., development and manufacturing experience), external knowledge (e.g., scientific and technical publications, including vendors' data, literature, and peer-reviewed publications), or the application of established scientific principles (e.g., chemistry, physics, and engineering principles).

Process characterization of viral clearance

Viral clearance studies in which nonspecific *model* viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

Process evaluation studies of viral clearance

Viral clearance studies in which *relevant* and/or specific *model* viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

Process robustness of viral clearance

The term *robustness* is used to describe one or both of the different characteristics. One characteristic is the ability of a process or process step to tolerate variability of materials and changes of the process without negative effect on clearing a virus. The other characteristic is the ability to clear a wide range of specific and nonspecific model viruses.

Production cells

Cell substrate used to manufacture product.

Production virus

A production virus is a process-related virus and may include a helper virus or a viral vector for protein expression.

[2] The Food and Drug Administration (FDA) acknowledges that the terms *platform* and *platform technology* have been used by both industry and FDA to describe technologies in ways differing from the term *designated platform technology* described in section 506K of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 356k). Use of the terms *platform* or *platform technology* within this guidance does not constitute a determination that the technology would meet that statutory definition and eligibility criteria for purposes of the platform technology designation program.

Helper virus

A virus that provides helper functions allowing an otherwise replication-deficient coinfecting virus to replicate.

Viral vector for protein expression

A recombinant virus, such as baculovirus, that can be used to express a recombinant protein or a virus-like particle or to produce a viral vector.

Purified bulk

The term *purified bulk* refers to material at the end of the purification process. While in most cases this represents drug substance, the purified material without inactive ingredients can be used instead to avoid interference with testing assays.

Supplementary test method

A test method used to provide additional data to support the conventional testing.

Unprocessed bulk

One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the bioreactor.

Viral clearance

Removal of viral particles or inactivation of viral infectivity.

Viral vector

A recombinant virus that may be applied in vivo as a medicinal product or applied ex vivo for other advanced therapeutic applications.

Viral vector-derived product

A product encoded and expressed by a viral vector, where the recombinant virus is referred to as a viral vector for production, such as a baculovirus.

Virus

Intracellularly replicating infectious agents that are potentially pathogenic, that possess only a single type of nucleic acid (either RNA or DNA), that are unable to grow and undergo binary fission, and that multiply in the form of their genetic material.

Nonspecific model virus

A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general (i.e., to characterize the robustness of the downstream process).

Relevant virus

Virus used in the process evaluation studies that is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

Replication competent virus (RCV)

Recombination of the viral vector with transcomplementing viral sequences generating an RCV.

Specific model virus

Virus, which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

Virus-like particles

Structures that morphologically appear to be related to known viruses. May or may not contain the viral genome.

Working cell bank (WCB)

The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

Working virus seed (WVS)

A WVS (stock, lot, or bank) is produced from the MVS.

REFERENCES (10)

International Council for Harmonisation draft guidances for industry *Q2(R2) Validation of Analytical Procedures* (August 2022) and *Q14 Analytical Procedure Development* (August 2022)[1]

International Council for Harmonisation guidance for industry *Q11 Development and Manufacture of Drug Substances* (November 2012)

International Council for Harmonisation guidance for industry *Q13 Continuous Manufacturing of Drug Substances and Drug Products* (March 2023)

[1] When final, these guidances will represent the FDA's current thinking on these topics. For the most recent version of a guidance, check the FDA guidance web page at https://www.fda.gov/regulatory-information/search-fda-guidance-documents.

TABLE 1. VIRUS TESTS RECOMMENDED FOR CHARACTERIZATION OF CELL SUBSTRATES

	MCB	*WCB*	*Cells at the LIVCA*
Tests for Retroviruses and Other Endogenous Viruses			
Retrovirus tests[a]	+	-	+
Other endogenous virus tests[b]	as appropriate[b]	-	as appropriate[b]
Tests for Adventitious Viruses			
In vitro assays or NGS[e]	+	+[c]	+[c]
In vivo assays or NGS[e]	+[d]	-	+[d]
Tests for specific viruses[f]	as appropriate[f]	-	-

MCB = master cell bank; WCB = working cell bank; LIVCA = limit of in vitro cell age; NGS = next generation sequencing.

a. Refer to section III.B.1 (3.2.1) for additional details.
b. As appropriate for cell lines that are known to contain such agents.
c. The in vitro virus assay is performed directly on the WCB or on LIVCA cells directly derived from this WCB.
d. The in vivo assay can be performed based on risk assessment. If residual risk remains, retention of the test or replacement with nontargeted NGS can be considered to detect viruses that may have been introduced during establishment of the MCB or during culture of the cells at the LIVCA stage. Refer to section III.B.3 (3.2.3) for details.
e. Nontargeted NGS can replace the in vivo assay (see section III.B.3 (3.2.3)) and supplement or replace the in vitro assay (see section III.B.2 (3.2.2)).
f. Testing is based on risk assessment including the origin and history of the cell substrate, and potential exposure to human- or animal-derived raw materials. Methods such as cell culture-based infectivity assays, antibody production tests (MAP, HAP, RAP), virus-specific NAT or other molecular methods (e.g., NGS) can be used. Refer to section III.B.4 (3.2.4) for details. This can include testing for species-specific viruses, for example, arboviruses in insect cells, and bovine or porcine viruses if serum or trypsin are used, respectively. Refer to Table 4 (Cases B, C, and E) for action steps to be taken for virus detection in cell substrates used for production.

