Add the following:

▲⟨1239⟩ VACCINES FOR HUMAN USE—VIRAL VACCINES

INTRODUCTION

Vaccines for the prevention of at least 15 human viral diseases have been commercialized in the United States (see *Table 1*); others have been licensed outside the US. Together, these vaccines have formed a primary public health measure for the prevention of these viral diseases worldwide. For the purposes of this chapter, a viral vaccine is a vaccine that prevents a viral disease; this includes products derived from cultured viruses and those derived from recombinant expression of viral antigens. General considerations for both viral and bacterial vaccines are provided in *Vaccines for Human Use—General Considerations* ⟨1235⟩.

Table 1. Viral Vaccines Licensed in US for Prevention of Human Diseases

Live and Attenuated Viral Vaccines	Nonreplicating Viral Vaccines[a]
	Inactivated or Killed
• Adenovirus types 4 and 7[a] • Influenza A and B • Measles • Mumps • Rotavirus • Rubella • Smallpox • Yellow fever • Varicella-zoster (separately, chickenpox and shingles) • Dengue • Ebola Zaire	• Hepatitis A • Poliomyelitis types 1, 2, and 3 • Rabies • Japanese encephalitis
	Split or Subunit Virus Vaccines: Recombinant or Virus-Derived
	• Influenza A and B (virus-derived) • Influenza A and B (recombinant)
	Virus-Like Particle Vaccines
	• Hepatitis B • Human papillomavirus bivalent (types 16 and 18) • Human papillomavirus quadrivalent (types 6, 11, 16, and 18) • Human papillomavirus 9-valent (types 6, 11, 16, 18, 31, 33, 42, 52, and 58)
	Nonreplicating Viral Vaccines
	• Human papillomavirus 9-valent (types 6, 11, 16, 18, 31, 33, 42, 52, and 58)

[a] Typically referred to as inactivated.

General Categories of Viral Vaccines

There are currently two general categories of viral vaccines that have received regulatory approval: 1) live and attenuated and 2) nonreplicating. The nonreplicating viral vaccines contain three subgroups: virus-derived (killed/inactivated), split/subunit vaccines, and virus-like particles (VLP). Other strategies, including DNA and RNA-based vaccines, are under development. The characteristics, advantages, and challenges of viral vaccines are listed in *Table 2*.

Table 2. Characteristics, Advantages, and Challenges of Viral Vaccines

Live and Attenuated Viral Vaccines		
Characteristics	**Advantages**	**Challenges**
• Able to replicate in the host • Attenuated in pathogenicity	• May elicit broader immune responses than other vaccine types (i.e., both humoral and cellular) • May require fewer doses • Highly effective • Protection is generally longer lasting	• Limited understanding of mechanism of action and stability of attenuation • Ensuring stability • Characterization • Manufacturing • Possible transmission to another person via "shedding" • Controlling reactogenicity
Nonreplicating, Subunit, and Recombinant VLP Viral Vaccines		
Characteristics	**Advantages**	**Challenges**
• Unable to replicate in the host • Not pathogenic • Elicit mostly humoral immune responses	• Cannot multiply or revert to pathogenicity • Generally less reactogenic • Not transmissible to another person • More stable than live viral vaccines	• Ensuring sufficient immunogenic potency • May require adjuvant to enhance immune responses • Stimulating cell-mediated immune responses • Variable efficacy

LIVE AND ATTENUATED VIRAL VACCINES

Live viral vaccines contain live viruses or live viruses that have been attenuated (weakened) to reduce or eliminate their virulence (i.e., their ability to cause disease). Attenuation results from reassortment or from mutations induced by the passage of the virus in embryonated eggs, tissue culture using semi-permissive or atypical animal cells, or under altered growth conditions (e.g., at temperatures lower than the host's body temperature), or a combination of these mechanisms. Alternatively, live viral vaccines can be produced using recombinant technology. Attenuated vaccine strains are still immunogenic and replicate at a reduced rate compared to the wild type strain in their original host. The development of live viral vaccines is not standardized and the passage and propagation history varies between different vaccine viruses. Viruses for this group of vaccines are grown in human (e.g., MRC-5, WI-38) and non-human (e.g., primary chick embryo cells, Vero cells) cells, and do not undergo an inactivation step in their manufacturing process. Therefore, contamination by extraneous viruses and other adventitious agents already present in these substrates or introduced during manufacturing (e.g., raw material contamination) is a potential risk. This contamination risk requires a control strategy for manufacturing and testing that ensures vaccine purity, quality, and safety. Some live viral vaccines (e.g., yellow fever or live influenza) are produced using specific pathogen-free (SPF)

eggs. These eggs are produced by chicken flocks that are regularly screened for avian pathogens (e.g., avian leukosis virus) and are maintained using appropriate animal husbandry practices.

