

Guidance for Industry

Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases

DRAFT GUIDANCE

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For questions on the content of this guidance, contact the Office of Vaccines Research and Review at 301-827-5105.

U.S. Department of Health and Human Services
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TABLE OF CONTENTS

I. INTRODUCTION.....	1
II. OVERVIEW: CHARACTERIZATION AND QUALIFICATION OF CELL SUBSTRATES	2
A. BACKGROUND	2
B. PRODUCT-SPECIFIC PARAMETERS INFLUENCING CHARACTERIZATION AND QUALIFICATION OF CELL SUBSTRATES	3
1. Vaccine Purity.....	3
2. Potential Sources of Contamination.....	4
3. Quality Design	5
4. Use of Control-Cell Cultures	5
5. Assay Validation.....	5
III. CHARACTERIZATION AND QUALIFICATION OF CELL SUBSTRATES, VIRAL SEEDS, BIOLOGICAL RAW MATERIALS AND VACCINE PRODUCTION	6
A. PROPERTIES OF THE CELL SUBSTRATE	6
1. Properties relevant to cell substrate selection	6
2. Source	7
3. History (including identifying characteristics) and other important characteristics	7
4. Growth Characteristics.....	8
5. Expression Characteristics	9
6. Susceptibility to adventitious agents.....	9
7. Generation of Cell Substrate	9
B. CELL BANKING.....	10
1. Cell Banking Strategies and Methods.....	10
2. Qualification of Cell Banks and Primary Cells.....	11
3. Special Considerations for Primary Cells	12
4. Special considerations for Diploid Cell Strains	13
5. Special Considerations for Continuous Cell Lines	14
6. Additional considerations for cell lines that are tumorigenic or tumor-derived.....	14
7. End-of-Production Cells	15
C. VIRAL SEEDS	16
1. Master Viral Seed	16
2. Working Viral Seed	17
D. BIOLOGICAL RAW MATERIALS AND ANCILLARY REAGENTS	17
1. Serum	18
2. Trypsin	18
3. Amino Acids	18
4. Other Biological Reagents	18
E. CONSIDERATIONS IN TESTING AT DIFFERENT STAGES OF PRODUCTION	19
1. Cell Banks.....	19
2. Pre-Production Cells.....	19

Contains Nonbinding Recommendations

Draft – Not for Implementation

3.	Pre-filtered Harvest or End-of-Production Cells	19
4.	Control Cells	20
5.	Post-Filtered Harvest or Final Bulk	20
6.	Final Filled Product.....	20
IV.	DESCRIPTION OF QUALITY-CONTROL TEST METHODS	21
A.	TESTING FOR ADVENTITIOUS AGENTS.....	21
1.	In Vivo Tests.....	21
a.	Adult Mice	22
b.	Suckling Mice	22
c.	Guinea Pigs	23
d.	Rabbits	24
e.	Embryonated Chicken Eggs.....	24
f.	Antibody Production Tests	25
2.	In Vitro Tests for Viruses	26
a.	Cell Culture Safety Test.....	26
i.	Human Diploid Cells	26
ii.	Monkey Kidney Cells	26
b.	Transmission Electron Microscopy (TEM)	28
c.	Biochemical Tests for Retroviruses	28
d.	Infectivity Tests for Retroviruses.....	29
e.	PCR or Other Specific in vitro Tests	29
f.	Sourcing and Testing of Bovine-Derived Materials	30
g.	Testing of Porcine-Derived Reagents	30
3.	In Vitro tests for Non-Viral Agents	31
a.	Mycoplasma.....	31
b.	Bacterial and Fungal Sterility	34
c.	Mycobacteria Testing.....	34
B.	TESTING OF CELL PROPERTIES	35
1.	Tests for Tumorigenicity.....	35
2.	Testing for Oncogenicity	36
3.	Identity Testing of Cell Substrates.....	37
4.	Testing for Genetic Stability.....	38
C.	OTHER TESTS.....	38
1.	Testing for the Presence of Residual Cells	38
2.	Testing for Residual Cellular DNA	38
3.	General Safety Test (GST).....	39
V.	CONCLUSIONS	39
VI.	GLOSSARY.....	40
VII.	REFERENCE LIST.....	44

Contains Nonbinding Recommendations

Draft – Not for Implementation

Guidance for Industry

Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases

This draft guidance, when finalized, represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

We, the Food and Drug Administration (FDA), are providing you, manufacturers of viral vaccines, recommendations for the characterization and qualification of cell substrates and viral seeds used for the production of viral vaccines for human use. These recommendations may be used to support a Biologics License Application (BLA) or an application for an Investigational New Drug (IND).

This guidance applies to the production of viral vaccines for the prevention and treatment of infectious diseases regulated by the Office of Vaccines Research and Review (OVR) of the Center for Biologics Evaluation and Research (CBER).

This document is intended to replace the information pertaining to viral vaccines used for the prevention and treatment of infectious diseases that we provided in the document entitled "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals," dated 1993 (Ref. 1). This document also is intended to supplement recommendations on the production of viral vaccines for the prevention and treatment of infectious diseases, provided in International Conference on Harmonization (ICH) documents Q5A and Q5D (Refs. 2 and 3, respectively). For the production of biological products not covered under this guidance, we recommend that you refer to the "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals," dated 1993 (Ref. 1).

For the purpose of this document, viral vaccines are a heterogeneous class of preventive, and in some cases, therapeutic and medicinal products that when administered are intended to elicit immune responses that could prevent and/or lessen the severity of one or more infectious diseases. A viral vaccine may be a live attenuated preparation of viruses, inactivated (killed)

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whole or subunit virions, purified recombinant proteins, including those derived from recombinant deoxyribonucleic acid (DNA) in a host cell, synthetic antigens, polynucleotides (such as plasmid DNA vaccines), or live viral vectors expressing specific heterologous vaccine antigens. It may also be a combination of viral vaccines listed above. Antigens may be presented alone or delivered in combination with other antigens, adjuvants, additives and other excipients. Therapeutic vaccines for non-infectious diseases (e.g., certain cancer vaccines) and monoclonal antibodies used as immunogens (e.g., anti-idiotypic antibodies) are not considered here.

The scope of this guidance document is limited to cell substrates of human or animal origin and does not cover characterization of unicellular organisms, such as bacteria or yeast. This guidance also applies to the characterization and qualification of viral seeds. A glossary defining scientific terms in this guidance is located in Section VI below.

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

II. OVERVIEW: CHARACTERIZATION AND QUALIFICATION OF CELL SUBSTRATES

A. BACKGROUND

The number of different cell substrates used in currently licensed vaccines is limited. The emergence of new infectious diseases necessitates the need for development of new vaccines for agents such as human immunodeficiency virus (HIV), pandemic influenza virus strains, severe acute respiratory syndrome (SARS) virus, and agents of bioterrorism. In some cases, novel human and animal cell substrates might be needed to manufacture certain vaccines, including live attenuated viruses, live viral vectors expressing vaccine antigens, inactivated whole or subunit virions, purified recombinant proteins, and virus-like particles. Development of new-generation vaccines likely will involve a wider variety of cell substrates, both for the isolation of virus seeds and vectors, and for vaccine manufacture. Due to the absence or limited experience with new cell substrates, manufacturers should develop and apply the best technologies available to assure that these new cell substrates are safe.

Selection of a cell substrate influences the safety and purity of the biological product manufactured in it. You should evaluate cell substrates on a case-by-case basis, but characterization of any cell substrate used for the development and manufacture of a vaccine should address certain general issues that might affect the safety and purity of vaccine products manufactured in them. Examples of such issues include karyotype and tumorigenic phenotype of the cell substrate, the identity and genetic stability of

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the cell substrate and virus seeds, and the requirement that the vaccine product be free of extraneous infectious microorganisms and potential oncogenic agents. Tests to address these general issues affecting characterization and qualification of cell substrates are described in Section IV.

Qualification of a biological starting material includes an assessment of the material to demonstrate its safe use in manufacturing a final product. The qualification of cells intended for use in the manufacture of biologicals includes assessment of the:

- history and general characteristics of the cells;
- cell banking system; and
- characterization of the cells through quality-control testing.

In some situations, additional validation studies to demonstrate virus removal and/or inactivation by the manufacturing process might be necessary.

B. PRODUCT-SPECIFIC PARAMETERS INFLUENCING CHARACTERIZATION AND QUALIFICATION OF CELL SUBSTRATES

1. Vaccine Purity

The regulations in 21 CFR 610.13 state that “Products shall be free of extraneous material except that which is unavoidable in the manufacturing process described in the approved biologics license application.” In 21 CFR 600.3(r), purity is defined as the “relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product.” (see Section VI. Glossary). Because live attenuated viruses, whole inactivated virions, or virus-like particles often cannot be purified as rigorously as viral subunit vaccines, their potential for contamination might be greater than that of subunit vaccines. The generation of live viral vaccines often involves cell disruption. In addition, such vaccines often are minimally purified and are not subjected to any inactivation steps. Comprehensive testing and qualification of the biological starting materials and raw materials should be performed, and lot-by-lot testing for adventitious agents might be necessary, because it might not be possible for a manufacturer of live viral vaccines to validate clearance of any adventitious agents.

For more highly purified products where viral clearance can be achieved during downstream processing, you may place more reliance on process validation (Ref. 2). For inactivated vaccines, the process you use to inactivate your vaccine virus might not inactivate all adventitious agents that might be present (as occurred with early inactivated poliovirus vaccines (Ref. 4). Therefore, you should provide documentation of your validation for inactivation of adventitious agents. The choice of tests and the stages at which the tests are applied will depend on your inactivation process. The degree of viral clearance that is feasible might influence

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the sensitivity of the testing you should perform to demonstrate the absence of contaminants in your product.

You should validate any methods used to inactivate or clear potential viral contaminants during production of your vaccine including the starting materials used to produce it, as the purity of your product could be affected by the purity of reagents and biological raw materials you use to produce the vaccine. For example, inactivation of viruses by irradiation of serum could provide additional assurance regarding the purity of the final product. Certificates of Analysis (COA) for all reagents and biological raw materials used for vaccine production should be included in your submission.

If a seed was exposed to a known adventitious agent or if the passage history of a virus seed is unclear, you should purify viral seeds (e.g., by molecular cloning, serial passage in medium containing neutralizing antibody directed against the adventitious agent, or plaque purification). If your purification method is demonstrated to be capable of removing all adventitious agents from a viral seed to within an acceptable safety margin, this approach could be used to qualify a seed. If you are contemplating performing seed purification, you should discuss your proposed methodology and its validation with us as early in the process as is feasible.

2. Potential Sources of Contamination

It is important that you identify and examine all potential sources of contamination of your product. For example, the viral seed used in the production of many licensed viral vaccines is exposed to the following potential sources of contamination: the person or animal from which it was isolated; the cells and raw materials (e.g., serum or trypsin) used in its isolation and attenuation; materials used in banking and propagation of cells for viral seed growth; and other materials used during production and filling of the seed. You also should consider the species of origin of your cell substrates, viral seeds, and other biological starting materials in selecting your tests. You should consider any infectious viruses (including those that infect nonhuman species) as potential contaminants if there is the possibility of contact with your product at any time during development and production. You should test your product at appropriate stages of production for contaminating agents. Finally, you should consider the possibility of contamination from unusual sources, as exemplified by the reported presence of minute virus of mice (MVM) in some lots of recombinant proteins (Ref. 5). Testing might be needed to verify the absence of additional contaminating agents, particularly those agents whose propagation might be supported by your cell substrate.

