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INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED GUIDELINE

VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

Q5A(R2)

Draft version

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ICH HARMONISED GUIDELINE

VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

Q5A(R2)

ICH Consensus Guideline

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1. INTRODUCTION

This guideline concerns the testing and evaluation of the viral safety of biotechnology products, and it outlines what data should be submitted in marketing application and registration packages for those products. Biotechnology products include biotherapeutics and certain biological products derived from cell cultures initiated from characterised cell banks of human or animal origin (e.g., mammalian, avian, insect). In this document, the term "virus" excludes non-conventional transmissible agents like those associated with mammalian prions (e.g., bovine spongiform encephalopathy, scrapie). Applicants are encouraged to discuss bovine spongiform encephalopathy-associated issues with the appropriate regulatory authorities.

This document covers products produced from *in vitro* cell culture using recombinant DNA technologies such as interferons, monoclonal antibodies, and recombinant subunit vaccines. It also covers products derived from hybridoma cells grown *in vivo* as ascites: special considerations apply for these products, and Annex 1 contains additional information on testing cells propagated *in vivo*. The document also applies to certain genetically-engineered viral vectors and viral vector-derived products, which can undergo virus clearance without a negative impact on the product. These products may include viral vectors produced using transient transfection or from a stable cell line, or by infection using a recombinant virus. It also includes viral vector-derived recombinant proteins, for example, baculovirus-expressed Virus-Like Particles (VLPs), protein subunits and nanoparticle-based vaccines and therapeutics. Furthermore, the scope includes Adeno-Associated Virus (AAV) gene therapy vectors that depend on helper viruses such as baculovirus, herpes simplex virus or adenovirus for their production. Specific guidance on genetically engineered viral vectors and viral vector-derived products is provided in Annex 7. Inactivated viral vaccines and live attenuated viral vaccines containing self-replicating agents are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from exogenous introduction of adventitious virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, the safety of these products with regards to viral contamination can be reasonably ensured only 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 have evolved 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 infectious viruses; and
- Testing the product at appropriate steps of production for the absence of contaminating infectious viruses.

Some virus clearance steps used during production of genetically engineered viral vectors and viral vector-derived products may not be as effective as when used for recombinant proteins. In such cases, considerations for further risk reduction (e.g., treatment of raw materials, extensive testing for broad virus detection) can be applied (see Annex 7).

For statistical reasons, a quantitative virus assay's ability to detect low viral concentrations depends on sample size. Therefore, establishing that an infectious virus contaminant is absent from a product will depend not just on direct testing for the presence of a contaminant, but also on demonstrating that the purification regimen can remove or inactivate the viruses.

The type and extent of viral tests and viral clearance studies required 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 extent of cell bank characterisation and qualification; 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 purpose of this document is to provide a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers to ensure viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful to regulatory reviewers. This summary should

contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination.

2. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Virus contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes, including generation of a recombinant production cell line and/or cell line banking. Introduction of potential adventitious viruses from a Master Virus Seed (MVS) or Working Virus Seed (WVS) is discussed in Annex 7. Use of well characterised banks and MVS or WVS can reduce the risk of virus contamination. Furthermore, helper viruses used for the production of recombinant proteins, VLPs, or gene therapy viral vector products are also considered as process-related viral contaminants (see Annex 7).

2.1 Viruses that Could Occur in the Master Cell Bank

Cells may have latent or persistent virus (e.g., herpesvirus) and endogenous retrovirus, and those viruses can be transmitted vertically from one cell generation to the next. In such cases, the virus may be constitutively expressed or may unexpectedly become expressed as an infectious virus.

Viruses may 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 banking processes.

2.2 Adventitious Viruses that Could be Introduced During Production

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 virus or viral vector (including helper viruses used in their production) to induce expression of specific genes encoding a desired protein (see Annex 7), 3) the use of a contaminated raw material or reagent used during downstream purification, such as a monoclonal antibody coupled affinity resin for product selection or purification, 4) the use of a contaminated excipient during formulation, and 5) contamination from the environment, including storage of non-biological raw materials or

during cell culture and medium handling.

Monitoring cell culture parameters can 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 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.

When possible, cell culture media or media supplement treatments such as gamma irradiation, virus filtration, high temperature short time processing, or ultraviolet C irradiation can be used as additional virus risk mitigation measures.

3. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

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.

3.1 Suggested Virus Tests for Master Cell Bank, Working Cell Bank, and Cells at the Limit of *In Vitro* Cell Age Used for Production

Table 1 shows an example of virus tests to be performed only once at various cell levels, including MCB, WCB, and cells at the Limit of *In Vitro* Cell Age (LIVCA) that are used for production.

3.1.1 Master Cell Bank

Extensive screening for both endogenous and adventitious viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or non-human primate in origin, tests should be performed to detect viruses of human or non-human primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for adventitious viruses should include both broad and specific virus detection assays as described in Table 1. Introduction of new methodologies for detecting a broad range of adventitious viruses is encouraged. To ensure detection of contaminating viruses, the testing approach should be based on the origin and history of the cell line and the potential exposure to materials of human or animal origin during cell line generation and MCB expansion.

3.1.2 Working Cell Bank

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 used to test for the presence of adventitious viruses then similar tests may be omitted on the initial WCB. Antibody production tests are usually not recommended for the WCB. An alternative approach in which complete testing is carried out on each WCB rather than on the MCB would also be acceptable.

3.1.3 Cells at the Limit of In Vitro Cell Age Used for Production

The LIVCA established for production should be based on data derived from production cells expanded under pilot plant scale or commercial scale conditions to the proposed *in vitro* cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the LIVCA should be evaluated once for those endogenous viruses that may have been undetected in the MCB. Cells at the LIVCA are also referred to as end of production cells. The performance of suitable tests (as outlined in Table 1) at least once on cells at the LIVCA used for production would provide further assurance that the production process does not lead to activation of endogenous viruses or amplification of adventitious viruses, including slow-growing viruses. If any adventitious viruses are detected at this stage, the process should be checked carefully to determine the source of the contamination.

3.2 Recommended Virus Detection and Identification Assays

A number of assays can detect endogenous and adventitious viruses. Table 2 lists examples of such assays. These assays are recommended, but the list is not all-inclusive nor definitive. The most appropriate techniques may change with scientific progress; proposals for alternative techniques should be accompanied by adequate supporting data. Manufacturers are encouraged to discuss these alternatives with the appropriate regulatory authorities. A comprehensive testing strategy 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. The strategy should include additional assays as appropriate based on risk assessments of the cell substrate, raw materials, and reagents used. 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.

Next Generation Sequencing (NGS) and Nucleic Acid Amplification Techniques (NATs) such as Polymerase Chain Reaction (PCR) may be appropriate for broad and specific virus detection, respectively. The introduction of these tests may be done without a systematic head-to-head comparison with the currently recommended *in vitro* and *in vivo* assays. In particular, a head-to-head comparison is not recommended for *in vivo* assays to meet the intent of the global objective to replace, remove, and refine the use of animals. Because of the assay sensitivity and breadth of virus detection, NGS may also be used to replace cell-based infectivity assays, to overcome potential assay limitations, or to detect viruses without visible phenotypes in the assay system. Positive results should be investigated to determine whether detected nucleic acids are associated with an infectious virus.

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

3.2.1 Tests for Retroviruses

Tests for retroviruses should be performed for the MCB and for cells cultured up to or beyond the LIVCA used for production. These tests include infectivity assays by direct inoculation or co-cultivation, assays for Reverse Transcriptase (RT) activity, and evaluation of particles by Transmission Electron Microscopy (TEM).