NONREPLICATING VIRAL VACCINES

Inactivated or killed viral vaccines: Inactivated (or killed) viral vaccines are produced by culturing and isolating the virus and then killing it chemically or with heat. The goal is to destroy the viruses' replicative function while keeping the outer virion coat intact. Nonreplicating viral vaccines may or may not be purified after harvest and before inactivation. Although many such vaccines have been successful in controlling viral diseases, not all viral proteins are equally immunogenic, and an unbalanced presentation (greater number of less immunogenic proteins) can dampen the immune response to protective antigens. Modification of the primary protective antigens can induce atypical immune responses that may enhance disease upon natural infection. This phenomenon was observed for formalin-inactivated measles virus and respiratory syncytial virus in early vaccines developed in the 1960s. Because of this experience, the use of attenuated measles vaccines is now recommended.
Subunit or split virus vaccines: Subunit vaccines are composed of viral gene products, which may be extracted from a cultured virus or produced via recombinant technology, e.g., in insect cell lines using baculoviruses. Traditional inactivated influenza A and B vaccine components are grown in embryonated eggs; and antigens of interest [e.g. hemagglutinin (HA) and neuraminidase (NA)] are purified following membrane solubilization (splitting). Recombinant HA antigens can be produced using the baculovirus expression vector system (BEVS), leveraging the natural infection process of insect cells by baculoviruses. For example, in one such vaccine, r-hemagglutinin proteins are produced in a continuous insect cell line derived from Sf9 cells of the fall armyworm, *Spodoptera frugiperda* (related to moths, caterpillars, and butterflies).
Recombinant virus-like particle vaccines: VLP vaccines, in which recombinant viral antigens are assembled (or self-assembled) into particles or are incorporated into a lipid vesicle, have been developed for human papillomavirus (HPV) and hepatitis B vaccines. The most common recombinant platforms are yeast (*Saccharomyces cerevisiae* for hepatitis B) and insect cell lines (*Spodoptera frugiperda* or *Trichoplusia ni* for HPV). Although they are more immunogenic than the monomeric antigen, some currently manufactured VLP vaccines based on recombinant protein technology require the addition of adjuvants [e.g., aluminum salts, monophosphoryl lipid A (MPLA), squalene emulsions] to optimize immunogenicity. These vaccines have similar advantages and challenges to nonreplicating viral vaccines.

RAW MATERIALS AND MATERIALS OF ANIMAL ORIGIN

General guidelines for viral vaccines are found in ⟨1235⟩.

Culture ingredients must be selected carefully, especially for enveloped viruses since many of these can only be partially purified before losing infectivity or antigenicity. In such cases, the suitability of raw material for human administration can be a key criterion. A formal risk analysis of all raw materials should be performed to assess any risk of low-level contamination by unwanted entities. Media components for cell culture of viral vaccines (both during isolation and subsequent culture) need to be documented in detail. Chemically defined media without materials of animal origin are preferred. The cell culture medium should be suitable for the cells used in production. If human albumin is used, it must be licensed by the FDA for use in vaccines. The final product should be within specified limits of residual medium components such as serum, antibiotics, selection agents, or reagents added for growth enhancement. In the case of recombinant technology-based viral vaccines, fermentation media may consist of materials like proteins, sugars, inorganic trace elements, amino acids, and vitamins needed for cell growth. The protein component may be as simple as free casein (milk protein), or it can be as complex as extracts from bacterial, plant, or animal sources. The composition of a medium is often customized to optimize product quality attributes. Medium components that are known to cause allergic reactions should be avoided (e.g., eggs, gelatin).

For animal-derived raw materials it is particularly important to guard against the entry of adventitious agents into the process. Many vaccine viruses cannot be separated from environmental contaminants, and therefore prevention of environmental contaminant entry is required to ensure the safety of the product during the manufacturing operation. Animal raw materials are controlled in several ways. The origin of the herd(s) or flock(s) used is documented, controlled or isolated, and monitored to ensure the absence of disease. The material is harvested from the animals under controlled conditions using standardized, documented practices designed for pharmaceutical use to avoid contamination of the raw material with other viruses or microbes. The material is processed and stored accordingly and is often treated to destroy or remove any microbes that may have been introduced from the source or during processing (e.g., by sterile filtration, UV or gamma irradiation, detergents, heat, or a combination thereof). If additives from animal sources are added to the culture medium, they should be carefully sourced and certified to be free from contaminants and adventitious agents such as those that cause bovine spongiform encephalopathy or transmissible spongiform encephalopathy. To ensure an appropriate level of product safety, quantitative risk analysis with careful documentation should be performed on all factors that impact viral safety. For specific guidance on bovine serum quality attributes, sourcing, and testing, see *Bovine Serum* ⟨1024⟩ and *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩.

EGGS AND PRIMARY CELL LINES USED IN VIRAL VACCINE PRODUCTION

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. In the case of influenza vaccines, the vaccine virus is harvested from egg allantoic fluid. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. Therefore, residual egg or embryo components are special considerations in vaccine purification.

Egg-based vaccine production, like all biomass expansions, requires care and quality control of the virus seed lots and egg substrates to avoid contamination with other organisms. For example, chicken flocks used to prepare chicken embryo cells undergo extensive serological testing for adventitious agents before the flock can be used to prepare the cells. For vaccines

produced in embryonated eggs the eggs may be either SPF (for viral seed production) or may be from healthy flocks (for vaccine production) monitored using methods approved by local animal health authorities.

Appropriate records must be retained for documentation [21 CFR 610.18(d)].

Maintenance of Flocks

For in ovo cultured vaccines, eggs are derived from isolated healthy flocks of birds that are established for several generations and tested to ensure absence of common transmissible pathogens. Potential contaminants of the avian flocks (including those that infect nonhuman species) are routinely tested for and monitored. Flock origin and husbandry, isolation and management practices, health history, and testing should be documented clearly.

Handling of Eggs

The number of eggs used for vaccine production varies according to the validated process in the manufacturers' license. Upon arrival at the facility, the eggs are first candled for egg selection and held up to light to view the embryo to determine its viability and lack of contamination. The eggs are then sanitized and injected with the vaccine virus of interest, which is allowed to propagate until harvest. Embryo age, candling procedures, sanitization methods, and injection and incubation methods are all critical manipulations performed under cGMP.