TABLE 2. EXAMPLES OF THE USE AND LIMITATIONS OF ASSAYS THAT CAN BE USED TO TEST FOR VIRUSES

Test	*Test Article*	*Detection Capability*	*Assay Limitation*
Antibody Production Test	Lysate of cells and their culture medium	Specific viral antigens	Viruses failing to replicate and/or produce antibodies in animals. Not all virus infections result in a measurable antibody response.
In vivo virus assay	Lysate of cells and their culture medium	Limited range of viruses	Viruses failing to replicate or produce diseases in the test system. Assay interference.
In vitro virus assay		Broad range of viruses	Viruses failing to replicate or produce signs of infection in the test system. Assay interference.
1. Cell bank testing	Lysate of cells and their culture medium (for cocultivation, intact cells should be in the test article)		
2. Production testing	Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM:			Low sensitivity. Does not indicate whether virus is infectious.
Cell bank testing	Viable cells	Virus and virus-like particles including endogenous retroviruses	Qualitative assessment of viral particles.
Production testing	Cell-free material		Quantitative assay (for viral clearance assessment).
Reverse transcriptase (RT) assay (e.g., PERT assay)	Cell-free culture supernatant	Retroviral particles and RT activity	May need to distinguish retrovirus RT from cellular polymerases.
Cell bank testing Production testing			
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system.
Cocultivation	Viable cells	Infectious retroviruses	RV failing to replicate
Infectivity endpoint			See above under RV infectivity.
2. TEM endpoint			2. See above under TEM[a]
3. RT endpoint			3. See above under RT

Test	*Test Article*	*Detection Capability*	*Assay Limitation*
PCR Cell bank testing Production testing	ls, culture fluid, and other materials	Specific viral sequences	Primer sequences are present. Does not indicate whether virus is infectious.
NGS	ls, culture fluid, and other materials	Broad range of viral sequences	Positive result does not indicate whether virus is infectious and should be investigated.

a. In addition, may be difficult to distinguish test article from indicator cells.
TEM = transmission electron microscopy; PERT = product-enhanced reverse transcriptase; NGS = next generation sequencing.

TABLE 3. VIRUSES DETECTED IN ANTIBODY PRODUCTION TESTS[D]

MAP	*HAP*	*RAP*
Ectromelia Virus[b,c]	Lymphocytic Choriomeningitis Virus (LCM)[a,c]	Hantaan Virus[a,c]
Hantaan Virus[a,c]	Pneumonia Virus of Mice (PVM)[b,c]	Kilham Rat Virus (KRV)[b,c]
K Virus[b]	Reovirus Type 3 (Reo3)[a,c]	Mouse Encephalomyelitis Virus (Theilers, GDVII)[b]
Lactic Dehydrogenase Virus (LDM)[a,c]	Sendai Virus (SV)[a,c] SV5[a,c]	Pneumonia Virus of Mice (PVM)[b,c]
Lymphocytic Choriomeningitis Virus (LCM)[a,c]		Rat Coronavirus[b]
Minute Virus of Mice[b,c] Mouse		Reovirus Type 3 (Reo3)[a,c] Sendai
Adenovirus (MAV)[b,c]		Virus[a,c]
Mouse Cytomegalovirus (MCMV)[b,c]		Sialodacryoadenitis Virus (SDAV)[b]
Mouse Encephalomyelitis Virus (Theilers, GDVII)[b]		Toolan's H-1 Virus[b,c]
Mouse Hepatitis Virus (MHV)[b]		
Mouse Rotavirus (EDIM)[b,c]		
Pneumonia Virus of Mice (PVM)[b,c]		
Polyoma Virus[b]		
Reovirus Type 3 (Reo3)[a,c] Sendai		
Virus[a,c]		
Thymic Virus [b,e]		

MAP = mouse antibody production; HAP = hamster antibody production; RAP = rat antibody production.

a. Viruses for which there is evidence of capacity for infecting humans or primates.
b. Viruses for which there is no evidence of capacity for infecting humans.
c. Virus capable of replicating in vitro in cells of human or primate origin.
d. NAT such as PCR assays or targeted or non-targeted NGS or other molecular methods can be used instead.
e. Also called murid herpesvirus 3.