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

If a cell line is known to constitutively produce retroviral particles (as occurs in some cell lines derived from rodent, insect, and avian species), RT activity is expected and therefore a PCR-based RT assay may not be needed. TEM should be performed to examine the type of retroviral

particles (e.g., type-A and 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* and SC-1 cells for rodent retroviruses) with sensitive readout assays for retrovirus detection (e.g., a product-enhanced Reverse Transcriptase (RT) assay, a Sarcoma-Positive, Leukemia-Negative (S^+L^-) assay, or an XC plaque assay or a broad molecular assay).

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 Section 3.3 and Section 5.

Induction studies have not been found to be useful for cell lines that have been well characterised for endogenous retroviruses (e.g., Chinese Hamster Ovary (CHO), NS0, and Sp2/0). However, such studies may help to evaluate a new cell substrate for the presence of unknown endogenous retroviruses. 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.

3.2.2 In Vitro Cell Culture Infectivity Assays

In vitro tests are carried out by inoculating a test article (see Table 2) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. 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 panel of cell lines should include a cell line of the species of origin and a human and a non-human primate cell line susceptible to human viruses.

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. For cell line qualification, the test should be performed as a 14-day initial cell culture followed by a secondary passage with a 14-day duration followed by observation for both cytopathogenic and hemadsorbing/hemagglutinating viruses.

Alternatively, molecular virus detection methods may be used to supplement (e.g., when required to address certain limitations such as test article-mediated interference or toxicity) or replace the cell culture assays.

3.2.3 In Vivo Assays

NGS is encouraged as a replacement for *in vivo* assays because of the breadth of viruses it detects and because its use promotes the global objective to replace, reduce, and refine the use of animal testing. Use of NGS to replace *in vivo* assays may be justified by submitting a validation package. Based on risk assessment and on the overall testing strategy, the use of the *in vivo* assay may include inoculation of test article (see Table 2) into suckling mice, adult mice, and embryonated eggs. Additional animal species may be used depending on the nature and source of the cell lines being tested. The health of the animals should be monitored, and any abnormality should be investigated to establish the cause.

3.2.4 Antibody Production Tests

Antibody production 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 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 (SPF) 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.

Virus-specific PCR or targeted molecular methods can be used as a replacement assay for the animal testing described in Table 3.

3.2.5 Molecular Methods

Molecular methods can be used to supplement or replace *in vitro* cell culture-based and *in vivo* animal assays.

3.2.5.1 Nucleic Acid Amplification Techniques

Nucleic Acid Amplification Techniques (NATs) such as PCR-based methods are typically used singly or in a multiplex format to detect virus sequences from known viruses or known closely related virus families. Targeted NGS methods may also apply for sensitive detection of known viruses. These molecular methods can be used to supplement cell culture assays when there are limitations as a result 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. NAT methods also have the capacity to be adapted for more broad range virus detection

(e.g., degenerate PCR), but specificity may be reduced. Because of the assay specificity, multiple virus-specific PCR assays may be needed to detect the breadth of viruses that would be detected by a single more general biological assay. NAT assays should be appropriately qualified or validated for their intended use.

3.2.5.2 Next Generation Sequencing

New advanced molecular methods such as NGS (also known as high-throughput sequencing) are 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. For any NGS method used, a validation package should be provided to support its use for the application. This includes the method validation and assay or matrix-specific qualification, as suitable. Based on the potential safety concerns, the bioinformatic analysis can be targeted to specific viruses or can be agnostic for broad virus detection. NGS can replace the *in vivo* tests with broad virus detection for unknown or unexpected virus species. NGS can also supplement or replace the *in vitro* cell culture assays for detection of known and unknown or unexpected virus species. Furthermore, the assay may also be used for the detection of known viruses, and it can replace the HAP, MAP, and RAP tests and other virus-specific PCR assays.

Use of NGS should be considered particularly for characterisation or testing of a cell substrate and cell bank, for detection of known and unknown viruses, and in a viral seed or harvest if there is assay interference as a result of lack of effective neutralisation of the vector virus (see Annex 7) or toxicity due to the product or media components. In such applications, NGS can be used to detect viral sequences present in the cell DNA (genomics) or expressed as RNA in cells (transcriptomics), or it can be used to detect viral genome present in particles (viromics). The rationale for selecting these different strategies should be provided.

When applying NGS for sensitive detection of known viruses and/or broad detection of novel viruses, applicants should consider several critical steps in the NGS workflow. These include 1) sample treatment (when performed) and processing based on the type of sample material, 2) efficient viral nucleic acid extraction (including enveloped and nonenveloped particles) and library preparation, 3) selection of a suitable sequencing platform, and 4) comprehensive bioinformatics analysis against a database with diverse representation of viral sequences of different viral families. Steps for sample treatment and processing can be carried out to maximize virus detection.

Suitable standards or reference materials should be used for assay qualification and validation to evaluate performance of the different steps involved in the methodology and to demonstrate sensitivity, specificity, and breadth of virus detection. This can include using currently available reference virus reagents with distinct physical (size, enveloped and non-enveloped), 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. Furthermore, other standard types may be used to evaluate the specific technical and bioinformatic steps. Since NGS has a complex workflow, manufacturers are encouraged to have discussions with the appropriate regulatory authorities regarding expectations for method validation and data submission.

3.3 Acceptability of Cell Lines

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 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 risk-benefit 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.

4. TESTING FOR VIRUSES IN UNPROCESSED BULK

It is recommended that manufacturers develop programs to continuously assess adventitious viruses in production batches. The scope and extent of virus testing on the unprocessed bulk should be determined by considering 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.

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. 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. Appropriate testing for viruses should be performed on the unprocessed bulk. For perfusion or continuous manufacturing processes, cells may not be readily accessible (e.g., due to use of hollow fiber or similar microfiltration systems). In such cases, the unprocessed bulk would constitute fluids harvested from the bioreactor. The potential influence of cell separation technology and progressive filter fouling on the representativeness of these unprocessed bulk test samples should be considered. If unprocessed bulk is toxic in test cell cultures, initial partial processing (e.g., minimal sample dilution or alternative testing assays) can be considered (see Section 3.2). In certain instances, it may be more appropriate to test a mixture of both intact and disrupted cells and their cell culture supernatants that were removed from the production reactor before further processing. For processes that involve continuous harvest, the sampling strategy (including periodicity and composition of the samples) should be justified because adventitious viruses and endogenous virus particles can variate along the cell culture duration (see Section 7).

Adventitious virus testing should be routinely applied to each unprocessed bulk. This may include *in vitro* screening assays using several cell lines or broad molecular virus detection methods such as NGS (see Section 3.2). Based on the risk assessment (considering the cell substrate, use of animal-derived raw materials or reagents, and level of virus clearance of the process), the indicator cell cultures should be observed for at least 2 weeks. Detection for specific viruses or families of viruses may also be appropriate to include based on risk assessment (e.g., Minute virus of mice). When appropriate, a PCR or other molecular method may also be selected as rapid test methods can facilitate real-time decision making.

If any adventitious viruses are detected at the unprocessed bulk stage, the harvest should not be used for product manufacture unless justified. (See Section 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 continuous manufacturing processes, release of a final sublot requires documented absence of viral contamination for the period during which cultivation fluids were harvested for manufacture of that sublot. If an adventitious virus is detected, a procedure to segregate potentially contaminated material should be considered to mitigate wider production impact.