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3. Quality Design

Concepts of quality design consistent with Current Good Manufacturing Practices (cGMP) and Good Laboratory Practices (GLP) (see 21 CFR parts 211 and 58) are relevant to the selection of suitable cell substrates and viral seeds. If your cell substrate or viral isolate was not originally derived under concepts consistent with cGMP and GLP practices, additional documentation (e.g., donor and source of raw materials), testing and production steps might be necessary to support its use. This may include having some virus seeds re-derived or further purified to reduce the possibility of adventitious agent contamination.

4. Use of Control-Cell Cultures

If you are using primary cell cultures to propagate your vaccine virus, complete testing of the primary culture might not be feasible prior to inoculation of virus. In this situation, you should produce and test uninfected control-cell cultures that are derived in parallel with and handled in the same manner as the production culture. Control-cell cultures should be processed simultaneously with your production culture, but left uninfected and tested for the presence of adventitious agents by direct observation and testing of the cell sheet and by testing the culture fluid using appropriate tests (described in Section III.A and Table 1 in Section IV.E). Tests for adventitious agents should be performed at times at which you would perform similar tests on your manufactured product if it were possible to do so.

Use of control-cell cultures is important when your vaccine virus might interfere with the results of in-process testing of the product; for example, when the virus cannot easily be neutralized to permit testing for adventitious agents. You should propagate control-cell cultures under conditions similar to production for a suitable period to allow for possible reactivation and detection of latent or endogenous adventitious agents and poorly replicating adventitious agents. You should use a culture period of at least 14 days beyond the time of inoculation of the production vessels prior to testing. A longer period might be needed to detect some agents. Testing of control cells does not always eliminate the need for testing end-of-production cells, which might be required to demonstrate the absence of agents induced during vaccine manufacture.

5. Assay Validation

You should demonstrate the reliability of assays or tests used to evaluate your cell substrate in the context of intended use. Guidance on appropriate ways to validate an analytical assay may be obtained from the ICH Q2A and Q2B documents (Refs. 6 and 7, respectively). Further suggestions are provided by the U.S. Pharmacopoeia (USP) in Chapter 1225 (Ref. 8). Test methods should be validated according to the principles defined in these guidance documents.

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Certain “compendial” methods might not require full validation, as long as you verify their suitability under your actual conditions of use. Verification of suitability for your use may include a demonstration of comparability between your limits of detection and those for methods described in the published literature.

You should include appropriate statistical analysis in your assay validation. This may include assessment of assay accuracy, precision, limits of detection, limits of quantification, specificity, linearity and range, ruggedness and robustness, and system suitability. Further guidance in this regard may be obtained from the ICH Q2A document (Ref. 6).

III. CHARACTERIZATION AND QUALIFICATION OF CELL SUBSTRATES, VIRAL SEEDS, BIOLOGICAL RAW MATERIALS AND VACCINE PRODUCTION

In this section we describe strategies for cell banking, the stages of production and adventitious agent testing or characterization recommended at those stages, and the factors that could influence your choice of tests. Although your tests should address issues specific to your viral vaccine product, the following recommendations represent general guidance. To maximize the value of the recommended tests, it is important that you perform each test at the stage of production at which contamination is most likely to be detected.

A. PROPERTIES OF THE CELL SUBSTRATE

The cell substrate you choose for production of your viral vaccine should be well characterized. Your application should address the following issues.

1. Properties relevant to cell substrate selection

The safety and efficacy of the vaccine might depend on the characteristics of the cell substrate. Ideally, all stages of development of a potential vaccine candidate starting with the initial isolation of the viral strain from a screened and suitable donor through to a licensed vaccine should be performed using a well-characterized cell substrate.

Assuring that the vaccine virus strain remains stable during passage in cell culture is an important component of cell substrate selection. Different cell types may apply different selective pressures on the vaccine virus, which could alter its sequence and possibly its phenotype. For example, when Sabin poliovirus strains are grown in different cells, their likelihood of reversion to neurovirulence is different (Ref. 9). If attenuating mutations or other genetic markers (including expression of antigens relevant to immune response) of the vaccine virus are

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known, the influence of serial passage in the cell substrate on retention of these markers can be useful in characterizing the genetic stability of the vaccine virus.

Whatever starting materials are used for the generation of the cell substrate (e.g., parent cells or plasmids used for transfection), information about those starting materials and any characterization (e.g., sequence of the plasmid) that has been performed should be provided. If a sponsor starts with a primary cell to generate a novel cell substrate, complete information on donor screening and testing should be provided. See Sections III.A.2. for additional information on donor screening and testing.

2. Source

You should provide documentation of the species of origin and the tissue type of your cell substrate. You should also provide the medical history of the donor and the results of any screening and testing performed on the donor or on samples from the donor. Generally, human donors should be screened and tested in a manner comparable with whole blood or source plasma donors, as described in 21 CFR part 640. Animal donors should be screened and tested by applying similar concepts. They should be in general good health (except in cases where the cell substrate is derived from a tumor) and free from symptoms of infectious diseases. Ideally, animals intended to be donors should be quarantined prior to sacrifice or obtaining the sample to be used to generate the cell substrate (Ref. 10). Issues specific to bovine- and porcine-derived materials are discussed in sections III.A.2.f and III.A.2.g). The species of origin is highly relevant, because different animal species have different potential contaminants, and some animal cells might be capable of propagating human viruses that are of potential concern as adventitious agents. Tissue type is also an important element for you to describe and consider in deciding on testing strategies. For example, neuronal cells might harbor latent viruses (e.g., herpesviruses) or express infectious prion proteins (PrP) and should be evaluated for these potential adventitious agents. Additional considerations might apply to cells that are tumor-derived

3. History (including identifying characteristics) and other important characteristics

The history of your vaccine cell substrate must be identified according to 21 CFR 610.18(c)(1)(i) and should include: sources and testing of the raw materials to which it was exposed in its passage history; listing of any other agents grown in the facilities around the time of cell substrate passage; and the conditions under which it was passaged (including adherence to GLPs or cGMPs, if applicable). In some cases (e.g., Chinese hamster ovary [CHO] cells and Vero cells), multiple related cell lines or cell clones exist, and it is important to specify which of these related lines are being used, including the source you obtained it from and the passage (or population doubling) level at the time. You should provide documentation of all raw materials you used for the entire passage history, but it

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is particularly important for passages using bovine materials acquired during and after 1980, when BSE emerged in the U.K. You should document all known manipulations (adaptation, engineering, cloning) performed on the cell substrate since original isolation from a primary donor, whether they occurred prior to or after your receipt of the cells.

In addition, you must provide the following information for any cell substrates used for the production of viral vaccines (except that under 21 CFR 610.18(c)(3) these elements are not mandatory if you use primary cell cultures that are not subcultivated or that are subsequently subcultivated for only a very limited number of population doublings, in which case FDA still recommends that you provide them):

- results of all identity testing, including information about any characteristics (such as cytogenetic characteristics, required by 21 CFR 610.18(c)(1)(ii)) that could be used to identify the cells;
- results of all available adventitious agent testing (21 CFR 610.18(c)(1)(iv));

You should also provide the following:

- age, gender, and species of the donor;
- donor's medical history and the results of tests performed on the donor for the detection of adventitious agents;
- culture history of the cell line, including methods used for the isolation of the tissues from which the line was derived, passage history, medium used, and history of passage in animals;
- documentation of the history of human-derived and animal-derived materials used during passage of the cells; and
- documentation of any genetic material introduced into the cell substrate.

4. Growth Characteristics

You must characterize the *in vitro* growth characteristics (e.g., suspension or monolayer cultures, rate of growth) of your cell substrate (21 CFR 610.18(c)(1)(iii)), except if you use primary cell cultures exempted from that requirement by 21 CFR 610.18(c)(3). If the cells have a finite life expectancy, the total number of population doubling levels through senescence should be determined. You should perform tumorigenicity testing on continuous cell lines. A description of the tumorigenic property of cells is required for all diploid and non-diploid cells (21 CFR 610.18(c)(1)(ii)), but is not applicable to primary cell cultures that are not subcultivated or that are subsequently subcultivated for only a very limited number of population doublings (21 CFR 610.18(c)(3)).

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The microscopic appearance of your cell substrate should be stable from the master cell bank to the end-of-production cells. If there are specific markers that might be useful in characterizing your cell line (such as marker chromosomes, transfected genetic elements, specific surface markers, etc.), these should be analyzed for their stability at manufacturing passage levels.

5. Expression Characteristics

If your cell substrate is used to produce a recombinant protein, you should evaluate its ability to do so as a part of its characterization. This includes evaluating the copy number and stability of introduced nucleic acids and the quantity and quality of expressed proteins. In some cases, it might be appropriate for you to evaluate expression of other genes relevant to the cell phenotype. Some cell substrates might express (or might be induced to express) genes of endogenous or latent viruses. Such expression should also be described if it is associated with a safety concern or could be used to identify the cell. If viral sequences are expressed, you might need to assess their infectivity and potential interference with adventitious agent testing.

6. Susceptibility to adventitious agents

In developing comprehensive and appropriate testing strategies for adventitious agents, you should consider the susceptibility of the cell substrate to infection with viruses other than the vaccine strain. For example, because the cell substrates used for the production of licensed oral poliovirus vaccines (primary monkey kidney cells) were susceptible to contamination with certain simian viruses, such as SV40 or herpes B virus, specific tests were required to assay for these viruses in each lot. On the other hand, because human papillomaviruses cannot be propagated in cell culture, we do not generally recommend that sponsors assess virus production lot-by-lot for human papillomaviruses once the initial cell substrate and viral seed have been demonstrated to be free of these agents. The same concepts might be useful for you in developing new cell substrates and testing strategies for them.

7. Generation of Cell Substrate

You should characterize the cell substrate for the impact that the manipulations have had on the characteristics of the cell when you use cell substrates that have been generated by in vitro manipulations or engineered to express a viral antigen. For example, immortalization of primary cells with viral oncogenes to generate a complementing cell line for production of defective viruses or viral vectors will inevitably change the phenotype of the cell. Such manipulations might induce the expression of latent or endogenous viruses, and/or might cause the cells to become tumorigenic. In addition, a cell substrate that has been derived by cell cloning might have different characteristics from the parental cell line. Because it

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is derived from one or a few cells, it might not have characteristics representative of the original population from which it was cloned. Alternatively, a clone might be selected as the cell substrate because of its particular outlier characteristics, such as rapid propagation in culture or adaptation to particular cell culture conditions that modify its growth properties to enhance vaccine virus replication (e.g., development of suspension cell cultures from adherent cells). It is important that you thoroughly evaluate the characteristics of generated or engineered cell substrates, as it cannot be assumed that the parental cell characteristics were maintained following the manipulations used to generate the production cell substrate.

B. CELL BANKING

Cell banking assures that an adequate supply of equivalent, well-characterized cells exist for production over the expected lifetime of the product. In addition to providing a constant supply of biological starting material, cell banking provides you with the opportunity to undertake a comprehensive characterization of the cell substrate and to minimize the chance of adventitious agent contamination and/or to maximize the chance of detection of a contamination.

1. Cell Banking Strategies and Methods

Ordinarily, the cell bank system consists of two tiers: a master cell bank (MCB) and a working cell bank (WCB), often called a manufacturer's working cell bank (MWCB). The MCB represents a collection of cells of uniform composition derived from a single source prepared under defined culture conditions. Sometimes, a Parent Cell Bank, comprising vials of the progenitor cells to the Master Cell Bank, is also maintained.

The WCB is derived from one or more vials of cells from the MCB, which are expanded by serial subculture. The pooled cells are dispensed into individual vials and cryopreserved to form the WCB. One or more vials from the WCB are generally used for the production of a lot of a vaccine. If cells from more than one WCB vial are used, the cell suspensions should be pooled at the time of thawing. The population doubling level or passage level of cells used for production should not exceed an upper limit based on criteria established by you.