TABLE 4. RECOMMENDED ACTION PLAN IN RESPONSE TO THE RESULTS OF VIRUS TESTS ON CELLS OR UNPROCESSED BULK

	Case A	Case B	Case C[b]	Case D[b]	Case E[b]	Case F
STATUS						
Presence of adventitious virus[a]	-	-	+	+	+[c]	-
Virus-like particles[a]	-	-	-	-	+[c]	-
Retrovirus-like particles[a]	-	+	-	-	+[c]	-
Virus identified	not applicable	+	+	+	-	+
Virus infectious for humans	not applicable	-[d]	-[d]	+	unknown	+[i]
Presence of production virus	-	-	-	-	-	+
ACTION						
Process characterization of viral clearance using nonspecific *model* viruses	yes[e]	yes[e]	yes[e]	yes[e]	yes[e]	yes[e]
Process evaluation of viral clearance using *relevant* or specific *model* viruses	no	yes[f]	yes[f]	yes[f]	yes[g]	yes[i]
Test for virus in purified bulk	not applicable	no[j]	yes[h]	yes[h]	yes[h]	yes[i]

a. Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production that are contaminated with viruses generally should not be used unless justified by specific viral clearance and risk assessment. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB can be acceptable if appropriate viral clearance evaluation procedures are followed.
b. Source material that is contaminated with viruses, where they are known to be infectious and/or pathogenic in humans, should only be used under exceptional circumstances by demonstration of specific viral clearance and risk assessment.
c. Virus has been observed by either direct or indirect methods.
d. Believed to be nonpathogenic.
e. Characterization of clearance using nonspecific *model* viruses should be performed.
f. Process evaluation for *relevant* viruses or specific model viruses should be performed.
g. See text under Case E.
h. The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots/batches of purified bulk manufactured at pilot plant scale or commercial scale should be provided.
i. Virus may or may not be infectious for humans. Process evaluation for the production virus should be performed. If this is not possible, then a specific model virus should be used. When utilized in production, the production virus is quantified in the unprocessed bulk stage using at least three cell culture lots/batches to determine the target for viral clearance. In purified bulk, absence of detectable production virus is determined using an infectivity assay with *relevant* permissive cell lines for sensitive virus detection. Alternatively, molecular

methods can be used. Testing for the absence of residual production virus should be performed for each purified bulk, unless justified by robust clearance (see section VI.C (6.3)).

j. Please refer to section V (5), description of Case B.

APPENDIX A: THE CHOICE OF VIRUSES FOR VIRAL CLEARANCE STUDIES (ANNEX 1)

A. Examples of Useful *Model* Viruses (Annex 1.1)

1. Nonspecific *model* viruses representing a range of physicochemical structures:

   - A polyomavirus (e.g., SV40), an animal parvovirus, or some other small and physicochemically resistant, nonenveloped virus.

   - A parainfluenza virus or influenza virus, Sindbis virus, or some other medium-to-large, enveloped, RNA virus. Alternatively, another nonenveloped virus such as reovirus, SV40, or a picornavirus can be used to extend the spectrum of nonenveloped virus.

   - A herpesvirus (e.g., herpes simplex virus-1, a pseudorabies virus), or some other medium-to-large DNA virus.

   These viruses are examples only, and their use is not mandatory.

2. For cell substrates producing retroviral-like particles, murine retroviruses are commonly used as specific model viruses. It may be also possible to use endogenous murine or other rodent retrovirus particles.

B. Examples of Viruses That Have Been Used in Viral Clearance Studies (Annex 1.2)

Several viruses that have been used in viral clearance studies are listed in Table A-1. However, as these are merely examples, the use of any of the viruses in the table is not mandatory, and manufacturers are invited to consider other viruses, especially those which may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear three or more different viruses with differing characteristics.

Table A-1: Examples of Viruses That Have Been Used in Viral Clearance Studies

Virus	*Family*	*Genus*	*Natural host*	*Genome*	*Env*	*Size (nanometer)*	*Shape*	*Resistance* [a]
Vesicular Stomatitis Virus (VSV)[b]	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70x150	Bullet	Low
Parainfluenza Virus	Paramyxo	Respirovirus or Orthorubulavirus	Various	RNA	yes	100-200+	Pleo/Sphere	Low
Murine Leukemia Virus (MLV)	Retro	Gammaretrovirus	Mouse	RNA	yes	80-110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60-70	Spherical	Low
Bovine Viral Diarrhea Virus (BVDV)	Pesti	Pestivirus	Bovine	RNA	yes	50-70	Pleo/Sphere	Low
Pseudorabies Virus[c]	Herpes	Varicellovirus	Swine	DNA	yes	120-200	Spherical	Med
Autographa Californica Multiple Nucleopolyhedrovirus[c]	Baculo	Alphabaculovirus	Insect	DNA	yes	250-300	Polyhedral	Med
Adenovirus Type 2 or Type 5[c]	Adeno	Mastadenovirus	Human	DNA	no	70-90	Icosahedral	Med
Vesivirus 2117	Calici	Vesivirus	Unknown	RNA	no	27-40	Icosahedral	Med
Encephalomyocarditis Virus (EMCV)	Picorna	Cardiovirus	Mouse	RNA	no	25-30	Icosahedral	Med
Bovine Enterovirus (BEV)	Picorna	Enterovirus	Bovine	RNA	no	25-30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	RNA	no	60-80	Spherical	Med
SV40	Polyoma	Betapolyomavirus	Monkey	DNA	no	40-50	Icosahedral	Very high
Parvoviruses (canine, murine, porcine)[d]	Parvo	Protoparvovirus	Canine Mouse Porcine	DNA	no	18-24	Icosahedral	Very high

a. Resistance to physicochemical treatments based on studies of production processes. Resistance is relative to the specific treatment, and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment.
b. Specific *model* virus for rhabdovirus found in insect cells.
c. Specific model virus or *relevant* virus for helper virus or viral protein expression vector used for viral vector production.
d. Can be used as single worst-case model virus for larger spherical/icosahedral viruses and enveloped viruses for validation of virus filters.