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

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 characterisation of viral clearance from unprocessed bulk. The evaluation and characterisation of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterising the clearance of non-specific "model" viruses (described later). Definitions of relevant, specific, and non-specific "model" viruses are given in the glossary. Process evaluation requires 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 helpful in ensuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth time-dependent inactivation studies, demonstration of reproducibility of inactivation or removal, and evaluation of process parameters should be performed. When a manufacturing process is characterised for robustness of clearance using non-specific "model" viruses, particular attention should be paid to non-enveloped viruses in the study design. The extent of viral clearance in characterisation 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 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 characterisation of viral clearance and virus tests on purified bulk. Various cases are presented in the table and are described below. In all cases, characterisation of clearance using non-specific "model" viruses should be performed. The most common situations are Cases A and B. 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 or remove the virus in question from the manufacturing process.

Case A: When no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or the unprocessed bulk, virus removal and inactivation studies should be performed with

non-specific "model" viruses, as previously stated.

Case B: In rodent cell lines, if only a rodent retrovirus (or a retrovirus-like particle that is believed to be non-pathogenic, such as rodent A- and R-type particles) is present, the process evaluation using a specific "model" virus (such as a murine leukemia virus) should be performed. Purified bulk should be tested using suitable methods with high specificity and sensitivity for the detection of the virus in question. For marketing authorisation, data from at least 3 lots of purified bulk at pilot plant scale or commercial scale should be provided. Cell lines such as Chinese Hamster Ovary (CHO), C127, BHK and murine hybridoma cell lines 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 characterised and clearance has been demonstrated, it usually is not recommended to test for the presence of the non-infectious particles in the purified bulk or drug substance. Studies with non-specific "model" viruses, as in Case A, are appropriate. A similar approach may be relevant for insect cell lines (e.g., Sf9) that produce endogenous retroviral-like particles that have been extensively characterised.

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 (such as those identified in Table 3, footnote 2 except rodent retroviruses (Case B)), 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 authorisation, data from at least 3 lots of purified bulk manufactured at pilot plant scale or commercial scale should be provided.

Case D: If a known virus is infectious to humans (such as those viruses indicated in Table 3, footnote 1), is identified, the product should be 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 the purification and inactivation processes. 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 authorisation, data from at least 3 lots 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 helper virus is used in production, clearance of the virus should be demonstrated using the helper virus itself or a specific model virus (e.g., baculovirus, adenovirus, herpesvirus).

6. EVALUATION AND CHARACTERISATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterisation of the 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 to biological products derived from various source materials other than fully characterised 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.

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 a virus to the crude material or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not necessary to evaluate or characterise every step of a manufacturing process if adequate clearance is demonstrated by the use of 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 virus clearance. In general, in order to determine the amount of endogenous virus particles that enter the purification process, quantification should be performed on three cell cultures campaigns,

lots or batches. This data should be submitted as part of the marketing application or registration package.

The reduction of virus infectivity may 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 6.2.5).

Viral clearance evaluation studies are performed to 1) demonstrate the clearance of a virus known to be present in the MCB, or 2) ensure that adventitious viruses that could not be detected, or that might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale to show that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterise the ability to remove or inactivate other viruses should be conducted. The purpose of studies using viruses with a range of unknown or unexpected biochemical and biophysical properties is to characterise the robustness of the procedure 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 6.3). Such studies are not performed to evaluate a specific safety risk. Therefore, achieving a specific clearance value is not needed.

6.1 The Choice of Viruses for Evaluation and Characterisation of Virus Clearance

Viruses for clearance evaluation and process characterisation studies should be chosen to resemble viruses which 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 characterisation study provided in this guideline.

6.1.1 "Relevant" Viruses and "Model" Viruses

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) non-specific "model" viruses.

"Relevant" viruses are used in the process evaluation of viral clearance studies which are the

identified viruses or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The process for purification and/or inactivation 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 which 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 retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or non-infectious (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 a murine leukemia virus--a specific "model" virus in the case of cells of murine 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 or biochemical) should be qualified for its use. When human cell lines secreting monoclonal antibodies have been obtained by the immortalisation of B lymphocytes by Epstein-Barr Virus, the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterise the capacity of the manufacturing process to remove and/or inactivate viruses in general (i.e., to characterise the robustness of the clearance process), virus clearance characterisation studies should be performed with non-specific "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 characterisation of the cell lines and the production process.

Annex 2 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.

6.1.2 Other Considerations

Additional points to be considered:

- 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 every stage of manufacturing that is tested; and
- The health hazard that certain viruses may pose to the personnel performing the clearance studies should be considered.

6.2 Design and Implications of Virus Clearance Evaluation and Characterisation Studies

6.2.1 Facility and Staff

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 conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

6.2.2 Scaled-Down Production System

The validity of scaling down should be demonstrated. The level of purification of the scaled-down version should represent the production procedure as closely as possible. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Unavoidable deviations should be discussed with regard to their influence on the results.

6.2.3 Analysis of Step-Wise Elimination of Virus

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 and 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 in-process material at each step to be tested. In some cases, adding high titer virus to unpurified bulk and testing its concentration between steps is sufficient. 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 virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers, can be carried out as part of the overall process assessment. 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 may 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 Annex 3).

6.2.4 Determining Physical Removal versus Inactivation

Reduction in virus infectivity 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 or removal. If little clearance of infectivity is achieved by the production process and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. 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 removal achieved by each of these chromatographic steps should be distinguished.

6.2.5 Inactivation Assessment

For the assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be

recognised that virus inactivation is not a simple first order reaction and is usually more complex with a fast "phase 1" and a slow "phase 2". 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 non-specific "model" viruses are used or when specific "model" viruses are used as surrogates for virus particles such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least 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 may 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 should be performed to demonstrate that infectivity is indeed lost by inactivation.

6.2.6 Function and Regeneration of Columns

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Chromatography media/resin lifetime use should be indicated, and critical process parameters that impact viral clearance should be defined.

For protein A affinity capture chromatography, prior knowledge indicates that virus removal is not impacted 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 or cation exchange). Accordingly, to support repeated resin use for other chromatography types, equivalent prior knowledge including in-house experience and a detailed justification should be provided instead of product-specific viral clearance studies with end of lifetime resin.

Assurance should be provided so that any virus potentially retained by the production system would be adequately destroyed or removed before reusing the system. For example, evidence may be provided demonstrating that the cleaning and regeneration procedures inactivate or remove virus.

6.2.7 Specific Precautions

The following specific precautions should be considered:

- Care should be taken in preparing the 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 which can be reliably assayed;

- The study should include parallel control assays to assess the loss of infectivity of the virus due to 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. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale;

- Small differences in buffers, media, or reagents (for example) can substantially 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 or interference in assays used to determine the virus titer, as 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 anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behaviour 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;

- Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be considered when analysing the data. The effectiveness of virus elimination by a particular process may vary with the stage in manufacture at which it is used; and

- Overall reduction factors may be 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 due to inherent limitations or inadequate design of viral clearance studies.