When using a two-tiered banking system, we generally recommend that you perform an extensive characterization of the MCB. Because all WCBs would be derived from the well-characterized MCB, your WCBs may be tested in a more limited manner, focusing on adventitious agents to which the WCB could have been exposed during expansion from the MCB. The use of a cell banking system might allow you to perform more limited testing of individual vaccine lots, focusing on adventitious agents to which the cells could have been exposed during

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manufacturing from the WCB. Alternatively, some manufacturers might choose to extensively characterize each WCB *in lieu* of thorough characterization of the MCB. The testing strategy chosen will depend on your own circumstance, but you should characterize at least one bank extensively. Testing of the MCB will be a one-time testing. However, in the absence of thorough characterization of the MCB, we may request a thorough characterization on the WCB used to produce each lot.

You may use other cell banking systems, for example, when the original source of your cell substrate is limited and/or the need to maintain a low passage level restricts expansion of cell numbers. Regardless of what cell banking system you use, cells at some level of expansion should be characterized completely. In either case, the derivation and cell bank designations should be thoroughly described and explained.

We recommend that all cell banks be stored in either the liquid or vapor phase of liquid nitrogen. Storage of cell banks in the vapor phase of liquid nitrogen might reduce the potential for cross-contamination. You should document in your biologics license application the location, identity, and inventory of individual ampoules of cells. Cell recovery or viability data should be documented to demonstrate cell stability under the freezing and storage conditions. You should store the MCB and WCB in two or more separate areas within the production facility as well as at a distant site in order to avoid loss of the cell substrate due to local disaster or malfunction. Access to these cell banks by your staff should be limited and controlled.

2. Qualification of Cell Banks and Primary Cells

The purpose of qualifying cell banks is to demonstrate their suitability for use in vaccine production. This section includes recommendations on testing for different types of cells and stages of cell banking; they also apply to the use of primary cells. Not all categories of cells (primary, diploid, continuous, or tumor-derived) will be suitable for all types of vaccines (live, inactivated, recombinant, or highly purified). Characterization of a cell bank is dependent on its use, and your particular use should guide your determination of which of the recommended testing is necessary and whether additional testing might be necessary. Consideration of the choice of tests will be based on the assessment of the risks that the cell substrate might represent for the product type and its clinical indication.

Testing to qualify the MCB should be performed directly on the cell bank, except when it is more appropriate to test cell cultures derived from the cell bank.

Tests that you might perform on your MCB include tests for bacteria, fungi, mycoplasma, and viruses (e.g., *in vitro* and *in vivo* testing, specific tests for retroviruses, and specific tests for viruses known to exist in the species of origin or

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that could be acquired during serial passage in cell culture). Other specific tests might be warranted, depending on the passage history of the cell substrate. Many of the tests in tissue culture or animals were originally promulgated in 21 CFR part 630 and remained in the regulations until revision of the regulations in 1996 (61 FR 40153). FDA removed many of the specific tests from the regulations to allow manufacturers flexibility in choosing appropriate new tests that might become available. Details of these tests are presented in Section IV. If the species from which your cells were derived is susceptible to infection with *Mycobacterium tuberculosis*, an appropriate test should be performed for this agent as well. Depending on the source species, you may consider specific tests such as the test for avian leukosis virus for product produced in avian cells. If you are using primary monkey kidney cell cultures, for example, you should test for species-specific simian agents, such as SV40, and herpes B virus, and other simian agents, such as simian polyomaviruses and simian cytomegalovirus.

Either the MCB or all animal-derived reagents to which it was exposed should be shown not to contain infectious agents that could potentially have been present in the source animal. Some of the tests that are relevant in selected circumstances are described in Section IV, including the tests for bovine (Section IV.A.2.f) and porcine agents (Section IV.A.2.g). Additional considerations are described for serum in Section III.D.1 and for trypsin in Section III.D.2. In the case of bovine serum, the issue of TSE agents will need to be considered. Finally, testing should be performed in most circumstances to determine if the cells produce retroviruses or retrovirus particles. This testing is described in Sections IV.A.2.c and IV.A.2.d.

Extensive characterization of the MCB should be done once and should also address the considerations in Section III.B.1. Methods that may be used for identity testing of the MCB are discussed in Section IV.B.3.

3. Special Considerations for Primary Cells

Primary cells are obtained directly from the tissues of healthy animals. Major successes in the control of viral diseases, such as poliomyelitis, measles, mumps, and rubella, were made possible through widespread use of vaccines prepared in primary cell cultures, including those from chicken or duck embryos, the kidneys of monkeys, and other tissues. Cultures of monkey kidney cells have been used for the production around the world of IPV and OPV for prophylaxis of poliomyelitis for almost 50 years. Cultures of primary chick embryo fibroblasts or chicken eggs continue to be used to manufacture safe and effective influenza, measles, and mumps vaccines.

Primary cells are more likely to contain adventitious agents than banked, well-characterized cells. This concern with primary cells is mitigated by rigorous qualification of source animals and primary cell substrates. Animals from which primary cultures are established should be from specific-pathogen-free closed

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flocks, herds, or colonies, when feasible. The term “closed” refers to the maintenance of a group (flock, herd, and colony) free from introduction of new animals (new genetic material). Animals that are not from closed flocks, herds, or colonies should be quarantined and thoroughly evaluated for a period sufficient to detect signs of disease or infection before introducing them into the flock, herd, or colony. Animals should be screened serologically for appropriate adventitious agents to determine their suitability as a source for the primary cell substrate. Animal husbandry practices should be described in the application.

Embryonated chicken eggs used as the source of chick embryo tissue for the propagation of viral vaccines should be derived from flocks certified to be free of *Salmonella pullorum*, avian tuberculosis, fowl pox, Rous sarcoma virus, avian leucosis virus, reticuloendotheliosis virus, and other adventitious agents pathogenic for chickens. Certificates of Analysis (COA) should be submitted for documentation. If eggs are procured from flocks that are not certified, tests should be performed to demonstrate freedom of the vaccine from such agents.

Control cells are often used with primary cell substrates and are described in more detail in Section II.B.4.

4. Special considerations for Diploid Cell Strains

Diploid cell strains are established from primary cell cultures by expansion and cell banking. These types of cells have a finite life span and are not immortal like cell lines. Diploid cells usually retain a diploid or near diploid karyotype, a characteristic that also differs from cell lines, which are generally aneuploid or non-diploid.

You should determine the karyotype of your cell source, as it might be helpful to establish identity and to characterize a cell strain. Such analyses will establish the diploid character of your cells and determine its freedom from contamination with other cell lines. It might also be useful to monitor the genetic stability of the diploid cell strain throughout production. Other tests of identity (see Section IV) might be appropriate.

Animal tumorigenicity testing is not needed if you are using genetically unmodified diploid cell strains, such as MRC-5 and WI-38, and FRhL-2, because their extensive previous characterization and well-defined non-tumorigenic phenotype satisfy the requirement in 21 CFR 610.18(c)(1)(ii) that they be described with respect to tumorigenicity. However, tumorigenicity testing is needed for a novel diploid cell substrate because such testing is needed to satisfy the requirement in 21 CFR 610.18(c)(1)(ii) that you describe its tumorigenicity.

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5. Special Considerations for Continuous Cell Lines

Some continuous cell lines, including Vero cells and CHO cells, have been used as substrates for licensed biologicals. Cell lines might have biochemical, biological, and genetic characteristics that differ from primary or diploid cells (e.g., they are typically aneuploid and have accumulated genetic changes). Because the mechanism by which most cell lines become immortal is generally unknown, and because some cell lines form tumors when inoculated into immunodeficient rodents, there are additional concerns for continuous cells lines compared with diploid cells, including potential adventitious agents and residual DNA.

There has been considerable discussion internationally on general criteria for the acceptability of products prepared in cell lines (e.g., hormones, blood components, viral vaccines). These products should be purified to be free (see Section VI. Glossary) of adventitious agents and residual cells and should have low levels of cell-substrate DNA. When potential biological activity of residual cell substrate DNA is a concern, you should introduce procedures that extensively remove or degrade DNA. If you are considering the use of cell lines, we encourage you to develop and evaluate efficient methods for the purification of your product as an essential element of any product development program.

Because certain cell lines might express endogenous viruses (e.g., retroviruses), tests capable of detecting such agents should be carried out on cells grown under production conditions (See Section IV.A. Testing for Adventitious Agents). If specific contaminants have been identified as endogenous agents in the MCBs and WCBs you are using, it is essential that you demonstrate their clearance (inactivation and/or removal) to a defined level by the purification procedures you use in production. You may obtain further guidance on this topic from the ICH Q5A document (Ref. 2).

Under 21 CFR 610.18(c)(ii), you must describe cell lines with respect to tumorigenicity. Cell lines could acquire tumorigenic properties with increasing passage levels. It is therefore important that you limit the passage level of the cells used in production and that you characterize cells at or beyond this end-of-production limit. The maximum end-of-production passage level should be based on data derived from production cells expanded under comparable or analogous conditions to the production conditions. Cells from either the MCB or the WCB may be expanded for this evaluation. Tumorigenicity testing should be performed at or beyond this level. If you increase your defined limit for end-of-production, you should support the safety of this with data from cells that have been expanded to a passage level or population doubling level that is equal to or greater than your newly proposed end-of-production level.

6. Additional considerations for cell lines that are tumorigenic or tumor-derived

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You should perform additional testing if your cell lines are tumorigenic or derived from a tumor. You should assess cell lines that are tumorigenic or tumor-derived for potential oncogenic viruses and oncogenic substances (including nucleic acids), which could be associated with induction of a neoplastic process in a vaccine recipient. Test strategies for potential oncogenic viruses or oncogenic substances may be determined case-by-case, depending on the tissue type, source species, passage history, and extent of knowledge about the transforming event(s). (See section IV.B.1 for a discussion of tumorigenicity testing and section IV.B.2 for a discussion of oncogenicity testing.)

In cases where you know the transforming event (for example, if the cells were transformed by a known oncogene), your testing should demonstrate that your final product is free of the transforming agent. For example, if adenovirus sequences are used to transform a primary human cell to produce a cell line (e.g., 293 cells), then testing should demonstrate that the final product is free of the introduced viral sequences. Similarly, if a virus is used to transform cells, that virus and its genetic material should not be detectable in the final product using an assay with sensitivity sufficient to provide assurance of safety. Tumorigenic or tumor-derived cell lines for which the mechanism of transformation is unknown will require additional testing to ensure the absence of potential transforming and oncogenic agents. You should consult with CBER on which tests you should perform and which methods you should follow.

The testing recommended for qualification of cell substrates and cell banks should also be applied to rodent cell lines. However, because most rodent cell lines used for the production of biologicals are known to be tumorigenic, it is considered unnecessary to test rodent cell lines for tumorigenicity. A presumption is made that they will be tumorigenic, and the considerations described above apply. In addition, rodent cell lines are presumed to be capable of producing endogenous retroviruses. Assessment of the quantity and type of retroviruses produced should be performed. Infectivity assays for retroviruses are also recommended. You should only use rodent cell lines if your product can be sufficiently purified to demonstrate levels of viral clearance that assure the final product is not contaminated with retroviral particles. You may obtain additional guidance on viral clearance validation from the ICH Q5A document (Ref. 2).

7. End-of-Production Cells

You should characterize cells from the actual end-of-production of one or more lots or cells that are expanded to or beyond the end of production passage level. Such cells are referred to as End-of-Production Cells (EOPC). You should demonstrate the stability of the cell substrate characteristics using your EOPC. Your characterization should include growth characteristics, tumorigenic phenotype, expression of endogenous viruses, stability of expression of the inserted or engineered genes, and genetic stability.