These viruses are examples only, and their use is not mandatory.

APPENDIX B: STATISTICAL CONSIDERATIONS FOR ASSESSING VIRUS AND VIRUS REDUCTION FACTORS (ANNEX 2)

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them, and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods can be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include molecular-based methods or plaque assays in which each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system that are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately 0.5 $\log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision can be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of *relevant* and specific *model* viruses. If the 95 percent confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95 percent confidence limits for the reduction factor are:

$$\pm \sqrt{s^2 + a^2}$$

Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per liter (L)), it is evident that a sample of a few milliliters (mL) may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$$p = ((V\text{-}v)/V)^n$$

When V (L) is the overall volume of the material to be tested, v (L) is the volume of the sample and n is the absolute number of infectious particles statistically distributed in V.

If V >> v, this equation can be approximated by the Poisson distribution:

$p = e^{-cv}$

When c is the concentration of infectious particles per liter.

Or, c = ln p /-v

As an example, if a sample volume of 1 mL is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per liter are:

c	10	100	1000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per liter, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus, and the test is negative, the amount of virus that would be present in the total sample to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical because of material limitations.

APPENDIX C: CALCULATION OF REDUCTION FACTORS IN STUDIES TO DETERMINE VIRAL CLEARANCE (ANNEX 3)

The virus reduction factor of an individual removal or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the preprocessed material and the virus load in the postprocessed material that is ready for use in the next step of the process. If the following abbreviations are used:

Starting material:

Vol v'; titer $10^{a'}$; Virus load: (v')($10^{a'}$), Final material:

Vol v"; titer $10^{a"}$; Virus load: (v")($10^{a"}$),

The individual reduction factors Ri are calculated according to 10^{Ri} = (v')($10^{a'}$) / (v")($10^{a"}$)

This formula takes into account both the titers and volumes of the materials before and after the process step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than one.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale, which implies that, although residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

APPENDIX D: CALCULATION OF ESTIMATED PARTICLES PER DOSE (ANNEX 4)

This appendix is applicable to those viruses, such as endogenous retroviruses, for which an estimate of starting numbers can be made.

Example:

1) Assumptions

Measured or estimated concentration of virus entering the purification process (e.g., virus concentration in cell culture harvest) = 10^6/ (milliliter) mL

Calculated viral clearance factor = $>10^{15}$

Volume of culture harvest used to make a dose of product = 1 liter (10^3mL)

2) Calculation of Estimated Particles/Dose

$$\frac{(10^6 \text{ virus units/mL}) \times (10^3 \text{mL/dose})}{\text{Clearance factor } >10^{15}}$$

$$= 10^9 \text{ particles/dose}$$

$$\text{Clearance factor } >10^{15}$$

$$= <10^{-6} \text{ particles/dose}$$

Therefore, less than one particle per million doses would be expected.

The case above is typical for the reduction of endogenous retroviruses during the manufacture of monoclonal antibodies (mAbs) or other recombinant proteins from rodent cells (Case B). In a comprehensive risk assessment for a specific virus, additional factors should be considered, such as the host range of the virus, the infectivity and pathogenicity of the virus, measures to avoid contamination, testing, the route of administration, and the human infectious dose.

In the Case B scenario for Chinese hamster ovary (CHO) cells, since endogenous retroviruses have been extensively characterized as noninfectious, a value of less than or equal to 10^{-4} particles/dose is considered acceptable for retroviral-like particles for mAbs or other recombinant proteins if testing fails to identify the presence of infectious retroviruses.

APPENDIX E: EXAMPLES OF PRIOR KNOWLEDGE INCLUDING IN-HOUSE EXPERIENCE TO REDUCE PRODUCT-SPECIFIC VALIDATION EFFORT (ANNEX 5)

A. Introduction (Annex 5.1)

According to the general principles for a platform validation approach, robust viral clearance should be demonstrated across products from the same platform, and the procedure for viral clearance should follow established and well-characterized conditions. In addition, it should be shown that the composition of the process intermediate is comparable to the intermediates used in viral clearance studies unless prior knowledge indicates robustness of viral clearance with respect to process intermediate composition.

In this context, as opposed to product-specific process validation, platform validation is defined as the use of prior knowledge including in-house (applicant-owned data) experience with viral clearance from other products, to claim a reduction factor for a new similar product. In general, viral clearance claims for a new product based on prior knowledge including in-house experience should include a discussion of all relevant platform data available and the rationale to support the platform validation approach (see section VI.F (6.6) of the main guidance). Part of the prior knowledge and in-house data used to reduce product-specific validation could be provided as a comparison of the new product and its manufacturing process with other in-house products, related process conditions, and process intermediates.