6.3 Interpretation of Virus Clearance Studies

The object of assessing virus inactivation/removal is to evaluate and characterise process steps considered effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to ensure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

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 Transmission Electron Microscopy (TEM) or a quantitative Nucleic Acid Amplification Technique (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 Annex 4 for calculation of virus reduction factors and Annex 5 for calculation of estimated particles per dose. Manufacturers should recognise 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 appropriateness of the test viruses used;

- The design of the clearance studies;

- The log reduction achieved;

- The time-dependence of inactivation;
- The potential effects of variation in process parameters on virus inactivation/removal;
- The limits of assay sensitivities; and
- 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 virus contaminants. In this context, whenever feasible and not adversely affecting the product, implementing two distinct effective steps that complement each other in their mode of action is recommended. One of the manufacturing steps should effectively clear non-enveloped viruses. An effective virus removal step generally gives reproducible reduction of virus load in the order of 4 logs or more shown by at least two independent studies. However, it is recognised that steps giving a reproducible reduction in the order of 1 to 3 logs contribute towards viral safety and can be considered for assessment of overall virus reduction. Process steps dedicated to virus inactivation/removal such as Solvent/Detergent treatment, treatment with detergent alone, virus filtration (nanofiltration), or incubation at low pH, have been very successful in clearing a wide range of viruses. Using virus filters designed for removal of small viruses is also an effective virus clearance step for the smaller parvovirus or polyomarivruses. Finally, there is experience of efficient inactivation of Xenotropic Murine Leukemia Virus (XMuLV) and pseudorabies virus by incubation at low pH after protein A capture step for purification of monoclonal antibodies.

Acceptable overall clearance can be achieved by any of the following steps: multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Separation methods may be dependent on the extremely specific physico-chemical properties of viruses which influence their interaction with stationary phases for chromatography (e.g., resins or chromatography membranes) and precipitation properties, “model” viruses can be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well designed separation steps, such as chromatographic procedures, filtration steps and extractions, can be effective virus removal

steps provided that 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 titer of the order of 1 $\log_{10}$ or less would be considered negligible and could 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.

6.4 Limitations of Viral Clearance Studies

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. However, 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 behaviour 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 could 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 of multiple steps, particularly of steps with little reduction (e.g., below 1 $\log_{10}$), may overestimate the true potential for virus elimination. Addition of individual virus

reduction factors resulting from similar inactivation mechanisms during the manufacturing process may also overestimate overall virus 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, while 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 ml by a factor of 8 $\log_{10}$ leaves zero $\log_{10}$ per ml or one infectious unit per ml, taking into consideration the limit of detection of the assay; and

- Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.

6.5 Statistics

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 Annex 3).

6.6 Application of Prior Knowledge for Evaluation of Viral Clearance

As a general principle, viral clearance is evaluated by experiments when the virus is added to the product-specific in-process material of each step to be investigated. When a manufacturer is developing similar products by established and well-characterised 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 must be well-understood. The representativeness of the prior knowledge for the specific process step should be clearly justified. The prior knowledge comprised of external and in-house experience should cover the aspects outlined below:

- There should be an understanding of the mechanism underlying virus clearance;
- There should be an understanding of all process parameters that may affect viral clearance;
- It should be clear that interactions between virus and product do not affect viral clearance.

- The composition of a specific process intermediate may affect viral clearance. For some process steps, even small differences in buffers, media, reagents, level, and profile of impurities (for example) may substantially affect viral clearance. Therefore, the representativeness of the composition of the process intermediate(s) from other products should be justified. In addition, processing before the specific step for the new and the established product(s) should follow a similar strategy unless prior knowledge indicates robustness of virus clearance with respect to composition of the process intermediate; and
- The general limitations of viral clearance studies as outlined in Section 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 critical process parameters 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 comparability of the processes across manufacture of different products involved, comparability 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 technology.

The decision on the acceptability of virus clearance data without product-specific experiments is made on a case-by-case basis while considering the whole viral safety concept for a medicinal product, including the nature and characterisation 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 LRV claim using prior knowledge, the claim should be justified considering all LRVs from the relevant platform data. A conservative LRV claim is advised to avoid a risk for overestimating the reduction capacity of the process step.

Annex 6 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.

6.7 Re-Evaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated, as needed. For example, changes in production 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 virus 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 must be performed.

7. POINTS TO CONSIDER FOR CONTINUOUS MANUFACTURING PROCESSES

Continuous Manufacturing (CM) processes are dynamic systems consisting of integrated plural unit operations in which raw materials; process intermediates; and starting materials enter the manufacturing process continuously; and products are discharged throughout the manufacturing process. CM can be applied to some or all unit operations. 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 types of CM processes for the manufacture of therapeutic proteins can be found in ICH Q13 (Annex 3).

In terms of virus safety, technical aspects for CM may differ from those encountered in batch processes including concepts of detection and removal of viruses; material traceability; system dynamics; monitoring frequency start-up/shutdown; advanced process controls; process validation; process models; and continuous process verification.

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

control regarding process parameters, which are relevant for virus clearance is ensured even in dynamic processes (see Section 6.6).

7.1 Viral Safety in CM Processes

Control of viruses 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 ensure absence of viruses. Guidance on testing provided in Sections 3 and 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 the process is free of contamination during cell culture and other downstream steps.

7.2 General Considerations for Virus Clearance in CM

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

- The manufacturing process may be partially run in continuous or connected mode of operation and it is possible to use knowledge/experiences of virus clearance study design based on batch processes for the evaluation of unit operation if suitable;

- 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 inhomogeneity of input materials) should be assessed to cover any impact to the virus reduction capabilities;

- There should be appropriate process monitoring and sampling strategies in place to detect inadvertent disturbance or adventitious virus contamination. If conducting real time decision making, this should include a procedure to determine the impact of the disturbance or contamination on the output material quality and product. According to the impact, the diversion of the potential non-conforming material from the product stream or the disposition of the material produced should be taken into account; and

- The virus clearance study designs should consider potential impact of the following if applicable:

- Fluctuation of input material attributes (e.g., viral load, concentration and homogeneity of protein or impurities, and level of aggregation);
- Flow rate, temporal disturbance or pausing;
- Operational loading capacity;
- Multicolumn cycling.

CM also presents unique aspects to consider for virus safety.

7.2.1 Potential Risk Related to Longer Periods in Cell Culture Production

Fluctuations in the levels of endogenous retrovirus may occur over time in the production culture so an assessment should be made of the appropriate sampling point so as not to impact the dose risk factor calculation for the drug product (see Section 4 and Considerations in Section 3 for cell line qualification).

7.2.2 Approach to Virus Clearance Study

Although CM is expected to maintain a state of control, the manufacturing process will include periods when the process output may vary during start-up, termination, and temporary 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 may be addressed using best practices for clearance studies addressed elsewhere in this guideline. Considerations specific to CM would include:

- Chromatography
  - For the process of repeating sub-batches (e.g., multi-column), a batch process could serve as a scale-down model with well-justified target process conditions (e.g., flow rate, resin load vs column overload, resin cleanability);
  - Simultaneous validation of two or more connected unit operations could be an option according to the equipment design and system integration (e.g., bind and elute mode of Cation Exchange Chromatography (CIEX) and flow through mode of Anion Exchange Chromatography (AEX)), but only when all unit operations are to be validated for viral clearance. For connected unit operations, if the loading of the challenge material does not differ from batch operation, it is possible to evaluate with a conventional scale-down model;
- Low pH/solvent detergent inactivation

849 - Validation as a batch process could be appropriate with well-justified target
850 process conditions;
851 - For virus inactivation (e.g., pH and solvent/detergent) the control of relevant
852 dynamic process parameters should be ensured (e.g., pH, solvent/detergent
853 concentration, homogeneity and mixing, temperature, residence time);
854 - Care should be taken in evaluating/justifying the effect of scale (e.g.,
855 residence time distribution) when a scale-down model is applied for
856 inactivation in dynamic process;
857 • Virus filtration
858 - Validation as a batch process could be appropriate if settings of parameters
859 which have impact on virus clearance do not vary beyond ranges tested in
860 the virus clearance study (e.g., worst case setpoint); and
861 - Process controls should be defined to allow for filter changes and post-use
862 integrity testing while maintaining viral clearance capacity. This should
863 include not interrupting the continuous process and allowing material
864 diversion in the event of a filter failure.