Primary Avian Embryo Cell Lines

For vaccines produced in primary avian embryo cells, the practices described above should be followed and described up to the step of sanitization. Embryo retrieval, tissue dissection, and dissociation methods before in vitro culture should be conducted under highly controlled environmental conditions and with reagents of appropriate quality. The methods and raw materials used should be described. For these cultures, a substantial portion of cells is held aside uninfected for several weeks after the virus culture has been completed to ensure detection of any slow-growing microbes or viral agents. These uninfected control cell cultures are propagated and handled following the same process used for the virus-inoculated cells to help ensure that the cells used for vaccine production are free of adventitious agents.

Primary Cell Lines from Other Animal Sources

Primary cell cultures from tissues can be cultured in vitro and used for vaccine production. Animals used to generate the primary cells or cell bank must be screened for potential viruses or have their serum tested to detect specific antibodies (see *Virology Test Methods* ⟨1237⟩ for specific guidance). Use of controlled colonies, herds, or flocks is recommended to monitor for specific pathogens. The use of primary cells requires additional testing from each manufacturing run to ensure the absence of contamination by infectious agents. To support the reduction of animal use, diploid and continuous cell lines are recommended. Continuous cell lines can be characterized extensively (including tumorigenicity testing, if required), and the culture conditions can be optimized and standardized. The Cell Banking system will provide production consistency. Continuous cell lines that express endogenous virus should be tested with tests capable of detecting such agents (see FDA Guidance for Industry-Characterization of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications and ICH Q5A).

Human diploid cells are used in the manufacturing process of several vaccines. The two most commonly used diploid cell lines, MRC-5 and WI-38, are derived from human embryonic cells and have the normal diploid number of human chromosomes. They are widely used to manufacture vaccines because they have been shown to have no tumorigenic or oncogenic potential and have been shown to be susceptible to a wide range of human viruses. However, unlike continuous cell lines that can be passaged indefinitely, human diploid cell lines can only be passaged a limited number of times, after which they experience a rapid decline in their ability to proliferate. This issue is managed by freezing multiple aliquots of master and working cell banks.

CELL BANKS AND SEED LOT SYSTEMS

To produce vaccines in cell culture, two seed bank systems are required: one for the cell substrates and another for the viral seed lots. General information on the development, characterization, testing, and production of mammalian and bacterial cell banks and the related documentation requirements is provided in *Cell Banking* ⟨1042⟩ and ⟨1235⟩. Routine testing for master cell bank (MCB), working cell bank (WCB), control cells, and end of production cells (EOPC) is presented in *Table 3* below. A risk assessment for adventitious agents testing should be performed to determine if additional tests are needed (see note below *Table 3*). In cases where recombinant technology is used to develop the seed, the applicable guidance for information that should be supplied regarding the characterization and expression of such recombinant vectors can be found in *Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products* ⟨1048⟩. Stability of cell-line viability under cryopreservative storage conditions must be demonstrated using real-time testing; see *Cryopreservation of Cells* ⟨1044⟩ for guidance on cryopreservation of cell lines.

Viral Seed Lots

New master viral seed preparations are established under current good manufacturing practices (cGMP) conditions in a manner that is similar to the preparation of the cell banking process, as described in ⟨1235⟩ and ⟨1042⟩. The derivation and passage history of viral seeds (including the sourcing of all biological materials used: plasmids, animal-sourced raw materials, donor isolate and strain) are recorded in detail [21 Code of Federal Regulations (CFR) §610.18, *Cultures*]. Any manipulation of

the viral phenotype (e.g., cold adaptation, development of temperature sensitivity, or attenuation of virulence) or intentional genetic manipulations (e.g., reassortment or recombination) should be documented (*e.g.* viral sequence level).

Viral seeds are commonly differentiated into a master viral seed and working viral seeds or working viral stock. Manufacturers should assess the following characteristics of the viral seed stock (characteristics assessed will depend on type of seed stock/ production process *i.e.* egg or cell line):

- Growth characteristics on the intended production cell substrate
- Tissue tropism
- Genetic markers
- Identity (for recombinant vectors)
- Viability during storage
- Genetic stability through production
- Attenuation properties
- Purity
- Microbial contamination

Absence of adventitious agents; if attenuation or derivation is achieved by passage through different species, the viral seed should be assessed for absence of adventitious agents also common to those species as well as for adventitious agents for the species in raw materials used.

The master viral seed should be extensively characterized to demonstrate the stability of the relevant genotype to a passage level comparable to a production batch. Cultures from the working seed lot should have the same relevant characteristics as the master seed lot from which they are derived. For influenza vaccines, which may be reformulated with new virus antigens each year, certified seed lots can be obtained from national regulatory agencies. A working seed lot is derived from the master seed within a limited number of passages. The working seed is tested to ensure its purity, freedom from adventitious agents based on viral risk assessment, and appropriate biochemical properties. The working seed is used for vaccine production without intervening passages. Reagents used for viral seed processing must be of appropriate quality.

Tests should be performed for identity (e.g., sequencing the entire virus or a portion of it), adventitious agents, viral phenotype, genetic stability, and, agents that might be present in the seed as a result of its passage history if applicable. Viral phenotype can be further assessed for tissue tropism, attenuation properties, and temperature sensitivity. Not all of these tests may be necessary for every viral seed strain.