Process steps dedicated to viral clearance (e.g., inactivation by detergent, low pH and removal by virus filtration) are suitable for a platform validation approach.

Therefore, examples for application of prior knowledge to xenotropic murine leukemia virus (XMLV) inactivation by detergent and incubation at low pH, as well as virus removal by virus filtration are given below.

These examples are provided for illustrative purposes, only suggesting how the platform validation approach could be applied, and should not be used as a template or the sole basis for a regulatory submission.

Tables A-2 to A-4 summarize process parameters and their potential criticality for the individual process step according to the current understanding of a wide range of process conditions applied across industry. The actual effect of process parameters and intermediates on viral clearance should be assessed by prior knowledge and in-house experience.

Based on evolving process understanding, further process steps can be considered for platform validation in the future.

B. Inactivation by Solvent/Detergent or Detergent Alone (Annex 5.2)

Based on the mechanism of action, detergent concentration of solvent/detergent (SD) reagents or detergent alone is an important process parameter.

In addition, hydrophobic impurities such as lipids, cell debris, or components of cell culture media such as antifoaming agents can affect virus inactivation by challenging the detergent or SD mixture in solubilizing the virus lipid envelope and, therefore, should be assessed.

There is, so far, no indication that the interaction between virus and a specific therapeutic protein affects inactivation by detergent. Aggregates (e.g., cell debris, aggregated virus particles) can potentially entrap and protect viral particles from detergent access. Therefore, at manufacture, the product intermediate (e.g., harvested cell culture fluid (HCCF)) should be clarified from cells/cellular debris including a filtration step of less than or equal to 0.2 micrometer (µm) nominal pore size before detergent inactivation.

The following paragraph describes how to apply a platform validation approach to XMLV inactivation using SD or Triton X-100 as an example. The approach may also be applicable to alternate detergents shown to provide robust and efficient XMLV inactivation.

Triton X-100 is a nonionic detergent commonly used in membrane research to solubilize lipid bilayers. It inactivates enveloped viruses by solubilizing the virus lipid envelope thus rendering the virus noninfectious. Triton X-100 has been widely used for viral inactivation in manufacturing processes of plasma derived products for many years as well as in platform purification processes for monoclonal antibodies (mAbs) by addition to HCCF.

The European Chemicals Agency included Triton X-100 in the Authorisation List (at Annex XIV) because of the hormone-like activity of degradation compounds in the environment. Therefore, though widely used, the pharmaceutical industry is looking into alternate detergents. Other detergents with similar physicochemical properties are commercially available and achieve efficient XMLV inactivation. Inactivation by Triton X-100 is included as an example in this guidance because it is well characterized and a critical mass of knowledge regarding its effectiveness exists.

Because of the nonionic nature of Triton X-100, its effectiveness should not be sensitive to pH, to ionic strength, or to the nature of the counter ions in HCCF. Prior experience indicates effective XMLV inactivation in HCCF at 0.2 percent Triton X-100 concentration, at 15°C, and at 60-minute incubation across multiple products from platform processes covering a range of typical lipid and total protein content in HCCF. However, as indicated below, applying a Triton X-100 concentration of 0.5 percent is recommended to ensure effective and reliable inactivation when omitting product-specific experiments.

Table A-2 summarizes process parameters and their potential criticality for detergent-based inactivation of lipid-enveloped virus using Triton X-100 or SD reagents and murine leukemia virus (MLV) as an example.

Table A-2: Summary of Process Parameters or Factors and Their Potential Effect for Detergent Inactivation or SD Treatment

Process Parameter or Factor	*Potential Effect*	*Rationale*
SD or Triton X-100 concentration	high	Inactivating agent
Incubation time	high	Mechanism of inactivation is time-dependent
Temperature	high	Effect on inactivation kinetics
Pretreatment by ≤ 0.2 µm filtration	high	Removal from the starting intermediate of aggregates potentially entrapping and protecting viral particles from detergent access
Total lipid content or surrogate parameter in HCCF	low	Low effect observed with worst-case HCCF
Type of product	low	No effect on inactivation observed for mAbs, half antibody, fusion protein or recombinant protein
Total protein content	low	Low effect observed
pH	low	Triton X-100 is a nonionic detergent
Ionic strength	low	Triton X-100 is a nonionic detergent
Buffer salt in HCCF	low	Triton X-100 is a nonionic detergent
Potential interaction between virus particle and product	low	No effect on inactivation observed and disruption of lipid envelope lowers probability of interaction with product

SD = solvent/detergent; µm = micrometer; HCCF = harvested cell culture fluid; mAbs = monoclonal antibodies.

Thus, consistent with current process understanding for using Triton X-100 with clarified HCCF, at a concentration of greater than or equal to 0.5 percent Triton X-100 for greater than or equal to 60 minutes at greater than or equal to 15°C effectively inactivates XMLV for multiple cell culture-derived products. When using SD reagents, treatment with 1 percent Triton X-100 and 0.3 percent Tri-N-Butylphosphate for greater than or equal to 30 minutes or treatment with 1 percent polysorbate 80 and 0.3 percent Tri-N-Butylphosphate for greater than or equal to 6 hours at greater than or equal to 23°C effectively inactivates retroviruses. According to current process understanding, a platform validation approach can be applied for XMLV inactivation by SD treatment or treatment with Triton X-100 alone.