865 **8. SUMMARY**

866 This guideline suggests approaches for evaluating the risk of viral contamination and for the
867 removal of virus from product, thus contributing to the production of safe biotechnology
868 products derived from animal or human cell lines and emphasises the value of many strategies,
869 including:

870 • Thorough characterisation/screening of cell substrate starting material to identify
871 which, if any, viral contaminants are present;

872 • Assessment of potential risk by determination of the human cell tropism or
873 knowledge of human infections;

874 • Establishment of an appropriate program of testing for adventitious viruses in
875 unprocessed bulk;

876 • Careful design of viral clearance studies using different methods of virus inactivation
877 or removal in the same production process to achieve maximum viral clearance; and

- Performance of studies which assess virus inactivation and removal.

9. GLOSSARY

Adventitious Virus

See Virus.

Cell Substrate

Cells used to manufacture product.

End of Production Cells (EOPC)

Cells harvested (under conditions comparable to those used in production) from the MCB or WCB cultured to a passage level or population doubling level comparable to or beyond the highest level reached in production. End of production cells are cells at or beyond the LIVCA.

Endogenous Virus

See Virus.

***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.

Inactivation

Reduction of virus infectivity caused by chemical or physical treatment.

Master Cell Bank (MCB)

An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks.

Master Virus Seed (MVS)

A master virus seed (stock, lot, or bank) is a preparation of a vaccine virus, helper virus, or viral vector from which all future production will be derived.

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 (HTS) or massive parallel sequencing (MPS) or deep sequencing, multi-step nucleic acid-based technology with broad capabilities for agnostic detection of known and unknown adventitious agents. In some cases, NGS can be used for targeted detection of known viruses.

Platform Manufacturing (according to ICH Q11)

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 using predefined host cell, cell culture, and purification processes for which considerable experience already exists).

Platform Validation

Throughout this guideline, this term exclusively refers to platform validation of virus 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 Characterisation of Viral Clearance

928 Viral clearance studies in which non-specific "model" viruses are used to assess the robustness
929 of the manufacturing process to remove and/or inactivate viruses.

930 **Process Evaluation Studies of Viral Clearance**

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

933 **Process Robustness of Viral Clearance**

934 The term robustness is used to describe one of the two different characteristics. One
935 characteristic is the ability of a process or process step to tolerate variability of materials and
936 changes of the process without negative impact on clearing a virus. The other characteristic is
937 the ability to clear a wide range of specific and non-specific model viruses.

938 **Production Cells**

939 Cell substrate used to manufacture product.

940 **Supplementary Test Method**

941 A test method used to provide data to refine the conventional testing. It is a test method used to
942 overcome a limitation(s) in an existing test method, such as test article interference or toxicity.

943 **Unprocessed Bulk**

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

946 **Virus**

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

950 **Adventitious Virus**

951 Unintentionally introduced contaminant viruses.

952 **Endogenous Virus**

Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. In this guideline, intentionally introduced, non-integrated viruses such as Epstein-Barr Virus used to immortalise cell substrates or Bovine Papilloma Virus.

Helper Virus

In the context of this guideline, a helper virus is a virus or a virus vector that provides a function to enable expression or replication of the product.

Non-Specific Model Virus

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

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.

Viral Clearance

Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

Virus-Like Particles

Structures visible by electron microscopy which morphologically appear to be related to known viruses.

Virus Removal

Physical separation of virus particles from the intended product.

Viral Vector

A recombinant virus that may be applied *in vivo* as a medicinal product or applied *ex vivo* for other advanced therapeutic applications. The genetically engineered viral vector may require a helper virus for production.

Viral Vector-Derived Product

A product encoded and expressed by a recombinant virus. The genetically engineered viral vector may require a helper virus for production.

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 working virus seed (stock, lot, or bank) is produced from the MVS.

Table 1. Virus Tests Recommended to Be Performed Once at Various Cell Levels

	MCB	*WCB*[a]	*Cells at the LIVCA*[b]
Tests for Retroviruses and Other Endogenous Viruses			
Infectivity	+	-	+
Electron microscopy[c]	+[c]	-	+[c]
Reverse transcriptase[d]	+[d]	-	+[d]
Other virus-specific tests[e]	as appropriate[e]	-	as appropriate[e]
Tests for Non-Endogenous or Adventitious Viruses			
In vitro Assays or NGS[j]	+[f]	+[f]	+[f]
In vivo Assays or NGS[j]	+[g]	-[g]	+[g]
Antibody production tests or specific molecular assay[h, j]	+[h]	-	-
Other virus-specific tests[i]	+[i]	-	-

a. Section 3.1.2.

b. Cells at the limit: cells at the limit of *in vitro* cell age used for production (See Section 3.1.3).

c. May also detect other agents.

d. If a cell line is known to constitutively produce retroviral particles, the assay may not be needed.

e. As appropriate for cell lines that are known to have been infected by such agents.

f. The *in vitro* virus test is performed directly on the WCB or on LIVCA cells directly derived from this WCB. Tests for viruses using broad molecular methods (NGS) can be used as supplementary or replacement assays for the *in vitro* tests (cell culture and PCR) based on the risk assessment.

g. *In vivo* testing may be performed based on risk assessment. However, *in vivo* testing is not necessary for well-characterised cell lines such as CHO, NS0 and SP2/0, based on cell line history; prior knowledge; and other risk-based considerations. This includes 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 virus 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 first WCB or subsequent WCB if they are 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 a molecular method for broad virus detection (e.g., NGS or PCR) 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.

h. e.g., MAP, RAP, HAP, which is usually applicable for rodent cell lines. Virus specific PCR or targeted molecular methods can be used as a replacement assay to the animal testing. e.g., based on the origin and history of the cell line including associated raw materials and reagents.

i. e.g., based on the origin and history of the cell line including associated raw materials and reagents

j When applicable, NGS should be considered to replace the *in vivo* test and may be used to supplement or replace the *in vitro* and other virus specific tests based on assay suitability and risk assessment.

1016 **Table 2. Examples of the Use and Limitations of Assays Which Can Be Used to Test for Virus**

TEST	*TEST ARTICLE*	*DETECTION CAPABILITY*	*DETECTION LIMITATION*
Antibody Production	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system
in vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses	Viruses failing to replicate or produce diseases in the test system
in vitro virus screen for:		Broad range of viruses	Viruses failing to replicate or produce diseases in the test system
1. Cell bank characterisation	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
2. Production screen	2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM on:		Virus and virus-like particles	Qualitative assay with assessment of identity
1. Cell substrate 2. Cell culture supernatant	1. Viable cells 2. Cell-free culture supernatant		
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples
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
1. Infectivity endpoint			1. See above under RV infectivity
2. TEM endpoint			2. See above under TEM[a]
3. RT endpoint			3. See above under RT
PCR (Polymerase chain reaction)	Cells, culture fluid, and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious
NGS	Cells, culture fluid and other materials	Broad range of viruses	Positive result does not indicate whether virus is infectious and may require further investigation

1017 a. In addition, may be difficult to distinguish test article from indicator cells
1018