Tests for identity, microbial contamination, mycoplasma, and adventitious agents should be performed based on established compendial and regulatory requirements (regulatory requirements vary and may be different outside the US). Depending on past treatments of the virus isolate (raw materials, agents, and biological reagents that the seed was in contact with), specific tests for additional adventitious agents that could potentially be present must also be carried out on the master viral seed preparation based on a risk analysis approach. The master viral seed bank is used to produce a working viral seed bank. The subsequent testing of the working viral seed preparation is dependent on the extent of testing carried out on the master seed, as well as the process and raw materials used to produce the working viral seed preparation. In the case of live and attenuated viral vaccines, the following tests should be included either on master or working viral seed:

- Safety test (microbial contamination, mycoplasma, mycobacteria)
- Whole genome sequencing
- Phenotypic, genotypic or any other attenuation test
- Genetic stability of seed virus
- Neurovirulence test (if virus is neurotropic, e.g., monkey neurovirulence test for yellow fever virus)

[NOTE—Assessment of neurovirulence should be performed if the virus is known to be neurotropic. Manufacturers should consult with regulators about appropriate animal models, methods, and scoring systems for this assessment before they initiate such studies. For viruses that are neurovirulent or may revert to neurovirulence (e.g., polioviruses), it may be necessary to assess neurovirulence beyond the master seed.]

Table 3. Example of routine testing scheme for cell banks and viral seeds

TEST	MCB	WCB	Control Cells	Control Eggs	EOPC/ Extended CB	MVS	WVS
Identity	+	±	+	—	±	+	+
Sterility (bacterial/ fungal)	+	+	+	+	+	+	+
Mycobacteria	±	±	±	—	±	±	±
Mycoplasma	+	+	—	—	—	+	+
Spiroplasma[a]	+	+	—	—	—	+	+
Cytopathic effects	—	—	+	—	—	—	—
Hemadsorbing virus	—	—	+	—	—	—	—
Hemagglutinating agents	—	—	—	+	—	—	—
In vitro adventitious agents	+	+	±	—	+	+	+

Table 3. Example of routine testing scheme for cell banks and viral seeds *(continued)*

TEST	MCB	WCB	Control Cells	Control Eggs	EOPC/ Extended CB	MVS	WVS
In vivo adventitious agents[b]	+	—	±	—	+	+	+
Specific virus by NAT	+	+	—	—	—	+	+
Tests for virus using broad molecular methods[c]	+	+	—	—	+	+	+
Retroviruses/ retroviral elements	+	—	±	—	+	+	+
Transmission electron microscopy (TEM)	+	—	—	—	+	—	—
Antibody production assay	±	—	—	—	—	±	—
Bovine viruses[b]	±	±	±	—	±	±	±
Porcine viruses[b]	±	±	±	—	±	±	±
Avian viruses	—	—	—	—	—	+	+
Avian leucosis virus	—	—	+	+	—	—	—
Insect viruses[a]	+	+	—	—	—	+	+
Tumorigenicity	+	—	—	—	+	—	—
Oncogenicity	—	—	—	—	±	—	—
+ = *required stage for testing*							
± = *alternative stage for testing*							

[a] If insect cells or plant source raw materials used.
[b] If applicable.
[c] As alternatives to in vivo and specific NAT or as supplement/alternative to in vitro culture based on risk assessment as agreed with the competent authority.

Cell Culture

In some cases, the viral seeds may have a broad host range and therefore may require neutralization of the vaccine virus before they are tested for adventitious agent(s). The neutralization procedure must be demonstrated to not interfere with detection of adventitious agents. If possible, testing for adventitious agents should be done without neutralization to avoid an antiserum that may inadvertently neutralize an adventitious agent present in the seed.

A strategy for testing adventitious agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk. This strategy includes a full range of suitable tests able to detect different families of adventitious agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. The capacity of the manufacturing process to remove or inactivate viruses is evaluated as part of the risk assessment. The list of tests must be adapted depending on the extraneous agents that have the potential to contaminate the product: for in vitro tests, the risk assessment may allow, with the agreement of the competent authority, the use of other permissive cell lines or molecular biology methods depending on the manufacturing process and the incubation temperature for the growth of particular viruses. If in vivo tests are more relevant than in vitro tests for the detection of some adventitious viruses (e.g. suckling mice for the vesicular stomatitis virus and fertilized SPF eggs for the influenza virus) the decision to maintain or to introduce such in vivo assays in a testing strategy must be justified by the risk assessment.

New, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridization to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to in vivo tests and specific NAT or as a supplement/alternative to in vitro culture tests based on the risk assessment and with the agreement of the competent authority.

If the master viral seed is well characterized, the working viral seed may not require extensive characterization. For example, it may not be necessary to repeat testing for all the relevant viruses from the derivation history.

Seed Storage

To ensure consistent production of the viral vaccine product, a well-defined storage system is required. Viral seeds should be stored at temperatures to maintain their stability (i.e. in liquid nitrogen or ultra-low temperature freezers) and in more than one location within a facility or at a distant site (if possible) to decrease risk of loss in case of a catastrophic event. Manipulation

of all vials must be controlled and documented to ensure the consistency of viral seed stock inventory. Viral seeds are stored under validated temperatures that support their stability.

A strategy to demonstrate the stability of the master and working viral seed preparations under the frozen storage conditions should be established, preferentially using real-time testing. Virus replication (infectious titer) can be determined during routine production. Trending the infectivity titer will alert the manufacturer to any stability issues and the need to produce a new working viral seed preparation. The master viral seed preparation should also be monitored in real time during the preparation of a new working viral seed. In the event that the master viral seed is not required for an extended period of time, a stability protocol with defined testing intervals should be implemented. If the infectious titers of the working viral seed are not decreasing, and the viral seed was produced and stored in a manner similar to the master viral seed preparation, stability of the master viral seed preparation may be inferred, and its testing may be reduced to conserve the supply.