C. Incubation at Low pH (Annex 5.3)

Low pH treatment inactivates enveloped viruses by denaturing proteins located in the viral envelope, thus preventing the adsorption to and infection of cells. Low pH treatment of the capture chromatography product pool has been widely used for retrovirus inactivation in manufacturing processes of cell culture-derived products such as mAbs.

Inactivation efficiency depends on the concentration of hydrogen ions as the inactivating agent, measured as pH, incubation time and temperature, and buffer matrix. Extremely high ionic strength may affect inactivation efficiency as well.

Table A-3 summarizes process parameters and their potential effect on low pH inactivation of XMLV.

Table A-3: Summary of Process Parameters or Factors and Their Potential Effect for Low pH Inactivation and Effect on XMLV

Process Parameter or Factor	*Potential Effect*	*Rationale*
pH	high	Inactivating agent
Incubation time	high	Mechanism of inactivation is time-dependent
Temperature	high	Effect on inactivation kinetics
Buffer matrix	high	Available data show that inactivation robustness depends on buffer matrix
Product concentration	low	No effect observed under the conditions outlined below
Formation of protein aggregates	low	No effect observed under the conditions outlined below
Type of product	low	No effect on inactivation observed for mAbs, half antibody, bispecific antibody, fusion protein or recombinant protein
NaCl concentration[a]	low	No effect if ≤ 500 mmol/L sodium chloride
Potential interaction between virus particle and product	low	No effect on inactivation observed

a. To date, data on influence of ionic strength of other buffers is limited.
mAbs = monoclonal antibodies; NaCl = sodium chloride; mmol = millimole; L = liter.

Consistent with current process understanding, low pH treatment at less than or equal to pH 3.6, greater than or equal to 15°C for greater than or equal to 30 minutes at less than or equal to 500 millimoles/liter sodium chloride concentration is effectively inactivating XMLV. Acetate and citrate buffer are most commonly used and allow for robust XMLV inactivation.

According to the current process understanding, a platform validation approach can be applied for XMLV inactivation by low pH treatment.

D. Virus Filtration (Annex 5.4)

The primary mechanism of action of virus filtration is size-based particle removal. In general, volumetric throughput of the product intermediate as well as the volumetric throughput of the buffer used for flushing filters and pressure including pressure/flow interruptions are potentially critical parameters in virus filtration.

A potential interaction of virus particles with the product is not critical when the virus particle size is much larger than the distribution of filter pore size. However, when the virus particle size and pore size are similar, the influence of the potential interaction on virus retention is not fully understood.

The rest of this section focuses on using prior knowledge and in-house experience in virus filtration of other products to claim large virus removal by small and large virus-retentive filters. Prior knowledge can be used for both large and small virus-retentive filters, but this section focuses on small virus-retentive filters because they are more commonly used.

Factors that affect efficient retrovirus removal by small virus-retentive filters are well understood with respect to variation of process parameters such as filter type (model and characteristics), flow- or pressure-controlled filtration mode, tangential or dead-end flow filtration, and pressure interruptions. Based on predictability and robustness of virus removal, this process step is considered suitable for a platform validation approach.

For virus removal using small virus-retentive filters, one option is to apply parvovirus reduction factors for larger spherical/icosahedral viruses and enveloped viruses thus avoiding the need to perform validation studies with larger viruses. However, sometimes this could result in underestimating virus removal capacity (e.g., large virus removal capacity) as a result of parvovirus passage. Given the size-based mechanism of action, and industry's experience of robust complete retrovirus removal with small virus-retentive filters, companies could use their in-house data from parvovirus and large virus removal to build a platform large viral clearance claim for commonly used small virus-retentive filters.

According to the size-based removal mechanism, the theoretical risk of virus passage through a small virus-retentive filter is higher for small viruses than for large viruses.

A thorough understanding of the effect of pressure/flow interruptions, as well as volume throughput and postfiltration flush volume reflecting manufacturing conditions should be in place. It should be considered that there may be a limited negative impact of low pressure/flow or pressure/flow interruptions on specific virus filter types.

If using prior knowledge and in-house experience from other products to claim parvovirus removal, at least one confirmatory product-specific validation run using a parvovirus should be performed considering worst-case conditions (see Table A-4). The brand/model of virus-retentive filter is important for virus reduction and its robustness with respect to the effect of process parameters and should be considered when designing platform data.

Table A-4 summarizes process parameters and their potential effect on parvovirus retention using small virus-retentive filters.