Table 3. Viruses Detected in Antibody Production Tests

MAP[4]	*HAP*[4]	*RAP*[4]
Ectromelia Virus[2,3]	Lymphocytic Choriomeningitis Virus (LCM)[1,3-]	Hantaan Virus[1,3]
Hantaan Virus[1,3]	Pneumonia Virus of Mice (PVM)[2,3]	Kilham Rat Virus (KRV)[2,3]
K Virus [2]	Reovirus Type 3 (Reo3)[1,3]	Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]
Lactic Dehydrogenase Virus (LDM)[1,3]	Sendai Virus (SV)[1,3]	Pneumonia Virus of Mice (PVM)[2,3]
Lymphocytic Choriomeningitis Virus (LCM)[1,3,]	SV5	Rat Coronavirus (RCV)[2]
Minute Virus of Mice [2,3]		Reovirus Type 3 (Reo3)[1,3]
Mouse Adenovirus (MAV)[2,3]		Sendai Virus[1,3]
Mouse Cytomegalovirus (MCMV)[2,3]		Sialodacryoadenitis Virus (SDAV) [2]
Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]		Toolan's H-1 Virus [2,3]
Mouse Hepatitis Virus (MHV)[2]		
Mouse Rotavirus (EDIM)[2,3]		
Pneumonia Virus of Mice (PVM)[2,3]		
Polyoma Virus [2]		
Reovirus Type 3 (Reo3)[1,3]		
Sendai Virus[1,3]		
Thymic Virus [2]		

1. Viruses for which there is evidence of capacity for infecting humans or primates.
2. Viruses for which there is no evidence of capacity for infecting humans.
3. Virus capable of replicating *in vitro* in cells of human or primate origin.
4. NAT such as PCR assays or other targeted molecular methods can be used for replacing specific rodent virus testing.

Table 4. Recommended Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk

	Case A	Case B	Case C[2]	Case D[2]	Case E[2]	Case F
STATUS						
Presence of virus[1]	-	-	+	+	(+)[3]	-
Virus-like particles[1]	-	-	-	-	(+)[3]	-
Retrovirus-like particles[1]	-	+	-	-	(+)[3]	-
Virus identified	not applicable	+	+	+	-	+
Virus infectious for humans	not applicable	-[4]	-[4]	+	unknown	(+)[9]
Presence of helper virus	-	-	-	-	-	+
ACTION						
Process characterisation of viral clearance using non-specific "model" viruses	yes[5]	yes[5]	yes[5]	yes[5]	yes[7]	yes[5]
Process evaluation of viral clearance using "relevant" or specific "model" viruses	no	yes[6]	yes[6]	yes[6]	yes[7]	yes[9]
Test for virus in purified bulk	not applicable	no	yes[8]	yes[8]	yes[8]	yes[9]

1. 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 may be acceptable if appropriate viral clearance evaluation procedures are followed.
2. Source material that is contaminated with viruses, whether 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.
3. Virus has been observed by either direct or indirect methods.
4. Believed to be non-pathogenic.
5. Characterisation of clearance using non-specific "model" viruses should be performed.
6. Process evaluation for "relevant" viruses or specific "model" viruses should be performed.
7. See text under Case E.
8. 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 authorisation, data from at least 3 lots or batches of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.
9. Virus may or may not be infectious for humans. Process evaluation for the helper virus (recombinant or wild type) should be performed. If this is not possible, then a specific model virus should be used.). When utilised in production, the helper virus is quantified in the unprocessed bulk stage using at least three cell culture campaigns to determine the target for virus clearance. Following purification, absence of detectable helper virus is determined using an infectivity assay with relevant

permissive cell lines for sensitive virus detection. Alternatively, molecular methods may be used. Absence of the residual helper virus should be confirmed for each purified bulk.

ANNEX 1: PRODUCTS DERIVED FROM CHARACTERISED CELL BANKS WHICH WERE SUBSEQUENTLY GROWN *IN VIVO*

For products manufactured from fluids harvested from animals inoculated with cells from characterised banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well-defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in Table 3, should be performed. Quarantine procedures for newly arrived and diseased animals should be described and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This can be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available onsite or within easy access. The extent to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum and the site and their route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacturing unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in Section 4 of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as *in vivo* testing in adult and suckling mice.

ANNEX 2: THE CHOICE OF VIRUSES FOR VIRAL CLEARANCE STUDIES

A. Examples of Useful "Model" Viruses

a. Non-specific "model" viruses representing a range of physico-chemical structures:

- SV40 (*Macaca mulatta polyomavirus*), animal parvovirus or some other small, non-enveloped viruses;
- a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
- a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

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

b. 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

Several viruses which 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 at least three different viruses with differing characteristics.

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

Virus	*Family*	*Genus*	*Natural host*	*Genome*	*Env*	*Size (nm)*	*Shape*	*Resistance* ***a***
Vesicular Stomatitis Virus b	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70x150	Bullet	Low
Parainfluenza Virus	Paramyxo	Paramyxovirus	Various	RNA	yes	100-200+	Pleo/Sphere	Low
MuLV	Retro	gammaretrovirus	Mouse	RNA	yes	80-110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60-70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50-70	Pleo-Sphere	Low
Pseudorabies Virus b,c	Herpes	Varicellovirus	Swine	DNA	yes	120-200	Spherical	Med
Autographa california multiple nucleopolyhedrovirus c	Baculo	Alphabaculovirus	Insect	DNA	yes	250-300	Polyhedral	Med
Adenovirus Type 2 or Type 5 c	Adeno	Adenovirus	Human	DNA	no	70-90	Icosahedral	Med
Vesivirus 2711	Calici	Vesivirus		RNA	no	27-40	Icosahedral	Med
Encephalomyo-carditis Virus (EMCV)	Picorna	Cardiovirus	Mouse	RNA	no	25-30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	RNA	no	60-80	Spherical	Med
SV40	Papova	Polyomavirus	Monkey	DNA	no	40-50	Icosahedral	Very high
Parvoviruses (canine, murine, porcine) d	Parvo	Parvovirus	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. Relevant model for rhabdovirus found in insect cells
c. Specific models or relevant virus for helper virus used for viral vector production
d. May be used as single worst-case model virus for larger spherical/icosahedral viruses and enveloped viruses at validation of virus filters.
These viruses are examples only, and their use is not mandatory.

ANNEX 3: STATISTICAL CONSIDERATIONS FOR ASSESSING VIRUS AND VIRUS REDUCTION FACTORS

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 (TCID) 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% confidence limits for results of within-assay variation normally should be on the order of ± 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 may be acceptable with appropriate justification.

4. The 95% 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% 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% confidence limits for the reduction factor are

$$\sqrt{S^2 + a^2}$$

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), it is evident that a sample of a few millilitres may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$p = ((V-v)/V)^n$

when V (liter) is the overall volume of the material to be tested, v (liter) 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% 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 which would have to 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% are desirable. However, in some instances, this may not be practical due to material limitations.

ANNEX 4: CALCULATION OF REDUCTION FACTORS IN STUDIES TO DETERMINE VIRAL CLEARANCE

The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material and the virus load in the post-purification 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 purification 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 1.

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, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

ANNEX 5: CALCULATION OF ESTIMATED PARTICLES PER DOSE

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

Example:

I. Assumptions

Measured or estimated concentration of virus in cell culture harvest = 10^6/ml

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

Volume of culture harvest needed to make a dose of product = 1 litre (10^3ml)

II. Calculation of Estimated Particles/Dose

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

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

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

$$= <10^{-6} \text{ particles/dose1}$$

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 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 pathogenicity of the virus, measures to avoid contamination, testing measures, the route of administration, and the human infectious dose.

In the Case B scenario for Chinese Hamster Ovary (CHO) cells, a safety margin of $<10^{-4}$ particles/dose is considered acceptable for Retroviral-Like Particles (RVLPs) for recombinant proteins if *in vitro* testing fails to identify the presence of infectious retroviruses.