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C. VIRAL SEEDS

The methods used to bank stocks of vaccine viral seeds are similar to those used in cell banking. As with cell banks, you should document passage history and derivation history of viral seeds. Your description should include donor screening, testing and donor medical history. Any manipulation of the viral phenotype, such as cold-adaptation, development of temperature-sensitivity, or attenuation of virulence, should be well documented and described. Any genetic manipulations, such as reassortment or recombination, should also be well documented and described, including determining the nucleic acid sequences and sourcing of each biological starting material (e.g., plasmids, parental viruses).

These vaccine virus banks are commonly referred to as the master viral seed (MVS) and working viral seed (WVS). Viral seeds should be stored in liquid nitrogen and in more than one location within a manufacturing facility or at a distant site for security reasons. You should assess your viral seed for its growth characteristics on your production cell substrate, tissue tropism, genetic markers, viability during storage, genetic stability through production, attenuation (if applicable), and its absence of adventitious agents. If attenuation or derivation is achieved by passage through different cell types from different species your viral seed should be assessed for absence of adventitious agents from all species that they might have been exposed to from isolation, through passage, and during production, including those that might be present in the raw materials used at each of these stages.

1. Master Viral Seed

You should extensively characterize your MVSs. In addition, you should demonstrate the stability of genotype and phenotype for a number of passages beyond the level used in your production. For example, if your MVS is considered to be at passage 47, your WVS at passage 48, your production cultures inoculated with WVS at passage 49, and your production process allows for only a single round of viral replication, then it would be appropriate for you to assess stability of your vaccine virus at passages 47 through 51 (5 passages).

You should perform tests for identity (which could necessitate sequencing the entire vaccine virus), bacterial and fungal sterility, the presence of mycoplasmas, *Mycobacterium tuberculosis* (if appropriate), adventitious viruses (in vitro and in vivo tests), the viral phenotype (e.g., tissue tropism, attenuation properties, temperature sensitivity), and genetic stability. You should also consider specific tests for agents that might be present in the seed due to its passage history.

In some cases, the virulence and the broad host range of your vaccine virus might complicate in vitro and in vivo adventitious agent testing. Testing for adventitious

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agents might then require neutralization of viral seed. Preferably, neutralizing antibodies should be monoclonal and prepared in a species other than the cells in which the MVS was prepared. In addition, due to the potential for cross-neutralization of adventitious human viruses, neutralizing antibodies should generally not be prepared from human or primate serum. It is important that you demonstrate that the neutralization procedure does not interfere with detection of adventitious viruses. Sometimes, it is not possible to efficiently neutralize a viral seed. In such cases, you may choose alternative strategies, including testing smaller quantities of seed, subculture onto fresh target cells in the in vitro adventitious agent test, or introduction of additional tests (e.g., PCR, Antibody Production assays (Ref. 11)).

Assessment of neurovirulence is often appropriate, and we recommend that you consult with CBER on appropriate animal models, methods, and scoring systems for this assessment before you initiate such studies. For viruses that are neurovirulent or might revert to neurovirulence (e.g., polioviruses), it might be necessary to assess neurovirulence not only on the MVS or an end-of-production passage level virus stock, but also on the product lot-by-lot.

2. Working Viral Seed

You may subject working virus seeds (WVSs) to less rigorous characterization than the MVSs from which they were derived. Once you have demonstrated your MVS to be free of adventitious agents from the species to which your vaccine virus had been exposed during its isolation and passage history, then you only need to show that your WVS is free of adventitious agents from the species you used to generate the WVS (e.g., production cells and raw materials used in propagation and processing).

D. BIOLOGICAL RAW MATERIALS AND ANCILLARY REAGENTS

You should test your raw materials and ancillary reagents and certify them for use in your vaccine production, including during the production of your cell banks and viral seeds. Raw materials derived from animals or humans should be tested and certified to be free from adventitious agents of the species from which they are derived. You should document whether you are relying on the vendor's COA or whether you are testing the raw materials.

If your documentation for the raw materials used in the passage of your cell substrate is not adequate, you should test the cell substrate itself for adventitious agents of the relevant species. For human-derived raw materials and reagents, your documentation should include sourcing from appropriately screened and tested donors (using 21 CFR 640.3 and 640.63 as guidelines) or use of products that are already licensed for human use. Testing and sourcing of bovine-derived materials and special considerations for

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porcine-derived materials are discussed in Section III. All biological raw materials should be free of adventitious agents, including bacterial and fungal agents, cultivatable and non-cultivatable mycoplasmas, mycobacteria, and viruses. If your process includes removal or inactivation of potential infectious contaminants from biological raw materials, you should validate this process.

1. Serum

The serum used in vaccine production (including the production of the cell banks and viral seeds), such as bovine serum used in culture medium or human serum albumin used to stabilize vaccine virus, should be tested and certified. Human serum albumin should be from a source licensed for human use in the U.S. or derived from donors who have been appropriately screened and tested (21 CFR 640.3 or 640.63). Bovine serum should be free of adventitious agents, including bacterial and fungal agents, mycoplasmas, mycobacteria, and bovine viruses (Section IV.A.2.f).

Additionally, you should consult with CBER to ensure that you take the appropriate actions to minimize BSE risk.

2. Trypsin

You should clearly identify the species from which the trypsin used in your vaccine production (including the production of the cell banks and viral seeds) is derived. If bovine trypsin is used, the concerns identified in Section IV.A.2.f, "Testing and Sourcing of Bovine-Derived Materials," apply. If porcine trypsin is used, it should be tested in accordance with the recommendations described in Section IV.A.2.g, "Testing of Porcine-Derived Reagents."

3. Amino Acids

You should document the source of the amino acids used in growth medium or in production. You should also consult with CBER to ensure that you take the appropriate actions to minimize BSE risk.

4. Other Biological Reagents

You should control and test other biological reagents used during manufacturing, such as transferrin, insulin, or other growth factors used in growth medium, as appropriate for their species of origin. If derived from humans, the ancillary product should either be licensed in the U.S. or derived from donors for whom appropriate screening and testing have been applied (if appropriate, comparable with blood or source plasma donors, see 21 CFR 640.3 or 640.63).

Under 21 CFR 610.15(c), you may not add penicillin to the production substrate of

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viral vaccines. Also, other beta-lactam antibiotics should not be present in production cell cultures.

E. CONSIDERATIONS IN TESTING AT DIFFERENT STAGES OF PRODUCTION

See Appendix 1 for a list of tests to consider at each stage of production for a viral vaccine.

1. Cell Banks

Qualification of cell banks is discussed in Section III.B.

2. Pre-Production Cells

An identity test may be performed on cells directly prior to production.

3. Pre-filtered Harvest or End-of-Production Cells

In general, the stage at which adventitious agents are most likely to be found is the stage at which the most extensive adventitious agent testing should be performed for each product. For many viral vaccines, the pre-filtered harvest is the stage of manufacture that is most concentrated and at which the least processing has been performed. For these reasons, this might be the best stage of production for testing for adventitious agents.

In addition to testing the viral or vaccine bulk harvest for cultivatable mycoplasmas, as required under 21 CFR 610.30 requires, you should also test the viral or vaccine bulk harvest for non-cultivable mycoplasmas (and spiroplasmas, if appropriate), and adventitious viruses by in vitro and in vivo methods. This might include a test for hemadsorbing viruses. If the production process is capable of inducing expression of retroviruses, PCR-based RT testing or infectivity studies might also be recommended at this stage. If appropriate, a test or tests for *Mycobacterium tuberculosis* should be performed.

As discussed for the MVS (Section III.C.1), if the assay system used for in vitro or in vivo adventitious virus testing is capable of supporting replication of the vaccine virus, and if the vaccine virus replicates to levels that interfere with the adventitious agent tests, then it might be necessary to neutralize the vaccine virus prior to performing these tests. If neutralization is difficult to achieve, the other considerations in Section III.C.1 might be applicable.

If cells survive the production process (e.g., if the vaccine virus does not result in a lytic infection), end-of-production cells may also be tested for adventitious agents.

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If multiple harvests are performed for a single vaccine lot, testing should be performed on each individual harvest in order to avoid dilution of a potentially contaminated harvest with uncontaminated harvests.

4. Control Cells

Recommendations for testing of control cells are discussed in Section II.B.3 and 4.

5. Post-Filtered Harvest or Final Bulk

The post-filtered harvest or final bulk should be tested for bacterial and fungal sterility. Other testing might be appropriate for the safety, identity, purity, potency, and quality of the final bulk. The appropriate test will depend on the product. These include testing for levels of residual cellular proteins and cellular nucleic acids. If the cell line used to produce the vaccine is known to produce viruses other than the vaccine virus, the clearance of these viruses should be demonstrated in the production processes. Guidance regarding viral clearance may be sought from the ICH Q5A document (see Ref. 2). If processes are not validated for clearance of residual cells, a test might be recommended at this stage.

6. Final Filled Product

Under 21 CFR 610.12, the sterility of bulk and final filled biologics must be demonstrated by specific tests listed in that section, except for products listed in subsection (g), including products granted an exemption under subsection (g)(4)(ii). Such an exemption may be granted for oral products, but those products should still be assessed by the microbial limits test for bioburden (Ref. 13). Under 21 CFR 610.11a, each lot of inactivated influenza vaccine must be assayed for endotoxin in comparison to a reference preparation provided by FDA. Other final filled products should also be assessed for endotoxin levels. In addition, a general safety test for detecting extraneous toxic contaminants, under 21 CFR 610.11, is required on final filled containers, except as described in 21 CFR 610.11(g). Other tests should be performed to assess the safety, identity, purity, potency, and quality of the final filled product. These tests are determined case-by-case depending on the product and should be described in your IND and BLA submissions.

When downstream processing provides clearance, concentration or dilution of the product, the final filled product stage might not be the best stage for performing certain tests (adventitious agents).

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IV. DESCRIPTION OF QUALITY-CONTROL TEST METHODS

A. TESTING FOR ADVENTITIOUS AGENTS

Your biological starting materials should be characterized to ensure that they are free from extraneous infectious organisms such as bacteria, fungi, cultivatable and non-cultivable mycoplasmas and spiroplasma, mycobacteria, viruses, rickettsia, protozoa, and the agent(s) responsible for transmissible spongiform encephalopathies (TSEs), where possible. For a substance to be considered free of a contaminant, your assay should demonstrate, at a predefined level of sensitivity, that a certain quantity of the substance is free of that contaminant. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate the absence of that contaminant. If an adventitious agent is known to be present in your cell substrate or viral seed, then you should demonstrate that your production process is sufficiently robust to eliminate or inactivate the agent within an appropriate margin of safety. You should avoid exposure of your product to agents that are known to be infectious for humans (other than the vaccine virus) and/or to agents for which there are no appropriately sensitive validated testing procedures. You should provide documentation indicating that you have appropriate and sufficient controls in place to avoid such exposure.

Many of the recommended tests described later in this document were originally promulgated in 21 CFR part 630 but were removed when the regulations were revised in 1996 (61 FR 40153). FDA removed many of the specific tests from the regulations to allow manufacturers flexibility in choosing appropriate new tests that might become available. For each of the suggested adventitious agent tests, alternatives such as those recommended by the World Health Organization (WHO) or the European Pharmacopoeia (EP) might be considered if justified with data showing sensitivity comparable to the recommended test. Different inoculation strategies for testing cell bank lysates and viral harvests may be acceptable when justified in the context of the entire testing program.

The appropriate test for adventitious agents will vary depending on a variety of factors, including the origin of the cell substrate and its history. We encourage manufacturers to consult with us regarding the appropriate tests. See sections II and III for additional information on factors to take into account in selecting appropriate tests.