Table A-4. Summary of Process Parameters or Factors and Their Potential Effect for Parvoviral Clearance by Small Virus-Retentive Filters

Process Parameter or Factor	*Potential Effect*	*Rationale*
Volumetric throughput of product intermediate loaded onto the virus filter	high	High volumetric/protein throughput is considered worst-case with effects varying for specific membrane types.
Volumetric throughput of the buffer used for flushing filters	high	High throughput is considered worst-case with effects varying for specific membrane types.
Pressure/flow	high	Pressure/flow should not exceed the upper limit for filter operation. Low pressure/flow can be worse case for a specific membrane type. Pressure/flow interruption (if occurring during filtration or at switching from filtration of product intermediate to filter flush) should be considered.
Type of product	low	No effects on viral clearance observed for mAbs, half antibody, bispecific antibody, fusion protein, or recombinant protein
Product concentration	low	No negative effects on viral clearance observed.
pH	low	No negative effects on viral clearance caused by size-based removal. May have limited negative effects at lower pH values (< pH 5) with varying effects for specific membrane types.
Ionic strength	low	Limited effects on viral clearance have been observed.
Buffer matrix	low	Limited effects on viral clearance have been observed.
Potential interaction between virus particle and product	low	Specific interaction between virus and antibody can enhance virus retention.

APPENDIX F: GENETICALLY ENGINEERED VIRAL VECTORS AND VIRAL VECTOR-DERIVED PRODUCTS (ANNEX 6)

A. Introduction (Annex 6.1)

Advances in biotechnology have led to the emergence of new and advanced production platforms expressing new product types manufactured using characterized cell banks of human or animal origin (i.e., avian, mammalian, or insect). The scope of Appendix F (Annex 6) includes genetically engineered viral vectors and viral vector-derived products that can be produced using helper virus and viral vectors for protein expression (collectively referred to as production viruses) or from stable or transient transfected cell lines. The products included here are those amenable to viral clearance based on the physicochemical properties of the product. These products include protein subunits and virus-like particles (VLPs) that are produced using baculovirus/insect cells, nanoparticle-based protein vaccines, and viral vector products such as adeno-associated virus (AAV). These vectors can be applied in vivo or ex vivo.

Helper virus independent products can be manufactured using stable or transient transfected cell lines or by infection with a viral vector for expression of recombinant protein or VLP (e.g., recombinant baculovirus). Helper virus dependent products require a helper virus to enable expression of the product or replication of the viral vector (e.g., AAVs that require a virus such as herpes simplex virus or adenovirus).

The potential sources for virus contamination for a biopharmaceutical product are described in section II (2) of the main guidance. Additional viral contamination risks such as those introduced by the expression system and the potential for contamination with replication competent virus should be considered. The susceptibility of the cell substrate to adventitious viruses should be carefully considered when assessing the potential for extrinsic contamination during product manufacture. The use of well-characterized cell banks and virus seeds can reduce the risk of virus contamination. Production viruses are considered process-related impurities.

Viral safety and contamination controls of new product types should be assured through the application of a comprehensive program of material sourcing, virus testing at appropriate steps of the manufacturing process, and removal and/or inactivation of adventitious viruses and production viruses by the manufacturing process. If viral clearance is limited, viral safety should focus on the testing and control of the raw materials and reagents and the manufacturing process.

Accordingly, a risk-based approach should be applied for demonstrating viral safety of the product.

B. Testing for Viruses (Annex 6.2)

Extensive testing and characterization for both endogenous viruses and adventitious viruses should be performed at suitable stages of manufacturing to support the overall product safety. Based on the product type and its associated risk factors, the testing scheme should apply across the product life cycle. Table A-5 below provides an outline of the tests to be performed at various stages during production. The tests applied for virus seeds, unprocessed bulk (harvest), and purified bulk/drug substance are described. The testing and characterization

schemes for the cell substrates used for viral vector production are referred to in Table 1 in the main guidance; additional considerations that may apply for these product types are also indicated in Table A-5. In cases where the producer cells cannot survive the production process, limit of in vitro cell age (LIVCA) applies to untransfected cells.

The type and extent of testing depends on a risk assessment considering the specific risk factors associated with the cell substrate and the manufacturing process. Factors that should be considered include the origin, passage history and characteristics of the cell substrate and viral seeds/vector, the source and production of the raw materials and reagents, and cell culture process used, the use of production viruses, and the capacity of the manufacturing process to inactivate and/or remove viruses.

Table A-5: Virus Tests That Should Be Performed at Applicable Stages

Test	MCB, WCB, Cells at the LIVCA	Virus Seeds[i]	Unprocessed Bulk (harvest)	Drug Substance (purified bulk)
Test for Adventitious Viruses				
In vitro assays or NGS [a,b]	See Table 1 of main guidance	+[g]	+[g]	-
In vivo assays or NGS[b]		+[g]	-[g, j]	-
Tests for specific virus[c]		+[h]	+	-
Test for Retrovirus and Endogenous Virus, Production Virus and Replication Competent Virus, As Applicable				
Retrovirus and endogenous virus	See Table 1 of main guidance	+	-[d, j]	-
Production virus	-	-	+[e]	+[e]
Replication competent virus	-	+[f]	+[f]	+[f]

MCB = master cell bank; WCB = working cell bank; LIVCA = limit of in vitro cell age; NGS = next generation sequencing.