ANNEX 6: EXAMPLES OF PRIOR KNOWLEDGE INCLUDING IN-HOUSE EXPERIENCE TO REDUCE PRODUCT-SPECIFIC VALIDATION EFFORT

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

In this context, platform validation is defined as the use of prior knowledge including in-house (applicant-owned data) experience with viral reduction data from other products, to claim a reduction factor for a new similar product. In general, a virus clearance claim for a new product based on prior knowledge including in-house experience should include a discussion of all the data available and the rationale to support the platform validation approach (see Section 6.6). 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 product intermediates.

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

Therefore, examples for application of prior knowledge to XMuLV inactivation/removal by detergent and incubation at low pH as well as virus filtration are given below.

These mock examples are provided for illustrative purposes, only suggest 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 impact of process parameters and intermediates on XMuLV clearance should be assessed by prior knowledge and in-house experience.

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

Inactivation by Solvent/Detergent (SD) or Detergent Alone

Based on the mechanism of action, detergent concentration of 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 impact 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 or 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 ≤0.2 µm nominal pore size before detergent inactivation.

The following paragraph describes how to apply a platform validation approach to XMuLV 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 XMuLV inactivation.

Triton X-100 is a non-ionic detergent commonly used in membrane research to solubilize lipid bilayers. It inactivates enveloped viruses by solubilizing the virus lipid envelope thus rendering the virus non-infectious. 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 (MAb) by addition to HCCF.

The European Chemicals Agency included Triton X-100 in the Authorisation List (Annex XIV) due to 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 XMuLV inactivation.

Because of the non-ionic 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 XMuLV inactivation in HCFF at 0.2 % 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

concentration of 0.5% 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.

Table A-2: Summary of Process Parameters and Their Potential Impact for Detergent Inactivation

Process parameter	*Potential Impact*	*Rationale*
SD or Triton X-100 concentration	High	Inactivating agent
Incubation time	High	Mechanism of inactivation is time-dependent
Temperature	High	Impact on inactivation kinetics
Pre-treatment 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 impact observed with worse-case HCCF
Type of product	Low	No impact on inactivation observed for MAb, half antibody, fusion protein or recombinant protein
Total protein content in HCCF	Low	Low impact observed with worse-case HCCF
pH	Low	Triton X-100 is a non-ionic detergent
Ionic strength	Low	See above
Buffer salt in HCCF	Low	See above
Potential interaction between virus particle and product	Low	No impact on inactivation observed and disruption of lipid envelope lowers probability of interaction with product

Thus, consistent with current process understanding ≥ 0.5% Triton X-100 treatment of clarified HCCF for ≥ 60 minutes at ≥ 15°C effectively inactivates XMuLV for multiple cell-culture derived products. Treatment with 1% Triton X-100 and 0.3% Tri-N-Butylphosphate (TNPB) for ≥ 30min or treatment with 1% polysorbate 80 and 0.3%TNBP for ≥6h at ≥23°C effectively inactivates retroviruses. According to current process understanding, a platform validation approach may be applied for XMuLV inactivation by SD treatment or treatment with Triton X-100 alone.

Incubation at Low pH

Low pH treatment inactivates enveloped viruses by denaturing proteins located in the viral envelope, thus disrupting the lipid envelope. 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 monoclonal antibodies (MAb).

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 impact inactivation efficiency as well.

Table A-3 summarizes process parameters and their potential impact for low pH inactivation of XMuLV.

Table A-3: Summary of Process Parameters and Their Potential Impact for low pH inactivation and impact on XMuLV

Process Parameter	*Potential Impact*	*Rationale*
pH	high	Inactivating agent
Incubation time	high	Mechanism of inactivation is time-dependent
Temperature	high	Impact on inactivation kinetics
Buffer matrix	high	Available data show that inactivation robustness depends on buffer matrix
Product concentration	low	No impact on inactivation observed
Type of product	low	No impact on inactivation observed for MAb, half antibody, bispecific antibody, fusion protein or recombinant protein
NaCl concentration (a)	low	No impact if ≤ 500 mmol/L sodium chloride
Potential interaction between virus particle and product	low	No impact on inactivation observed.

(a): to date, data on influence of ionic strength of other buffers is limited.

Consistent with current process understanding low pH treatment at ≤ pH 3.6, ≥ 15°C for ≥ 30 min at ≤ 500 mmol/L sodium chloride concentration is effectively inactivating XMuLV. Acetate and citrate buffer are most commonly used and allow for robust XMuLV inactivation.

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

Virus Filtration

The 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 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 flow dynamics and virus retention is not fully understood.

This section focuses on using prior knowledge and in-house experience in virus filtration of other products to claim retrovirus removal by small and large virus-retentive filters.

Factors that impact efficient retrovirus removal by small-virus filters are well understood with respect to variation of process parameters such as membrane type, flow- or pressure-controlled filtration mode, 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 filters, one option is to apply parvovirus log reduction values for larger spherical/icosahedral viruses and enveloped viruses. However, sometimes this could result in underestimating virus removal capacity (e.g., retrovirus 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 filters, companies could use their in-house data from parvovirus and retrovirus removal to build a platform retrovirus clearance claim for commonly used small virus 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 retroviruses.

A thorough understanding of the impact of pressure interruptions, as well as volume throughput and filter flush volume reflecting good manufacturing practice conditions should be conserved.

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.

The type of virus filter is important for virus reduction and its robustness with respect to impact of process parameters and should be considered when designing platform data.

Table A-4. Summary of Process Parameters and Their Potential Impact for Parvovirus Clearance by Small Virus-Retentive Filters

Process Parameter	*Potential Impact*	*Rationale*
Volumetric throughput of product intermediate loaded on the virus filter	High	Low level parvovirus passage has been observed depending on the specific filter type
Volumetric throughput of the buffer used for flushing filters	High	Low level parvovirus passage has been observed
Pressure	high	Pressure should not exceed the upper limit for filter operation. Low pressure can be worse case for a specific membrane type. Pressure interruption (if occurring during filtration or at switching from filtration of product intermediate to filter flush) should be considered.
Type of product	low	No impact on virus clearance observed for MAb, half antibody, bispecific antibody, fusion protein or recombinant protein
Product concentration	low	No negative impact on virus clearance observed
pH	low	No negative impact on virus clearance due to size-based removal
Ionic strength	low	Limited impact on virus clearance has been observed
Buffer matrix	low	Limited impact on virus clearance has been observed
Potential interaction between virus particle and product	low	Specific interaction between virus and antibody can enhance virus retention

ANNEX 7: GENETICALLY-ENGINEERED VIRAL VECTORS AND VIRAL VECTOR-DERIVED PRODUCTS

7.1 Introduction

Advances in biotechnology have led to an emergence of new and advanced production platforms expressing new product types manufactured using characterised cell banks of human or animal origin (i.e., avian, mammalian, or insect). The scope of Annex 7 includes helper-virus dependent and helper-virus independent genetically-engineered viral vectors and viral vector-derived products that are amenable to virus clearance based on considerations of the physicochemical properties of the product. These products include Virus-Like Particles (VLPs) and protein subunits that are produced using baculovirus/insect cells, nanoparticle-based vaccines, and viral-vector products such as AAV. These medicinal products may be applied *in vivo* or *ex vivo*.

Helper-virus independent products are manufactured using stably transformed or transiently transfected cell lines or by infection with a protein-expression virus vector (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., adeno-associated virus or recombinant proteins that are expressed using a helper virus such as herpes simplex virus or adenovirus).