Recombinant Cell Lines

To date, the production of recombinant viral antigens has used yeast and insect cell lines. The requirements, as for any other recombinant expression system, are outlined in ⟨1235⟩. For cell lines containing exogenously assembled expression constructs (recombinant cell lines), characterization of nucleotide and amino acid sequences should be performed. Extensive characterization of cell banks should use the most relevant qualified technology. Chapters ⟨1042⟩ and ⟨1048⟩ provide advice on the characterization of the engineered cell line and expression construct.

PROPAGATION AND HARVEST

Manufacturing of live and inactivated viral vaccines using eukaryotic cell culture is a two-phase production process. The first phase is the expansion of the cell cultures used as a substrate for viral replication. The second phase includes the initial virus infection and subsequent replication and virus production. Note that production of nonreplicating, recombinant viral antigens in yeast or insect cell lines does not involve infection of the growing cell and the requirements for these vaccines are essentially the same as the requirements for other recombinant proteins, described in the relevant compendial guidance for recombinant protein products.

CELL SUBSTRATE GROWTH PHASE

The cell substrate expansion process for viral production is the phase designed to prepare the cells in an appropriate physiological state to sustain virus growth. Cell substrates often require complex animal-derived supplements such as serum. The source and testing requirements of bovine serum are subject to regulatory requirements (also see ⟨1024⟩).

VIRUS PRODUCTION PHASE

Relatively few cell types have been used as substrates in US-licensed viral vaccines. Vaccine manufacturers have optimized nutrient requirements, growth factors, and serum concentration to support robust growth and strong virus productivity for these cell lines.

In Ovo Cultivation

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. The culture of viruses in ovo is fairly straightforward. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. In the case of influenza vaccines, the vaccine virus is harvested from egg allantoic fluid. Egg-based vaccine production, like all biomass expansions, requires care and quality control of the viral seed lots and egg substrates to avoid contamination with other organisms. Flock genetics, nutrition, and age can all affect in ovo virus propagation, as can egg sanitization procedures that may compromise the allantoic membrane cells. Optimization of the parameters should be documented to control the viral production process. Common egg culture variables include: egg age, amount of virus inoculated per egg, mode of inoculation, and harvest timing. Depending on the virus strain, the incubation temperature can also be critical. These factors have to be optimized for each individual serotype and master/working seed (for influenza viruses in particular), and the impact of all these factors should be investigated and documented.

Establishment of Primary Cell Cultures

Procedures for recovery of primary cells should be described from organ recovery through dispersed cell suspension, with attention paid to the reproducibility of the cell substrate's properties in culture. This analysis may involve subsequent growth reproducibility, or if minimal growth is involved, it should be aimed at reproducibility of metabolism and viral receptivity and productivity. In cases where whole organism harvest is involved (e.g., whole avian embryo dissociation), only a fraction of the resulting cells may be susceptible to viral infection. Other cells from the harvest provide a supporting matrix and other factors and can potentially inhibit virus replication. Preferably, modern analytical methods would be used to characterize the spectrum of cells harvested. Under conditions suitable for viral replication, little or no cell growth is expected.

Cell Culture

Culture for live virus vaccine production generally consists of two phases: a cell growth phase and a virus production phase. These processes are often complicated due to the diversity of cell types and their general dependence on a surface for growth. Virus properties—including replication cycle speed, yield of virus from the cells, and stability of infectious virus or antigen under culture conditions—also exert major influences on process technology, configuration, and operation in the manufacturing setting. Reproducibility of the cell processing steps is critical, and therefore good documentation of each manipulation ensures consistency of key parameters for product manufacturing. The virus culture process begins with the establishment of a receptive cell mass of sufficient size to produce a batch, which is typically about 10^5–10^6 doses of vaccine. Generally, about 10^6–10^8 cells are cryopreserved in one or more aliquots, whereas about 10^8–10^{12} cells may be required for a production batch. Historically, processes have also used secondary or tertiary cultures, in which primary tissues are grown for several cell divisions to either increase the biomass size and batch size, or to minimize the amount of primary tissue required per batch, or both.

Recovery of cells from cryopreservation involves thawing the cells and diluting out the cryoprotective (banking) medium such that maximum cell viability is preserved and reproducible cell growth is ensured. Practices should be documented and justified on the basis of development experiments; typical parameters investigated are thaw rates and temperatures, dilution rates, and dilution factors. A protocol must be devised that results in reproducible cell numbers in a state conducive to virus production. This requires passaging the cells and providing a larger surface area for growth for attachment-dependent cells. Cell expansion output:input ratios for a passage are typically 2:1–20:1, depending on the cell type and growth medium. In certain contexts, cells require a minimum threshold concentration to provide each other with growth factors, and this requirement may reduce the passage ratio. Other cell types, or media that provide these growth factors, can allow much lower inocula and larger ratios.

Two typical objectives for expansion passaging reproducibility are: 1) ensuring that cells experience a minimal “lag phase” (sometimes caused by the need for sufficient production and accumulation of growth factors and cellular attachment factors) and 2) ensuring that cells do not grow to the point of stagnation/death (often caused by nutrient exhaustion, or by crowding on the surface that causes “contact inhibition” of growth). Either of these issues can lead to a lag phase in the next passage. Early cell expansion is usually performed in a separate production area to avoid the potential for cross-contamination. The earliest, small-volume passages are often conducted in open laboratory vessels (e.g., flasks, roller bottles). However, it is highly desirable to use closed containers to facilitate aseptic handling of containers and to minimize or eliminate operator exposure risk. Control of the environment and aseptic handling practices are written into the procedures for use during development and are maintained during manufacturing.