1. In Vivo Tests

In the development of viral vaccines, in vivo adventitious agent testing includes inoculation of adult and suckling mice and inoculation of embryonated chicken eggs. Animal antibody-production tests may be performed when the potential for exposure to rodent viruses exists, for example, through exposure to rodents or rodent cells. These tests also may be performed in other species to detect species-

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specific viruses. For example, inoculation of rabbits or guinea pigs may also be used to detect additional agents, when appropriate.

a. Adult Mice

This test detects adventitious viruses including lymphocytic choriomeningitis virus (LCMV), coxsackieviruses, flaviviruses, and rabies virus.

Each of at least 20 adult mice weighing 15-20 grams should be inoculated intraperitoneally with 0.5 mL and intracerebrally with 0.03 mL of the material to be tested. The mice should be observed daily for 21 days. Each mouse that dies after the first 24 hours of the test, or is sacrificed because of illness, should be necropsied and examined for evidence of viral infection by gross observation and intraperitoneal and intracerebral inoculation of appropriate homogenized tissue into at least five additional mice, which should each be observed daily for 21 days. The material may be used only if at least 80% of the originally inoculated mice and at least 80% of each subsequently inoculated group of mice remain healthy and survive the observation period, and if none of the mice show evidence of a transmissible agent or other viral infection, other than agents known to be a component of the tested material (i.e., vaccine strains of virus, when relevant).

When this test is used to evaluate cell substrates, a lysate of cells and their culture medium (equivalent to 10^7 cells per mL) should be inoculated. When virus seeds are tested, it might be necessary to neutralize the vaccine virus prior to inoculation into mice. If the vaccine virus needs to be neutralized in order to perform a valid test, the source of the antiserum and its influence on test results should be considered; in general, antiserum used for neutralization should not be of human or simian origin or from the same cell type used for production of the vaccine virus.

b. Suckling Mice

This test detects adventitious agents including many human viruses, such as coxsackievirus types A and B (type B is also detectable in cell culture) and other picornaviruses such as polioviruses and echoviruses, alphaviruses, bunyaviruses, (including phleboviruses and nairoviruses), arenaviruses, flaviviruses, rabies, and herpesviruses (such as herpes simplex virus). This test can also detect many murine agents.

Each of at least 20 suckling mice less than 24 hours old should be inoculated intraperitoneally with 0.1 mL and intracerebrally with 0.01 mL of the material to be tested. The mice should be observed daily for at least 14 days. Each mouse that dies after the first 24 hours of the test, or is sacrificed because of

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illness, should be necropsied and examined for evidence of viral infection by gross observation and intraperitoneal and intracerebral inoculation of appropriate tissue into at least five additional mice, which should each be observed daily for 14 days. In addition, a blind passage (via intraperitoneal and intracerebral inoculation into at least 5 additional mice) should be made of a single pool of the emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test. The material may be used only if at least 80% of the originally inoculated mice and at least 80% of each group of subsequently inoculated mice remain healthy and survive the entire observation period and if none of the mice show evidence of a transmissible agent or other viral infection, other than agents known to be a component of the tested material (i.e., vaccine strains of virus, when relevant).

When this test is used to evaluate cell substrates, a lysate of cells and their culture medium (equivalent to 10^7 cells per mL) should be inoculated. When virus seeds are tested, it might be necessary to neutralize the vaccine virus prior to inoculation into mice. If the vaccine virus needs to be neutralized in order to perform a valid test, the origin of the antiserum and its influence on test results should be considered as noted above.

c. Guinea Pigs

This test detects *Mycobacterium tuberculosis* and adventitious viruses including paramyxoviruses (including Sendai virus), reoviruses, and filoviruses.

Each of at least 5 guinea pigs each weighing 350-450 grams should be inoculated intraperitoneally with 5 mL and intracerebrally with 0.1 mL of each material to be tested. The animals should be observed daily for at least 42 days. Each animal that dies after the first 24 hours of the test, or is sacrificed because of illness, should be necropsied. All remaining animals should be sacrificed and necropsied at the end of the observation period. The material may be used only if at least 80% of the originally inoculated animals remain healthy and survive the observation period and if none of the animals shows evidence of a transmissible agent or other viral infection, other than agents known to be a component of the tested material (i.e., vaccine strains of virus, when relevant).

When this test is used to evaluate cell substrates, a lysate of cells and their culture medium (equivalent to 10^7 cells per mL) should be inoculated. When virus seeds or production harvests are tested, it might be necessary to neutralize the vaccine strain prior to inoculation into animals.

In vitro methods, such as culture and PCR, are also acceptable for identifying *Mycobacterium tuberculosis* when validated.

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d. Rabbits

This test detects simian herpes B virus.

Each of at least 5 healthy rabbits each weighing 1500-2500 grams should be inoculated intradermally in multiple sites with a total of 1.0 mL of the material to be tested and subcutaneously with 2.0 mL of the material to be tested. The animals should be observed daily for at least 30 days. Each animal that dies after the first 24 hours of the test, or is sacrificed because of illness, should be necropsied. The material may be used only if at least 80% of the originally inoculated animals remain healthy and survive the observation period, and if none of the animals show evidence of a transmissible agent or other viral infection, including lesions at the site of inoculation, other than agents known to be a component of the tested material (i.e., vaccine strains of virus, when relevant).

When this test is used to evaluate cell substrates, a lysate of cells and their culture medium (equivalent to 10^7 cells per mL) should be inoculated. When virus seeds are tested, it might be necessary to neutralize the vaccine strain prior to inoculation into animals.

Alternative tests for herpes B virus might also be acceptable if justified.

e. Embryonated Chicken Eggs

This test detects adventitious agents including:

- by the allantoic route: orthomyxoviruses (influenza virus) and paramyxoviruses (mumps, measles, parainfluenza viruses), alphaviruses, and vesiculoviruses; and
- by the yolk sac route: herpesviruses, poxviruses, rhabdoviruses, as well as rickettsiae, mycoplasmas, and bacteria.

A sample volume, equivalent to at least 100 doses, or 10 mL, whichever represents a greater volume, should be used in egg testing. At least 10 embryonated eggs, 10 to 11 days old, should be inoculated by the allantoic route using 0.5 mL per egg. Following incubation at 35°C for 72 hours, the allantoic fluids should be harvested, pooled, and passaged by the same route into fresh, embryonated eggs, 10 to 11 days old, using 0.5 mL per egg and incubated at 35°C for 72 hours. Both the initial pool and the passaged harvest should be tested for the presence of hemagglutinating agents with red cells from guinea pigs, humans (type O), and an avian species. The tested material

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passes the test if all the embryos appear normal and there is no evidence of hemagglutinating agents.

At least 10 additional embryonated eggs, 6 to 7 days old, should be inoculated by the yolk sac route using 0.5 mL per egg. Following incubation at 35°C for at least 9 days, the yolk sacs should be harvested and pooled. A 10% suspension of yolk sacs should be passaged by the same route into the yolk sacs of fresh embryonated eggs, 6 to 7 days old, using 0.5 mL of inoculum per egg and incubated at 35°C for at least 9 days. The material passes the test if all the embryos in both the initial test and the passage appear normal.

When this test is used to evaluate cell substrates, a lysate of cells and their culture medium (equivalent to 10^7 cells per mL) should be inoculated. When virus seeds are tested, it might be necessary to neutralize the vaccine strain prior to inoculation into animals. If vaccine virus needs to be neutralized in order to perform a valid test, then antisera used for neutralization should not be of human, simian, or avian origin.

f. Antibody Production Tests

Antibody production tests are performed in the species-appropriate model (often rodents such as hamsters, rats or mice, but sometimes chickens) when the potential exists for exposure to rodent viruses. The test article is inoculated into specific-pathogen-free (SPF) animals that are subsequently tested for antibodies to specific agents. Agents detected in hamster antibody production (HAP) test include: lymphocytic choriomeningitis virus (LCMV), pneumonia virus of mice (PVM), reovirus type 3 (Reo3), Sendai virus, and simian virus 5 (SV5). The agents detected in rat antibody production (RAP) test include: Hantaan virus, Kilham rat virus (KRV), LCMV, mouse adenovirus, mouse encephalomyelitis virus (Theilers, GDVII), PVM, rat coronavirus (RCV), Reo3, sialodacryoadenitis virus (SDAV), Sendai virus, and Toolan virus (HI). The agents detected in mouse antibody production (MAP) test include: ectromelia virus, mouse rotavirus (EDIM) Hantaan virus, LCMV, lactic dehydrogenase virus (LDM), minute virus of mice (MVM), mouse adenovirus (MAV), murine cytomegalovirus (MCMV), mouse encephalomyelitis (Theilers, GDVII), mouse hepatitis virus (MHV), PVM, polyoma virus, Reo3, Sendai virus, thymic virus, and K virus (a mouse pneumonitis virus related to polyomaviruses).

A specific in vivo test for LCMV may be warranted when specific concerns about LCMV exist. This in vivo test involves intracerebral inoculation of mice used in the MAP test with live virulent LCMV to detect interference from non-lethal strains. Animals infected during the MAP test with non-virulent strains of LCMV will survive this challenge. If you use alternative

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methods, such as PCR, you should provide data showing sensitivity comparable to that of the suggested test.

Antibody tests such as “Test for Extraneous Agents Using Chicks” (Ref. 14), might also be useful in certain cases where the vaccine virus interferes with in vitro culture assays for adventitious virus testing.

2. In Vitro Tests for Viruses

a. Cell Culture Safety Test

Cell culture safety tests can detect a variety of adventitious viruses that include cytopathic viruses, hemadsorption viruses, and hemagglutinating viruses. You should select cells appropriate for such tests and relevant to potential exposures of your materials including:

- monolayer cultures of the same species and tissue type as that used for production;
- monolayer cultures of human diploid cells; and
- monolayer cultures of monkey kidney cells.

Human diploid cells and monkey kidney cells are commonly used and are often susceptible to infection by many of the viruses listed below. Specialized cell culture testing might be needed to detect growth of other viruses.

i. Human Diploid Cells

Human viruses: herpesviruses (including herpes simplex virus, varicella zoster virus, and cytomegalovirus), adenoviruses, coronaviruses, reoviruses, alphaviruses, rubella, flaviviruses, rabies, enteroviruses, certain strains of hepatitis A virus, poliovirus, coxsackie B virus, echovirus, rhinoviruses, orthomyxoviruses, and paramyxoviruses.

Simian viruses: SCMV (simian cytomegalovirus).

ii. Monkey Kidney Cells

Human viruses: enteroviruses, coxsackie B viruses, echoviruses, orthomyxoviruses, paramyxoviruses, herpes simplex virus, poxviruses, polyomaviruses, rotavirus, alphaviruses, rubella, flaviviruses, rabies viruses, vesiculoviruses, filoviruses, influenza viruses, bunyaviruses (including phleboviruses and nairoviruses), arenaviruses, and reoviruses, polioviruses, rhinoviruses, adenoviruses (some strains).

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Simian viruses: herpes B virus.

Methods:

An appropriate volume should be inoculated onto monolayer cultures of at least 3 cell types (see preceding discussion for appropriate cell types). The sample to be tested should be diluted as little as possible. The cell cultures should be observed for at least two weeks. After two weeks of observation, supernatants or lysates are subcultured onto fresh cells and observed for at least an additional two weeks. This subculture onto fresh cells might help to distinguish between non-specific CPE and virus-induced CPE, as toxic effects of the initial specimen or length in culture will be diluted, whereas virus-induced CPE should be amplified. If the possibility of contamination with human or simian cytomegaloviruses is a consideration, the first set of human cell cultures should be observed for at least four weeks unless a shorter incubation period is shown to be equally sensitive. A test is considered satisfactory only if there is no evidence of adventitious agents and at least 80% of the cultures are available for observation at the end of the observation period.