The potential sources for virus contamination for a biopharmaceutical product are described in Section 2 of the main guideline. Additional contamination risks such as those introduced by the expression system and the potential for contamination with replication competent virus should be considered. The susceptibilities 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-characterised cell banks and virus seeds can reduce the risk of virus contamination. Furthermore, helper viruses used for production are considered process-related viral contaminants.

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 manufacture and removal and/or inactivation of adventitious viruses and helper viruses by the manufacturing process. If virus clearance is limited, virus 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.

7.2 Testing for Viruses

Extensive testing and characterisation for both endogenous and adventitious viral contamination 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 lifecycle. Table A-5 below provides an outline of the tests to be performed at various steps during production. The tests applied for virus seeds, vector harvest, and drug substance are described. Although the testing and characterisation scheme proposed for the cell substrates used for viral vector production are broadly aligned with Table 1 in the main guidance document, additional considerations may apply for these product types, and are therefore specified within Table A-5 below for completeness.

The type and extent of testing depends on the 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 vector, the raw materials and reagents and culture methods used, the reliance on helper virus(es), and the capacity of the manufacturing process to inactivate and/or remove viruses.

Table A-5: Tests that Should Be Performed at Applicable Manufacturing Stages

Test	*MCB, WCB, Cells at the LIVCA*	*Virus Seed* [k]	*Unprocessed Bulk (Harvest)*	*Drug Substance*
Test for adventitious or endogenous viruses				
[a, b,] *In vitro* assays or NGS	[i,] See Table 1 of main guideline	+[h]	+[h]	-
[b,] *In vivo* assays or NGS		+ [h]	- [h,l]	-
[c] other virus specific tests		[l]	[l]	-
[d] Antibody production assays or specific molecular assay		+[j,l]	-	-
Tests for Endogenous, Helper and Replication Competent Viruses, as applicable				
[e]retroviruses	[i,] See Table 1 of main guideline	+	+[l]	-
[f]residual helper Viruses	NA	-	+	+ [l]
[g]replication competent viruses	+	+	(+)	(+)

[a]Testing should be performed on permissive cell lines, based on risk assessment. The indicator cells cultures should be observed for at least 2 weeks, with a further secondary passage of 2 weeks of observation. Include testing for haemadsorbing and

hemagglutinating viruses. For products produced in insect cell lines, the testing should include a permissive cell line for arboviruses (e.g., BHK cells). If viral vectors and viral vector-derived products cannot be neutralised, a validated alternative assay can be used. Testing should be performed on the virus seed and the unprocessed bulk harvest before downstream processing. In some cases, the unprocessed bulk harvest may be the same as the drug substance.

[b]When applicable, broad NGS should be considered to replace the *in vivo* adventitious virus tests and may be used to supplement or replace the *in vitro* tests based on assay suitability and risk assessment.

[c]Species-specific virus detection (e.g., NAT and cell culture or targeted NGS) is determined based on risk assessment of the cell substrate, raw materials or reagents, and manufacturing process. This may include human or rodent species-specific viruses, arboviruses in insect cells, and bovine or porcine viruses if serum components or trypsin are used.

[d]Antibody production test (MAP, HAP, RAP) or virus specific NAT or targeted NGS may be performed based on risk assessment of the cell substrate, raw materials, or reagents and manufacturing process.

[e] The presence of retrovirus using reverse transcriptase assays at the MCB and virus seed stage should be considered. If the MCB or virus seed is positive for reverse transcriptase activity, follow-up should include quantification of potential retroviral particles in the unprocessed bulk harvest from at least three cell culture campaigns to determine the target level for virus clearance. In addition, a PCR-based RT assay (PBRT) assay, for example, the product-enhanced reverse transcriptase assay (PERT), should be performed on the Unprocessed Bulk (harvest), based on risk assessment.

[f]When used in production, the helper virus should be quantified at the unprocessed bulk stage using at least three cell culture campaigns to determine the target for virus clearance. Following purification, absence of detectable helper virus should be determined using infectivity assay with relevant permissive cell lines for sensitive virus detection. Alternatively molecular methods may be used. Absence of residual helper virus should be confirmed for each purified bulk (Case F, Table 4).

[g]Replication Competent Virus (RCV) may develop at any step during manufacturing (e.g., at initial transfection or transduction steps and through production). Current recommendations include testing for RCV at multiple stages of manufacture to detect for recombination or for the vector virus to revert to parental or wild type phenotype. The manufacturing stages and test methods are when applicable and product dependent. For example, RCV testing is performed on cells and supernatant derived from the stably-transfected vector producer or packaging MCB and LIVCA and during the qualification of the virus seed or cell bank. Tests for RCV apply during production, with testing performed on vector producing cells and supernatant from each unprocessed bulk harvest or at each drug substance/final lot, when applicable. For example, replication-competent virus testing is typically performed at unprocessed bulk harvest to ensure detectability or drug substance step for Adeno-Associated Virus (AAV) based products indicated as (+) in the table.

[h]. When assay interference may occur control cells cultured in parallel are tested at the virus seed and unprocessed bulk harvest stages.

[i]. For cell lines of insect origin tests for species-specific viruses and arboviruses should be carried out. Refer to Table 4 (Case B, C, and E) for action steps to be taken for virus detection in cell substrates used for production.

[j]. Testing should be performed if cell substrate/cell bank not tested

[k]. Depending on the product type, the virus seed may be used to manufacture a vaccine virus, viral vector, or helper virus. The virus seed is 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 seed to ensure that the absence of adventitious virus may originate from the cell substrate and the absence of replication competent virus. Testing should be applied on the virus seed before processing. The Working Virus Seed (WVS) is derived directly from the MVS; a subset of adventitious agent testing therefore applies based on a risk assessment. An alternative approach in which complete testing is carried out on each WVS rather than on the MVS would also be acceptable.

[l] testing based on risk assessment

(+) alternative testing stage

NA not applicable

7.3 Virus Clearance

The risk of contamination with adventitious viruses and residues of viruses used during

production such as helper viruses and protein expression vectors should be mitigated following the general principles of this guideline to the extent possible.

The virus clearance should be validated using representative and qualified scale down systems.

The physicochemical characteristics of the viral vector and the viral vector-derived product will determine how virus clearance will apply within the product purification scheme. Virus-clearance validation should include model viruses representative of adventitious, endogenous, and if possible, the relevant helper virus. Sections 5 and 6 (including application of prior knowledge) as such apply, using the action plan for the selection of specific and non-specific model viruses described in Table 4. Common virus inactivation steps such as treatment with detergent or solvent/detergent may be suitable, when the product is compatible, such as non-enveloped viral vectors. Alternatively, virus filtration may be more suitable for small viral vector such as AAV or nanoparticle-based vaccines when virus removal can be based on the size exclusion. When appropriate, viral clearance studies should be performed to determine virus reduction factors for the relevant step(s) of the production process.

Examples include:

- Subunit proteins and VLPs produced using baculovirus/insect cells can be purified and high levels of virus log reduction factors can be achieved through the manufacturing process and are validated by viral clearance studies; and
- Some viral-vector products such as AAV are amenable to robust viral clearance steps, ensuring adventitious and helper virus clearance inactivation or removal.

The helper virus is considered a process-related viral contaminant. The manufacturing processes need to ensure an excess of helper virus clearance. Acceptable log-reduction factors can be based on risk assessment.

Since virus clearance steps during production may not achieve the same robustness as for recombinant proteins, the viral safety of these products relies also on closed processing, testing and other preventative controls (see Sections 2.2, 3, and 4).