The final cell expansion is typically an integral part of virus production, with goals of achieving the final biomass required for production and producing cells that are highly productive for virus. In batch or fed-batch cultures, a “cell density effect” may exist, whereby growth to higher cell concentrations correlates with lower virus productivity on a per-cell basis. This can be caused by many factors and should be characterized to ensure that the process operates in a robust and predictable manner.

Virus Culture

The final cell expansion and virus culture are typically a biphasic process in which the cells are grown in the final production vessel(s) and then are inoculated with virus. The viral inoculation often coincides with medium replacement or addition, as well as alteration of other culture parameters such as temperature. If cells are grown in a serum-containing medium, the medium for viral production is usually switched to a serum-free medium to minimize serum carryover into the harvest. If the virus is secreted into the medium and cannot be purified rigorously after harvest, extensive washing may be required to reduce residual serum to acceptable levels (see 21 CFR §610.15, *Constituent materials*). Documentation of washing effectiveness should be developed. Replacement of serum may be required for maximal virus yield, and this may entail the addition of other protein sources such as albumin, transferrin, or insulin.

Virus infection results are known to be sensitive to cell concentration; time of harvest; future cell substrate growth trajectory at the point of infection; and the “multiplicity of infection” (MOI), which is the number of infectious virus particles inoculated per cell. These factors, with the duration of viral culture, affect the quantities of infectious virus and total virus and can govern the quality of a vaccine via the ratio of infectious to total virus, the genetic stability, coupled with the degradation products that may have reduced immunogenicity. In addition to assessing titers over the duration of infection, it is useful to characterize virus stability under culture conditions with cell-free culture broth samples, because this can inform whether titer changes are due to changes in synthesis or degradation. High MOIs that occur over repeated passages are generally undesirable, especially for RNA viruses, because of the propagation of defective genomes that are trans-complemented by co-infection of a cell by another viral genome. Genetic stability of the vaccine virus should be well characterized. During development, the infection parameters need extensive characterization against all of the key quality attributes for the vaccine to ensure a robust process; thus, these parameters should be described.

Virus disposition upon replication is a significant determinant of the infection process configuration. Enveloped viruses are typically 1) secreted into the culture medium as they acquire their lipid envelope at the cell membrane or 2) fusing as secretory vesicles with the plasma membrane. For non-lytic viruses, multiple batch-wise harvests of the culture supernatant are common and re-feeding the culture upon harvest can promote higher yields. Perfusion processes are not used often because of product dilution and the difficulty, as well as added expense, of downstream concentration. For lytic viruses, destruction of the cells allows only for a single harvest; depending on the time elapsed from infection to lysis, feeding of the culture may maximize yields. Other viruses remain intracellular or cell associated, and processes for these may allow for extensive feeding to accumulate virus product. If the cell integrity is not compromised, washing to remove undesirable medium constituents may be performed at the end of the culture.

Many parameters of the culture environment also affect virus growth and stability, and it is expected that all relevant process parameters will be controlled to meet product quality expectations, as well as their acceptable ranges (“operating space”) as defined in the International Council for Harmonisation (ICH)’s *Q8(R2) Pharmaceutical development*. It is important to note that there is biological variability in cell growth, virus–host cell interactions are not well defined, and the analytics for potency and

other parameters are highly variable and should be defined. Consequently, despite efforts to characterize viral vaccines and their processes, it is not uncommon to experience root mean square variances of 50%–100% in process output, even for a well-controlled process, unlike some other biological medicines.

Control Cells During Production

Control cells are recommended for use when it is not feasible to directly test cells or product at various production stages. Control cells are tested for adventitious agents. For vaccines produced in cell cultures (e.g., live, attenuated, nonreplicating, and recombinant vaccines manufactured in insect cell lines), control cells may be evaluated for the presence of adventitious agents in conjunction with product testing using the same tests that are performed on the production culture and at the same time as a similar test would be performed on a manufactured product.

The national regulatory authority guidance-recommended volume of cell suspension used for seeding vaccine production culture should be used for the preparation of control cells. For static cell culture (using flask, roller bottle, cell stacks, or cell factory), the World Health Organization guideline recommends using a fraction equivalent to at least 5% of the total or 500 mL of cell suspension, or 100 million cells to prepare uninfected control cell cultures. For large scale production using a bioreactor, the volume of control cells should be as agreed with regulatory authority. The control cell culture should be treated similar to the production cell culture except the control is not infected with the vaccine virus under production; otherwise, the control cell culture should be maintained in conditions that are equivalent to those used for production culture, including the same batches of media and media changes, etc. The control cell should be incubated under the same condition of the production cell culture for 14 days or until the last virus harvest from production culture, whichever happens later. The control cells should be tested for identity and observed for cytopathic and morphological changes during this incubation period. For the test to be valid, at least 80% of control cells should not show any cytopathic or morphological changes during the observation period. Control cells should be tested for hemadsorbing viruses at the end of the observation period or at the time of viral harvest (whichever occurs later). At least 25% of the control cells are tested. If red blood cells to perform hemadsorption were stored, storage should be at 2°–8° and should not exceed 7 days. Calcium and magnesium ions should be absent from the medium for hemadsorption testing. The control cell culture supernatant should be tested for extraneous agents based on risk assessment aligned with the analytical testing strategy. For testing other extraneous agents, indicator cell lines (cell lines from simian origin and from human origin; production cell of a different batch if production cell is not of simian or human origin) should be inoculated with a control cell supernatant and incubated under the same conditions of the production cell culture for a period of 14 days. If any cytopathic changes due to extraneous agents occur in any of the cultures, the virus harvest produced from the batches of cells from which the control cells were taken should be discarded.