The volume to be tested should be determined based on your total culture or batch size and the number of cells you require to produce a dose of vaccine. Generally, you should use 10^7 cells or cell lysate equivalent to that number of cells when testing the cell substrate. When it is not possible to directly test cells or cell lysates, a corresponding quantity of cell culture supernatant should be tested. Historically, 500 doses or 50 mL of neutralized vaccine harvest, pool, or bulk (whichever represented more virus) were used as the inoculum onto each indicator cell culture. Under some circumstances, where neutralizing the vaccine strain has been difficult, smaller amounts were tested. When virus seeds are tested, it might also be necessary to neutralize the vaccine strain prior to inoculation onto indicator cells.

You may assess virus replication in cell-culture tests by various means, including development of cytopathic effect. If appropriate for your product, testing for hemadsorption or hemagglutination at the end of the observation period could facilitate the detection of non-cytopathic viruses. Hemadsorption refers to the ability of infected cells to bind to red blood cells (RBCs), while hemagglutination refers to the ability of infected cell supernatants to agglutinate RBCs. The test for hemadsorbing and hemagglutinating viruses is generally performed at the end of the observation period using guinea pig, chicken, and human type O RBCs, which are added independently to different portions of the cell cultures or to aliquots of the supernatants at 2-8°C for 30 minutes, and observed for hemadsorption and hemagglutination, respectively. This is followed by incubation with RBCs for 30 minutes at room

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temperature, followed by observation for hemadsorption/hemagglutination. Red blood cells from rhesus monkeys have occasionally been used for detection of certain respiratory viruses.

The test for hemadsorbing and hemagglutinating viruses may also be performed at the end of production, directly on cells used for production or on control cell cultures. Historically, one quarter to one third of the control vessels were tested for hemadsorption viruses.

b. Transmission Electron Microscopy (TEM)

TEM can detect viral particles in a cell substrate, including those from endogenous retroviruses. Under some circumstances, it might be appropriate to pre-treat cells with chemical or inducing agents to activate production of endogenous or latent viruses (Ref. 15). While TEM is fairly insensitive, it is a generic assay that can detect adventitious agents of many types. TEM can also be used to estimate the concentration of viral particles to support validation of viral clearance. Methods include negative staining and thin section. A discussion of these methods is provided in Bierley et al. (Ref. 16).

c. Biochemical Tests for Retroviruses

Retrovirus testing using reverse transcriptase (RT) assays should be performed on cell-free culture medium to detect retroviruses. RT assays can detect any retrovirus, as all retroviruses encode and contain RT. You should test cell substrates, viral seeds, and/or harvests of all viral vaccines produced in mammalian cell substrates (or that use or include mammalian-derived materials) using a highly sensitive PCR-based RT (PBRT) assay. If you plan to use conventional RT assays, you should provide a strong justification for using a less sensitive assay.

You should validate the performance of the PBRT for your product, especially with regard to the lower limit of detection for both manganese- and magnesium-dependent retroviral RTs, the specificity, and the reproducibility of the assay. The lower limit of detection should be comparable with the published literature (Ref. 17). If you obtain a positive result with the assay, you might need to perform infectivity studies to demonstrate whether the source of the positive result is an infectious retrovirus. The Center for Biologics Evaluation and Research (CBER) can provide you with further information on infectivity studies upon request.

The stage of manufacture at which you should perform the PBRT test is dependent on the manufacturing process of your product. For example, products manufactured from primary cells might need to be assessed lot-by-lot. In cases when control cell cultures are set up in parallel with production

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cells (Section II.B.4), PBRT testing of cell-free medium from the control cell culture should be performed. Products manufactured from an established PBRT-tested cell bank and PBRT-tested viral seed, generally, do not need to be tested lot-by-lot.

CBER recognizes that some products and reagents have RT activity that does not represent adventitious infectious retroviruses (*e.g.*, some avian cell substrates, such as chick embryo fibroblasts, chick embryo dermal cells, and eggs). For avian cells that have RT activity, you should perform testing to assure the absence of major avian retroviruses. Major avian retroviruses include exogenous retroviruses, such as avian leucosis virus (ALV), avian sarcoma virus, and reticuloendotheliosis virus, and infectious endogenous retroviruses, such as RAV-0. You may perform this testing directly on the cell substrate. In some cases, careful documentation of the health of a specific pathogen free flock might be adequate to assure the absence of these viruses. CBER can provide you with further information upon request.

d. Infectivity Tests for Retroviruses

For non-murine retroviruses, infectivity testing on appropriate indicator cells (selected for their susceptibility to different retrovirus types) might be appropriate. Such testing strategies would be determined case-by-case and should be discussed with CBER before implementation. You should test rodent cell substrates and viral seeds that are potentially contaminated with murine retroviruses for infectivity using *Mus dunnii* cells or other highly susceptible cells and/or in S⁺L⁻ assays (Ref. 18). S⁺L⁻ assays may be done on feline PG4 cells (to detect amphotropic viruses), mink cells (to detect xenotropic viruses) or murine D56 cells (to detect ecotropic viruses). You may use a broadly reactive monoclonal antibody to detect viruses cultured in these assays. Infectivity tests that are capable of detecting ALV (Ref. 19) and other avian retroviruses might be appropriate for cell substrates of avian origin. Often, infectivity tests may be augmented with sensitive RT assays. Under some circumstances, for example when tumorigenic cell substrates are proposed for use, it might be appropriate for you to pre-treat cells with chemical agents known to induce reactivation or replication of endogenous or latent viruses.

e. PCR or Other Specific in vitro Tests

In cases where viruses cannot be readily grown in culture, PCR might be the most effective tool to assess a cell substrate for contamination with such viruses. Due to the specificity of PCR, you might need to perform multiple PCR assays in order to detect the full range of viruses detectable in a single more general biological assay. You should consider the tissue source and medical history of the donor from which the cell line was derived in

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determining what testing is appropriate. Specific PCR tests for human pathogens that you should consider include assays for hepatitis A, B, and C viruses, enteroviruses, human HIV-1, HIV-2, HTLV-1, HTLV-2, circoviruses, parvovirus B19, papillomaviruses, human polyomaviruses, human adenoviruses, Epstein-Barr virus, human cytomegalovirus, and human herpes viruses 6, 7, and 8. Specific tests for simian viruses that could infect humans include simian polyomaviruses (e.g., SV40), simian foamy virus (SFV), simian immunodeficiency virus (SIV), simian retrovirus (SRV), and simian T-cell lymphotropic virus (STLV). You also should consider using PCR assays that detect several agents using degenerate or consensus primers provided the sensitivity of these assays is sufficient to assure product safety.

Although less sensitive, you may consider the use of Southern blot hybridization analysis of DNA extracted from the cell substrate or tissue culture to assess for contamination with some viruses. Either PCR or Southern hybridization may be used to detect non-cytopathic agents in tissue culture tests.

f. Sourcing and Testing of Bovine-Derived Materials

You should source bovine-derived reagents in a manner that minimizes the risk of contamination from bovine spongiform encephalopathy (BSE). We recommend that you consult with CBER for more information.

We recommend that you test bovine-derived reagents using assays at least as sensitive as the cell culture and fluorescent antibody testing recommended in 9 CFR 113.53 and 113.47. We recommend the use of Vero cells and bovine cell culture, such as bovine turbinate cells. Agents of concern from bovine sources include: bovine parainfluenza Type 3, infectious bovine rhinotracheitis virus, rabies virus, reovirus, bovine adenoviruses, bovine respiratory syncytial virus, bovine parvovirus, bluetongue virus, and bovine viral diarrhea virus (including non-cytopathic strains).

We may request testing for other potential contaminants of bovine materials depending upon exposure history and species of origin of a cell substrate or reagent including circoviruses, bovine herpesviruses, bovine polyomavirus, bunyaviruses, and bovine retroviruses (such as bovine immunodeficiency virus and bovine leukemia virus). Some of these agents might be detected in cell culture, while others might require additional tests.

g. Testing of Porcine-Derived Reagents

The testing required for porcine adventitious viruses in products intended for use in animals (9 CFR 113.47) represents our minimum recommendations for products intended for use in humans. Agents of concern from porcine cells listed in 9 CFR 113.47(b)(1) and (6) include: porcine parvovirus, bovine viral

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diarrhea virus (BVDV), reovirus, rabies, porcine adenovirus, transmissible gastroenteritis virus (TGE), and porcine hemagglutinating encephalitis virus. At a minimum, you should test porcine trypsin for porcine parvovirus.

Other porcine agents that might be of concern, depending upon exposure history and species of origin of your cell substrate or reagent, include circoviruses, enteroviruses, porcine respiratory and reproductive syndrome virus (PRRS), porcine cytomegalovirus, porcine influenza viruses, pseudorabies virus, swine pox virus, swine fever virus (hog cholera virus, African), vesicular stomatitis virus (VSV) (Ref. 20), Nipah virus and porcine retroviruses.

3. In Vitro tests for Non-Viral Agents

a. Mycoplasma

All licensed viral vaccines produced in cell substrates (and similar products) should be tested to ensure the absence of mycoplasma contamination. For most such products, testing is performed on the virus seed and/or master cell banks, cell substrate, and a representative portion of each working cell stock used for manufacture of the product. Each lot of product harvest concentrate should be tested prior to further processing, *e.g.*, prior to clarification, filtration, purification, and inactivation, unless testing at this stage of the manufacturing process is not feasible. Under 21 CFR 610.30, for virus vaccines produced from in vitro living cell cultures, you must test each virus harvest pool for mycoplasma prior to clarification or filtration in the case of live virus vaccines and prior to inactivation in the case of inactivated virus vaccines. Prior to testing, the product harvest concentrate sample must be stored either between 2 and 8°C for no longer than 24 hours or at -20°C or lower if stored for more than 24 hours. If stored for longer than 24 hours, we recommend that you store the sample at -60°C or lower to allow for greater recovery of mycoplasma.

Although mycoplasma testing prescribed in 21 CFR 610.30 specifies agar and broth media culture procedures, we now know that some fastidious strains of mycoplasma, especially *Mycoplasma hyorhinis*, might not be detected by the agar and broth media procedures, and therefore you should include an indicator cell culture procedure to detect such strains. We consider the testing described below to be acceptable alternatives to the method prescribed in 21 CFR 610.30. However, you must seek and obtain approval for these modifications in accordance with 21 CFR section 610.9(b). Additional information regarding mycoplasma testing is available (Ref. 21).

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Testing for Mycoplasma using both Alternative Agar and Broth Media, and Indicator Cell Culture Procedures

- Agar and Broth Media Procedure
 - (1) Each lot of agar and broth medium should be free of antibiotics except for penicillin, and each lot of medium should be examined for mycoplasma growth-promoting properties. To demonstrate the capability of the media to detect known mycoplasma contaminants, use the mycoplasma cultures specified below in (3)(i) as positive controls.
 - (2)
 - (i) Inoculate no less than 0.2 mL of the test sample (e.g., product harvest concentrate sample) in evenly distributed amounts over the surface of 2 or more agar plates of 1 medium formulation.
 - (ii) Inoculate no less than 10 mL of the product harvest concentrate sample into a flask containing 50 ml of broth medium which is incubated at $36 \pm 1^\circ \text{C}$.
 - (iii) Test 0.2 mL of the broth culture on the 3rd, 7th, and 14th days of incubation by subculture onto 2 or more agar plates of the same medium formulation as that used above in (i).
 - (iv) Incubate 2 of the initial isolation plates and 2 each of the three subculture plates in a 5 to 10 % carbon dioxide in nitrogen and/or hydrogen atmosphere containing less than 0.5% oxygen during the test incubation period.
 - (v) Incubate all culture agar plates for no fewer than 14 days at $36 \pm 1^\circ \text{C}$ and observe them microscopically at 100 times magnification or greater for growth of mycoplasma colonies.
 - (3)
 - (i) Include in each test at least 2 known mycoplasma species or strains as positive controls, 1 of which should be a dextrose fermenter (i.e., *M. pneumoniae* strain FH or equivalent species or strains) and 1 of which should be an arginine hydrolyzer (i.e., *M. orale* strain CH19299 or equivalent species or strain). Positive control cultures should be not more than 15 passages from isolation and should be used in a standard inoculum of 100 colony-forming units (CFU) or 100 color-changing units (CCU) or less.
 - (ii) Include uninoculated agar medium as a negative control.
 - (4) Interpret the results of the procedure according to the

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specification detailed in this section under Interpretation of Results.