a. A 28-day testing on permissive cells for MCB, WCB, LIVCA, MVS, and WVS should be performed with at least one subpassage at 2 weeks. For the unprocessed bulk, this can be reduced with justification to 14 days for products based on a risk assessment (considering the cell substrate, cultivation period for production, use of animal-derived raw materials or reagents, and level of viral clearance of the process). The indicator cell cultures should be monitored for cytopathic viruses, hemadsorbing and hemagglutinating viruses, consistent with existing regional regulations and guidance. For products produced in insect cell lines, the testing should include a permissive cell line for arboviruses (e.g., BHK cells). NGS can be used as an alternative assay and can be particularly useful if the viral vector or viral vector-derived product cannot be neutralized. Testing should be performed on the virus seed and the unprocessed bulk harvest. In some cases, the unprocessed bulk harvest may be the same as the drug substance.
b. Nontargeted NGS can replace the in vivo assay (see section III.B.3 (3.2.3) of the main guidance) and supplement or replace the in vitro assay (see section III.B.2 (3.2.2) of the main guidance).
c. Testing is based on risk assessment including the origin and history of the cell substrate, derivation of the virus seeds, and potential exposure to human- or animal-derived raw materials. Methods such as cell culture-based infectivity assays, antibody production tests (MAP, HAP, RAP), virus specific NAT or other molecular methods (e.g., NGS) can be used. Refer to section III.B.4 (3.2.4) of the main guidance for details. This can include testing for species-specific viruses, e.g., arboviruses in insect cells, and bovine or porcine viruses if serum or trypsin are used, respectively.
d. If either the MCB or virus seeds are positive for retrovirus, follow-up should include quantification of potential retroviral particles in the unprocessed bulk harvest from at least three lots/batches to determine the target level for viral clearance. For the MVS, if there is remaining residual risk, then testing of the unprocessed bulk harvest can be considered.

e. Quantification of the production virus should be performed at the unprocessed bulk stage using at least three cell culture lots/batches to determine the target for viral clearance. Absence of production virus should be demonstrated in the drug substance (purified bulk) using an infectivity assay with *relevant* permissive cell lines for sensitive virus detection. Alternatively molecular methods can be used for testing. Testing for the absence of residual production virus should be performed for each purified bulk, unless justified by robust excess clearance (see Case F, Table 4, in the main guidance).
f. Replication competent virus (RCV) may develop at any step during manufacturing. Recommendations include testing for RCV at multiple stages of manufacture. RCV testing is performed on the virus seed/bank and on each unprocessed bulk harvest or on each drug substance or drug product as applicable for maximum detection.
g. When interference may occur with the in vitro or in vivo assays, control cells cultured in parallel are tested at the virus seed and/or unprocessed bulk harvest stages. When NGS is used for testing the virus seeds and unprocessed bulk, testing of the control cell cultures is not needed.
h. Testing should be performed on the virus seeds if the cell bank has not been tested.
i. Testing should be applied on the virus seeds. Depending on the product type, the virus seed can be used to manufacture a vaccine virus, viral vector, or helper virus. The virus seeds are generated from an established cell line. Consistent with a risk-based approach, the virus testing should consider the origin of the cell line and raw materials and reagents used for preparation of the virus seeds, to ensure the absence of adventitious virus and the absence of replication competent virus. Because the WVS is derived directly from the MVS, a subset of adventitious virus testing applies based on a risk assessment. An alternative approach in which complete testing is carried out on each WVS rather than on the MVS could also be acceptable.
j. Testing based on risk assessment.

C. Viral Clearance (Annex 6.3)

The risk of contamination with adventitious viruses, endogenous viruses, and residual production viruses should be mitigated following the general principles of this guidance to the extent possible. Some viral vector products such as AAVs are amenable to viral clearance steps, assuring adventitious and production viral clearance (inactivation or removal).

The viral clearance should be evaluated using representative and qualified scale-down models. The physicochemical characteristics of the viral vector and the viral vector-derived product will determine how viral clearance will apply within the downstream process. Virus-clearance studies should include the production virus itself or a specific *model* virus (e.g., baculovirus, adenovirus, herpesvirus) and model viruses representative of adventitious and endogenous viruses (see Table A-1 in Appendix A (Annex 1)). Refer to sections V (5) and (6) of the main guidance for an action plan for the selection of specific and nonspecific model viruses described in Table 4 of the main guidance. The manufacturing processes should have robust clearance of the viral vector for protein expression or helper virus. Common virus inactivation steps such as treatment with detergent or solvent/detergent (SD) may be suitable, when the product is compatible, such as for nonenveloped viral vectors. Alternatively, virus filtration may be more suitable for small viral vectors such as AAV or nanoparticle-based vaccines when virus removal can be based on the size exclusion. Chromatography steps can provide viral clearance for viruses with different surface properties from the viral vector. When appropriate, viral clearance studies should be performed to determine virus reduction factors for the relevant step(s) of the production process. Examples include manufacture of subunit proteins and VLPs that are produced using baculovirus/insect cells, and which can be purified, and for which viral clearance can be achieved through the manufacturing process.

The viral safety of these products may also rely on closed processing, testing, and other preventative controls (see sections II.B.3 (2.2, 3) and IV (4) of the main guidance). Viral clearance steps during production may not achieve the same robustness as for recombinant proteins; therefore, viral safety of the products should be supported by a risk assessment.