If avian tissues are used for propagation of a vaccine virus, avian leucosis virus (ALV) tests should be carried out using control cell culture supernatant. This testing may be carried out directly on the cell substrate. In some cases, detailed documentation of the health of a specific pathogen free flock may be sufficient to ensure absence of ALV.

For live attenuated vaccines that are produced in eggs, a risk-based control strategy for extraneous agents should be used. For example, control eggs may be tested for extraneous viruses. A portion of uninoculated embryonated eggs from the same batch of eggs used for vaccine production should be incubated under the same condition as those maintained for production and for the same incubation period. Allantoic fluid from control eggs should be checked for hemagglutinating agents and extraneous agents e.g., ALVs.

Culture Process Technologies and Facility

Because of safety concerns regarding the use of tumorigenic cell types, the cells used for viral vaccine culture are almost uniformly dependent on an attachment surface for growth, with few anchorage-independent exceptions (e.g., Madin–Darby canine kidney cells for influenza). The attachment requirement creates a need to supply not only soluble nutrients but also an attachment surface for the common cell types (e.g., MRC-5 and Vero). Given the large variability in virus yields and production volumes, surface area requirements can differ substantially. This has created a situation in which a variety of technologies are used for cultivation. For small-scale operations, simple adaptation of laboratory vessels may suffice, and scale-up consists of scale-out (i.e., increasing the number of vessels). Beyond a certain point, aseptic risk precludes further scale-out. Intermediate scales of culture involve the use of specialized culture vessels, along with robotics where scale-out strategies are still used. Many of these technologies use disposable vessels and reactors with a focus on: 1) minimizing the risk of contamination and 2) the compatibility of product contact surfaces with the cells and virus. An extractable and leachable assessment should be performed for each disposable system of novel components used (see *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩).

Large-scale production is performed in engineered bioreactor systems that use agitation technology similar to the technology for bacterial vaccines and other biologicals. In the most common reactors using agitation, attachment-dependent cells use "microcarrier" technology, which consists of small beads of about 150-µm in diameter that are easily suspended by agitation and provide a very large surface area in a given volume of culture. Other configurations with packed or static beads are also used for these systems. The vessels used can be made of either disposable plastics or stainless steel. Compatibility of the material with the medium and the cells, and the non-impact of leaching, should be evaluated for either type of vessel, as well as for fittings and probes used in monitoring and controlling the cultures. For nondisposable equipment, cleaning and sterilization procedures must be established, validated, and well documented. For reactors with loose or suspended carriers, the carriers should be readily excluded from the product stream and should not shed pieces or chemicals that are not suitable for human administration.

For all systems, the environment should be designed appropriately, reflecting the need for aseptic manipulations if they are required for either culturing or bioreactor set-up. In addition, the potential for spills or breakage and the risk of operator exposure need to be taken into account, especially when culturing wild-type virus strains. In a multi-product facility, changeover procedures that minimize the potential for cross-contamination and validation of inactivation should be established and

documented. Operator training and facility management procedures must be established for handling cultures and avoiding contamination of the product, the facility, and the operators themselves.

PURIFICATION

A general overview of purification of vaccines is presented in ⟨1235⟩. Special considerations are observed depending on the types of vaccines and production system used, as discussed below.

For live, attenuated, and nonreplicating virus vaccines, the desired antigen is a virus entity that is amplified from a working viral seed, in a complex biological substrate, and generally with minimal downstream processing to prepare the drug substance. Although nonreplicating viral vaccines also require viral seed expansion, virus inactivation and purification are more complex to ensure complete removal of infectious virus, removal of inactivating agents, and maintenance of the desired antigenic properties. Many factors should be considered in developing a downstream purification strategy, with the understanding that all processing steps will result in some loss of viable virus. Downstream processing steps are designed to: 1) remove or reduce cellular debris (e.g., host cell protein and DNA) from the vaccine harvest; 2) remove impurities or reduce their levels; 3) concentrate the virus; 4) add a virus stabilizer; and 5) remove or reduce potential microbial contamination from the virus preparation to meet a specified level of adventitious agent removal by filtration. The ability to do this may be limited in the case of live virus vaccines.

If a continuous cell line (e.g., Vero) is used for vaccine production, a validated filtration step is necessary to separate the virus from intact cells. The quantity and size of any residual host cell DNA should also be determined (see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩). Currently, NMT 10 ng of host cell DNA is permitted per dose of a parenterally administered vaccine, and regulatory agencies continue to consider the level of risk posed by host cell DNA for vaccines that are administered by other routes (e.g., nasal or oral) on a case-by-case basis. Multiple purification methods to reduce the size and amount of residual host cell DNA present in the vaccine are desirable and include steps such as treatment with DNase, diafiltration, ultrafiltration, and column chromatography.

Viral Inactivation

Because of the pathogenic nature of the viruses (e.g., hepatitis A, Japanese encephalitis, rabies, and poliomyelitis), many viral antigens must be inactivated while maintaining their antigenic properties. Inactivation may involve the use of chemicals, detergents and surfactants, or irradiation, or various combinations thereof. Removal or reduction of the inactivating chemicals, detergents, and surfactants from the antigen to acceptable levels may be achieved by centrifugation, chromatography, ultrafiltration, diafiltration, or combinations of the above. The viral inactivation process should be optimized, validated, and well-documented. Inactivation is confirmed by carrying out a test for residual infectious viral particles on each lot.