- Indicator Cell Culture Procedure
 - (1) Using a Vero cell culture substrate, pre-test the procedure by using the mycoplasma cultures specified below in (3) (i) as positive controls to demonstrate the capability of the cell substrate to detect known fastidious mycoplasmal contaminants. An equivalent indicator cell substrate might be acceptable if data demonstrate at least equal sensitivity for the detection of known mycoplasmal contaminants.
 - (2) (i) Inoculate no less than 1 mL of the test sample (e.g., product harvest concentrate sample) to 2 or more indicator cell cultures grown on cover slips in dishes or equivalent containers.

(ii) Incubate the cell cultures for 3 to 5 days at $36 \pm 1^\circ\text{C}$ in a 5% carbon dioxide atmosphere. Examine the cell cultures for the presence of mycoplasmas by epifluorescence microscopy using a DNA-binding fluorochrome, such as bisbenzimidazole or an equivalent stain.
 - (3) (i) Include in each test two known mycoplasma species or strains as positive controls (*i.e.*, *M. hyorhinis* strain DBS 1050, *M. orale* strain CH19299, or equivalent species and strains), using an inoculum of 100 CFU or 100 CCU or fewer.

(ii) Include as a negative control a non-infected indicator cell culture.
 - (4) Interpret the results of the procedure according to the specifications detailed in this section under Interpretation of Results.
- Interpretation of Results
 - (1) For the agar and broth media procedure, compare the appearance of the media inoculated with the product to that of the positive and negative controls.
 - (2) For the indicator cell culture procedure, using 600 times magnifications or greater, compare the microscopic appearance of the cultures inoculated with the product to that of the positive and negative cell controls.

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(3) Marked cytopathic effects or nuclear chromatin fragmentation caused by vaccine virus infection that affect the interpretation of the results can be minimized by using a specific neutralizing viral antiserum or a nonpermissive cell culture substrate. The antisera should also be added to the positive and negative controls.

(4) The product is considered satisfactory for manufacture if both the agar and/or broth media procedure and the indicator cell culture procedure show no evidence of mycoplasma contamination (*i.e.*, no growth) and thus resemble the negative control(s) for Agar and Broth and Indicator Cell Procedures.

(5) If mycoplasmas are recovered, you should perform confirmatory testing to establish the species

Testing for Mycoplasma Using PCR-based Assays

PCR-based assays may be used to detect mycoplasma, provided that such an assay can be shown to be comparable to the agar and broth media procedure and the indicator cell culture procedure. In some cases, culture-based procedures cannot be used due to an inability to completely neutralize vaccine viruses, thus necessitating the use of PCR-based assays to test for mycoplasma in these products.

b. Bacterial and Fungal Sterility

You must perform the test for bacterial and fungal sterility described in 21 CFR 610.12 or an equivalent method that fulfills the requirements of 21 CFR 610.9.

c. Mycobacteria Testing

You should use the culture methods for detection of mycobacteria described in the next paragraph to test your culture filtrate in its most concentrated form. The previously described guinea pig tests have also been used to detect mycobacteria. If you use alternative tests, including PCR assays, they should be shown to be sufficiently sensitive to test for the presence of mycobacteria.

A 2.0 mL sample of the filtrate should be inoculated onto Lowenstein – Jensen’s egg medium or other media demonstrated to be equally capable of supporting growth. An appropriate positive-control test should be conducted simultaneously with the sample under test, and the test should be shown to be capable of supporting the growth of small numbers of the mycobacteria. All

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the test vessels should be incubated at a suitable temperature for a period of 6 weeks under conditions that will prevent drying of the medium, after which the cultures should be examined for evidence of mycobacterial colonies. The filtrate is satisfactory for use if the test shows no evidence of mycobacteria.

B. TESTING OF CELL PROPERTIES

1. Tests for Tumorigenicity

Tumorigenicity is defined as the process by which cells form tumors when inoculated into animals (generally a syngeneic, an immunosuppressed allogeneic or an immunosuppressed xenogeneic host) (see Section VI. Glossary).

Tumorigenicity is a characteristic of the immortalized cells themselves, rather than of agents or components present in them.

Tumorigenic cells have not traditionally been used for the production of prophylactic viral vaccines, primarily because of theoretical concerns that components within tumorigenic cells could induce tumors in vaccine recipients. These concerns include the potential presence of exogenous agents, such as oncogenic viruses, and the potential risk from endogenous materials, such as endogenous viruses or oncogenic nucleic acids. In addition, intact human cells derived from human tumors have been shown to form tumors in allogeneic humans.

The goal in tumorigenicity testing is to determine whether your cell substrate is capable of forming tumors after inoculation into animals. The TPD₅₀ (tumor-producing dose in 50% of animals) and capacity to form metastases are characteristic properties of a cell line, and these characteristics might be used to further define the tumorigenic phenotype of a cell line. Considerations associated with tumorigenicity testing include: 1) choice of appropriate animal models; 2) definition of a positive result; 3) determination of the appropriate duration of testing; 4) determination of appropriate numbers of cells to be tested; and 5) selection of appropriate controls.

You should use an animal model that is known to be susceptible to tumor formation by tumorigenic cells. Because immunocompromised adult and newborn rodents are relatively sensitive for revealing a tumorigenic phenotype, you should consider these animal models. Thus, the most commonly used animals for tumorigenicity testing are nude (nu/nu) mice because they are T-cell deficient. Newborn nude mice appear to be more susceptible to tumor formation than adult nude mice (Ref. 22), suggesting that newborn nude mice might be the best choice to use when identification of a weakly tumorigenic phenotype is important. You might choose to use another animal model if it has been shown to have comparable sensitivity to the nude mouse model.

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Regardless of the test system you use, the animals should be observed and palpated at regular and frequent intervals for the formation of nodules at the site of injection. Any nodules formed should be measured in two dimensions. We consider progressive tumor formation at the site of injection a positive result. Some cell types might cause tumors at distant sites as well (Ref. 22), and these should be reported. Animals with nodules that begin to regress during the period of observation should not be sacrificed, as nodules that spontaneously regress do not represent progressively growing tumors and are not indicative of a tumorigenic phenotype. You may euthanize any animals with tumors before the end of the study if the tumor interferes with the comfort of the animal.

You should perform a necropsy on each animal when it dies, is euthanized, or at the end of the observation period. The necropsy should include examination for gross evidence of tumor formation at the site of inoculation and in other organs such as lymph nodes, lungs, brain, spleen, kidneys, and liver. All tumor-like lesions, detectable regional lymph nodes, the site of inoculation, and the lungs are to be examined histologically. In some cases, it might be necessary for you to use molecular or immunological methods to distinguish cell-substrate-related from spontaneous tumors.

The inoculum per animal should consist of 10^7 reference cells or test cells suspended in 0.2 mL (0.1 mL for newborn animals) volume of serum-free medium administered by the subcutaneous route. You should inoculate at least ten animals, each with 10^7 test cells that are at or beyond the end-of-production passage level and at least ten with your positive control tumor cells. At least 9 out of 10 animals injected with positive control cells (e.g., HeLa cells or other cells with comparable tumorigenicity) should show progressively growing tumors in order for your test to be valid.

Selection of the appropriate duration of testing requires that you balance the increased sensitivity that might be obtained using a longer test, against the likelihood of false-positive results due to spontaneous tumor formation. Weakly tumorigenic cells might require between 4 and 7 months to form tumors in nude mice. Thus, extended observation periods might be necessary in some cases. CBER can provide you with further information on tumorigenicity testing and the appropriate observation periods.

2. Testing for Oncogenicity

Oncogenicity is defined as the process by which agents immortalize cells and endow them with the capacity to form tumors (see Section VI. Glossary). It is important to ensure that a cell substrate does not contain potentially oncogenic components that could contaminate your product. If your vaccine is manufactured in a cell substrate that was derived from a tumor, or that has

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developed a tumorigenic phenotype through an unknown mechanism, it might carry a higher theoretical risk of containing oncogenic substances.

If the presence of an oncogenic virus is suspected because of the cell phenotype or the origin of your cell substrate, it might be appropriate for you to perform oncogenicity testing in animals using lysates of the cell substrate. For cell substrates with a tumorigenic phenotype, it might be appropriate for you to perform oncogenicity testing in animals using DNA from the cell substrate, in order to provide assurance that residual DNA is non-oncogenic. Oncogenicity testing might also be appropriate for products with high quantities of residual cellular DNA. Existing assays for the presence of oncogenic agents in certain cell substrates might not be adequate to provide sufficient assurance of the safety of your vaccine for clinical use. You should consult with CBER if you have any questions on the necessity of these tests and the methods to be followed.

3. Identity Testing of Cell Substrates

Identity testing is required by the general requirements for laboratory controls (21 CFR 211.160(b)) and the general standards for biological products (21 CFR 610.18(b)). Identity tests assure that a licensed viral vaccine is as stated on the manufacturer's label. Identity tests also provide extra assurance in facilities or laboratories that are propagating multiple cell lines or viruses. It is important that identity tests be performed on cells used and generated in manufacturing. Some of the tests you may use for identity testing are described in the paragraphs that follow.

Biochemical tests such as PAGE analysis is one method to determine the species from which the cell substrate was derived. Comparison of the migration patterns of the cellular enzymes malate dehydrogenase, glucose-6-phosphate dehydrogenase, nucleoside phosphorylase and lactate dehydrogenase is another method (Ref. 23).

Cytogenetic analysis can be used to determine the karyotype of the cell substrate as a means of identifying the species, gender, ploidy, and, grossly, genetic stability. You should examine 100-200 metaphase cells. However, it might be appropriate for you to examine more, depending on the heterogeneity of the karyotypes of your product.

DNA fingerprinting is the most commonly used test of genetic identity and has been used to document cell origin and to determine whether cell contamination has occurred (Ref. 24). Specific methods for this purpose can be found in Stacey, et al. (Ref. 25).

Tests such as Southern blot hybridization, PCR, growth under selective conditions, expression of a reporter gene or other gene of interest, etc., may be applied to

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distinguish an engineered cell line both from the parent cell line and from other cell lines. Some examples can be found in Stacey, et al., (Ref. 26). You may also use other tests that provide information relevant to identity of cells, such as HLA typing.

4. Testing for Genetic Stability

You should establish processes and controls that ensure manufacturing consistency. This concept extends to the genetic stability of your cell substrate from the establishment of the master cell bank through and perhaps beyond the end of production. For an engineered cell line, the inserted gene of interest should remain intact and at the same copy number, and be expressed at equivalent levels throughout production. Also, a diploid cell strain should remain diploid throughout. If such characteristics are not stable, then you might be requested to show that the instability does not adversely impact manufacturing or product consistency. You may obtain further guidance on this topic from the ICH Q2B document (Ref. 7). For methods to assess a cell substrate's genetic stability, you may find guidance in the ICH Q5B and Q5D documents (Refs. 27 and 3, respectively).

C. OTHER TESTS

1. Testing for the Presence of Residual Cells

You should test your final vaccine product for the presence of residual cells. Processes, such as filtration, should be implemented and validated to ensure that intact cells are not present in the final product. Validation that residual cell removal processes are robust is important for immortalized cells. Determining the extent to which intact cells are cleared by these processes is an important part of this validation.

2. Testing for Residual Cellular DNA

Residual DNA might be a risk to your final product because of oncogenic and/or infectivity potential. There are several potential mechanisms by which residual DNA could be oncogenic, including the integration and expression of encoded oncogenes or insertional mutagenesis following DNA integration. Residual DNA also might be capable of transmitting viral infections if retroviral proviruses, integrated copies of DNA viruses, or extrachromosomal genomes are present.

The risks of oncogenicity and infectivity of your cell-substrate DNA can be lessened by decreasing its biological activity. This can be accomplished by decreasing the amount of residual DNA and reducing the size of the DNA (e.g., by DNase treatment or other methods) to below the size of a functional gene.

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Chemical inactivation can decrease both the size and biological activity of DNA. If DNA removal, digestion, or inactivation is undertaken, you should validate your methods.

You should measure the amount and size distribution of residual DNA in your final product. For widely used human diploid cell strains, such as MRC-5 and WI-38 cells, measurement of residual DNA might be unnecessary because we do not consider residual DNA from these human diploid cells to be a safety issue. We might require limitation of the amount of residual DNA, depending on the potential risks associated with that DNA, for human diploid or primary cell types for which there is less experience. You should limit residual DNA for continuous non-tumorigenic cells, such as low-passage Vero cells, to less than 10 ng/dose for parenteral inoculation as recommended by WHO (Ref. 28). If you are using cells with tumorigenic phenotypes or other characteristics that give rise to special concerns, more stringent limitation of residual DNA quantities might be needed to assure product safety.

3. General Safety Test (GST)

The requirements of a GST are described in 21 CFR 610.11. For vaccines, an exemption to the GST may be requested, as specified in 21 CFR 610.11(g)(2).

V. CONCLUSIONS

This guidance document provides to you, general advice on the selection, characterization, and qualification of cell substrates and viral seeds used to manufacture these products. We recognize that this is a complicated and challenging area. We welcome the opportunity to discuss cell substrate and adventitious agent issues with you, as early as feasible in your development of viral vaccines.

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VI. GLOSSARY

Because different guidance documents might use the same or similar terms differently, the following glossary is provided to define the terminology used in this document.

1. **ADVENTITIOUS AGENT:** A microorganism (including bacteria, fungi, mycoplasma/spiroplasma, mycobacteria, rickettsia, viruses, protozoa, parasites, TSE agent) that is inadvertently introduced into the production of a biological product.
2. **ANCILLARY PRODUCT:** Products that are used in the manufacture or production of a biological product that may or may not end up as part of the final product. Examples include: insulin, transferrin, growth factors, interferon, interleukins, other proteins, drugs or chemicals like dimethyl sulfoxide.
3. **ANEUPLOID:** Having an atypical number of chromosomes which is not an integer multiple of the haploid number.
4. **CELL BANK:** Vials of cells of uniform composition (although not necessarily clonal) derived from a single tissue or cell, aliquoted into appropriate storage containers, and stored under appropriate conditions, such as the vapor phase of liquid nitrogen.
5. **CELL LINE (CL):** Cells that have been propagated in culture since establishment of a primary culture and survival through crisis and senescence. Such surviving cells are immortal and will not senesce. Diploid cell strains have been established from primary cultures and expanded into cell banks but have not past through crisis and are not immortal. [The ATCC uses the abbreviation CCL to signify their Certified Cell Lines.]
6. **CHARACTERIZATION:** Determination of the properties of a cell substrate or cell bank.
7. **CONTROL CELLS:** Cells that are split off from the production culture and maintained in parallel under the same conditions and using the same reagents (e.g., culture medium) in order to perform quality control tests on cells that have not been exposed to the vaccine virus (which may interfere with some tests).
8. **DIPLOID:** Having the expected number of chromosomes for a species, (i.e., two of each autosomal chromosome and two sex chromosomes).
9. **ENDOGENOUS VIRUS:** A virus whose genome is present in an integrated form in a cell substrate by heredity. Endogenous viral sequences may or may not encode for an intact or infectious virus.
10. **END-OF-PRODUCTION CELLS (EOPC):** Cells harvested at the end of a production run or cells cultured from the MCB or WCB to a passage level or population doubling level comparable to or beyond the highest level reached in production.

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11. **END-OF-PRODUCTION PASSAGE LEVEL:** The maximal passage level achieved during manufacturing at final vaccine harvest. Cells may be evaluated at this level or beyond.
12. **FINAL BULK:** The stage of vaccine production directly prior to filling of individual vials.
13. **FREE OF and FREEDOM FROM:** For a substance to be considered free of a contaminant, an assay must demonstrate that a defined quantity of the substance to be negative for that contaminant to a defined level of sensitivity. The level of assay sensitivity is defined by the choice of assay and can be determined experimentally using standardized reagents. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate freedom from that contaminant.
14. **HARVEST:** At the end of vaccine virus propagation in cell culture, material is collected from which vaccine will be prepared. This material may be the culture supernatant, the cells themselves (often in disrupted form), or some combination thereof.
15. **IMMORTALIZATION:** The process by which cells with finite lifespan (e.g., primary cells, diploid cell strains) are converted to those with infinite lifespan.
16. **LATENT:** A virus that is present in a cell, without evidence of active replication, but with the potential to reactivate, is considered to be microbiologically latent.
17. **MANUFACTURER'S WORKING CELL BANK (MWCB) OR WORKING CELL BANK (WCB):** A cell bank derived by propagation of cells from MCB under defined conditions and used to initiate production cell cultures on a lot-by-lot basis.
18. **MASTER CELL BANK (MCB):** A bank of a cell substrate from which all subsequent cell banks used for vaccine production will be derived. The MCB represents a characterized collection of cells derived from a single tissue or cell.
19. **MASTER VIRUS SEED (MVS):** A viral seed of a selected vaccine virus from which all future vaccine production will be derived, either directly, or via Working Virus Seeds.
20. **ONCOGENICITY:** The property of certain biological agents (e.g., viruses) or materials (e.g., nucleic acids) that are capable of immortalizing cells and endowing them with the capacity to form tumors. Oncogenicity is distinct from tumorigenicity (See Tumorigenicity).
21. **PARENTAL VIRUS:** Virus that has been manipulated in some manner to generate a viral seed with characteristics needed for production.

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22. WHO Technical Report Series 878, 1998
23. PARENT CELL BANK: A few vials consisting of cells from which the Master Cell Bank was derived. Parental Cells may be manipulated to derive a cell substrate with desired characteristics.
24. PASSAGE LEVEL: The number of times, since establishment from a primary cell culture, a culture has been split or re-seeded.
25. POPULATION DOUBLING LEVEL: The number of times, since establishment from a primary cell culture, a culture has doubled in number of cells.
26. PRIMARY CELLS: Cells placed into culture immediately after an embryo, tissue, or organ is removed from an animal or human and homogenized, minced, or otherwise separated into a suspension of cells. Primary cells may be maintained in medium, but are not passaged (split).
27. PURITY: Relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product. (21 CFR 600.3(r))
28. QUALIFICATION: Determination of the suitability of a cell substrate for manufacturing based on its characterization.
29. TUMORIGENIC: A cell type is tumorigenic if it forms tumors when inoculated into animals (generally a syngeneic, an immunosuppressed allogeneic, or an immunosuppressed xenogeneic host). These tumors may be at the injection site or a different site and may also metastasize to other sites.
30. NON-TUMORIGENIC: A cell type is non-tumorigenic if it is shown not to form tumors in appropriate animal models.
31. TUMORIGENICITY: Tumorigenicity is the process by which immortalized cells form tumors when inoculated into animals (see Tumorigenic). Tumorigenicity is distinct from Oncogenicity (See Oncogenicity).
32. TUMORIGENICITY TESTING: An assay/test that determines whether or not immortalized cells are tumorigenic when injected into animals.
33. VALIDATION: Validation defines the performance characteristics of an analytical procedure, based on the demonstration that the procedure is suitable for its intended purpose or use. Validation of a process is the determination of what characteristics the process is capable of performing and the demonstration that the process uniformly performs to defined characteristics. Validation is generally performed in accordance with the relevant ICH guidelines.

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34. **VIRAL CLEARANCE:** The combination of the physical removal of viral particles and the reduction of viral infectivity through inactivation.
35. **VIRUS PRE-SEED:** A few vials consisting of vaccine virus from which the MVS was derived.
36. **VIRUS SEED or VIRAL SEED:** A live viral preparation of uniform composition (although not necessarily clonal) derived from a single culture process, aliquoted into appropriate storage containers, and stored under appropriate conditions.
37. **WORKING CELL BANK:** See “Manufacturer’s Working Cell Bank (MWCB)”.
38. **WORKING VIRUS SEED (WVS):** A viral seed derived by propagation of virus from the MVS under defined conditions and used to initiate production cell cultures lot-by-lot.

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Appendix 1

TABLE 1. Example of a Testing Scheme for Manufacture of a Viral Vaccine.

Multi-stage testing with a variety of different assays is used to assure product safety and ensure product quality. This includes testing all the biological materials in vaccine manufacture, especially the initial components, which are the virus seed and the cell substrate. Different stages for testing are selected based on the maximum likelihood of adventitious agent detection and effect on product quality. This includes the Harvest and Final Virus Seed; the Master Cell Bank and Control Cell Culture (in certain cases); and the Vaccine Bulk and Final product. Additional tests may be included on a case-by-case basis depending upon the cell substrate and other biological materials as wells as production conditions for vaccine manufacture.

Table 1:

VIRUS SEED:	
Harvest	Sterility
	Mycoplasma/Spiroplasma
	Mycobacteria
	Appearance
	Identity
	Potency/Activity
	Infectious titer
	In vitro adventitious agent testing
	In vivo adventitious agent testing
	Bovine viruses (if applicable)
	Porcine viruses (if applicable)
	RT assay
	Specific agent testing (if applicable)
Final	Sterility
	Identity
	Potency/Activity
	Infectious titer
	Attenuation (if applicable)
	Stability of phenotype/genotype (if applicable)
CONTROL CELL CULTURES:	Observation
	Hemadsorption
	Sterility
	Mycoplasma/Spiroplasma
	RT assay

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	In vitro adventitious agents
	In vivo adventitious agents
	Bovine viruses (if applicable)
	Porcine viruses (if applicable)
	Tests for specific agents (as applicable)
	ALV (if applicable)
	Mycobacteria
CELL BANKS (Diploid Cell Strains or Continuous Cell Lines):	
Master Cell Bank	Identity
	Sterility
	Mycoplasma/Spiroplasma
	In vivo adventitious viruses
	In vitro adventitious viruses
	TEM
	Bovine viruses (if applicable)
	Porcine viruses (if applicable)
	Tests for specific agents (as applicable)
	Tumorigenicity (except rodent cells)
	Oncogenicity (if applicable)
	RT assay
	Mycobacterium (if applicable)
	Antibody testing (MAP, HAP, RAP, if applicable)
	S+L- assay for retrovirus (if applicable)
VACCINE BULK:	Sterility
	Mycoplasma/Spiroplasma
	Mycobacteria
	Appearance
	Identity
	Potency/Activity
	Infectious titer
	In vitro adventitious agent testing (or on control cells)
	In vivo adventitious agent testing (or on controls cells)
	Bovine viruses (or on control cells) (if applicable)
	Porcine viruses (or on control cells) (if applicable)
	RT assay

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	Specific agent testing (if applicable)
FINAL FILLED PRODUCT:	Sterility
	Mycoplasma/Spiroplasma
	Endotoxin
	General Safety Test (unless you receive an exemption under 21 CFR 610.11(g))
	Identity
	Appearance
	Residual cellular protein concentration
	Residual cellular DNA concentration
	Potency/Activity
	Stability
	Residual serum, antibiotics, benzonase, ancillary products (as applicable)
	pH
	Osmolality