Live, Attenuated, and Nonreplicating Viral Vaccines

To preserve the infectivity and antigenic integrity of the vaccine viruses while removing egg-derived components, relatively simple, mild methods (e.g., zonal sucrose gradient centrifugation and diafiltration) are used for vaccine virus concentration, purification, and buffer exchange. After removal of cellular debris, further purification can be accomplished by using zonal centrifugation on a density gradient or by using column chromatography. Nucleic acid in Vero cell-produced vaccines can be reduced by treatment with suitable endonucleases, and removed by ultrafiltration using a 50-kDa membrane or by ion-exchange chromatography. The resulting drug product may be stabilized via cryoprotectant or lyophilization formulation (e.g., combinations of materials such as sucrose, phosphate, glutamate, and processed gelatin as stabilizers), filtration (using a filter of 0.22-µm pore size), for low bioburden bulk, and may be stored frozen at a temperature validated to support stability.

Some viruses may be heat labile, e.g., varicella virus, therefore precaution should be taken throughout manufacturing and distribution to maintain viral viability.

Viruses for nonreplicating vaccines can be produced using non-SPF eggs because of required chemical inactivation steps in the manufacturing process. Because the vaccine virus needs to be retained intact while removing egg-derived components and inactivating chemicals, relatively mild purification and concentration methods (e.g., zonal sucrose gradient centrifugation) are used. If chemical agents are used in the process, they should be minimized in the final product to below prespecified levels.

In some cases (e.g., the Varicella live vaccine) viruses are not released in the culture medium but remain associated to the cell substrate. In this case, the infected cells constituting a single harvest are rinsed, removed from the attachment surface, and subsequently disrupted by sonication to release the virus.

Virus-Derived Subunit Vaccines

Although most seasonal influenza vaccines (influenza A and B) are prepared either in embryonated eggs or via cell culture, the derived virus is processed using different manufacturing methods to make the drug substance. The drug substances can be live or attenuated viruses (whole virus), a detergent-solubilized "split vaccine", or a subunit vaccine resulting from purification of the surface glycoproteins (e.g., HA and NA in the case of influenza virus) from the internal nucleocapsids. To produce the split virus and subunit vaccines, the whole virus is subjected to disruption with a surfactant, which solubilizes the viral membrane. For subunit vaccines, the internal subviral core of the virus is separated from the surface proteins on the basis of their differing sedimentation rates. With split-virus vaccines, the choice and use of surfactant ensures that the subviral core itself is disassembled. Diafiltration is normally used to remove the surfactant and other media components from the product, and then the process is terminated with a sterile filtration step to produce a monovalent bulk solution.

Viruses for split virus and purified subunit influenza vaccines are produced in embryonated eggs. Inactivation and purification of vaccine viruses are achieved by chemical treatment (e.g., formaldehyde or β-propiolactone) and zonal sucrose gradient

centrifugation, respectively. Split virus vaccines are prepared by the disruption of vaccine virus particles using a detergent (e.g., sodium deoxycholate) that preserves antigenic integrity.

Recombinant or VLP Vaccines

Recombinant proteins that are components of VLP vaccines, such as hepatitis B and HPV, undergo stronger, more stringent purification processes. As with nonreplicating viral vaccines, the purification methods must maintain the desired antigenic properties. After cell culture propagation of recombinant proteins or VLPs, the cells are harvested and purified by downstream processing. This may involve chromatography, precipitation, microfiltration, ultrafiltration, or other techniques that will be specific for the vaccine being produced by each manufacturer.

The first licensed human vaccine using recombinant DNA technology was the hepatitis B VLP vaccine. The hepatitis B VLP vaccine is derived from non-infectious hepatitis B virus particles. The vaccine drug substance consists of spherical particles of the recombinant hepatitis B virus surface antigen (HBsAg), a lipidated protein whose viral gene component was cloned into the yeast *Saccharomyces cerevisiae*. The HBsAg particles consist of approximately 30% lipid by mass. For hepatitis B VLP production it is necessary to express the particles in host cells that have the appropriate environment and lipid composition for the HBsAg to self-assemble into spherical particles of the correct size.

In the case of the HPV vaccine, formation of VLPs can coincidentally result in the incorporation of host cell components (e.g., DNA) into the quaternary structure of the molecular assembly, resulting in a class of impurities that has a tight association with the active pharmaceutical ingredient. As a result, in some cases modern approaches to VLP production include a disassembly step that dissociates impurities from the viral proteins. This procedure is followed by a reassembly step that reforms the VLPs in the absence of the host components. Liquid-phase extractions and chromatographic procedures can be used to provide high-purity components for use in vaccine products with no substantial risk of carrying over significant residual host components. One HPV vaccine is bivalent and consists of recombinant proteins expressed and purified from insect cells infected with recombinant baculovirus (the vaccine contains no viral genetic material). The second HPV vaccine is tetravalent and consists of recombinant proteins purified from yeast cells. After purification, the VLPs may be mixed or absorbed onto an adjuvant to increase the immune response.

Recombinant influenza virus can be produced in continuous insect cell cultures which are grown in a serum-free medium composed of chemically-defined lipids, vitamins, amino acids, and mineral salts. Each of the four HAs is expressed in this cell line using a baculovirus vector (*Autographa californica* nuclear polyhedrosis virus), extracted from the cells with Triton X-100 and further purified by column chromatography. The purified HAs are then blended and filled into single-dose syringes. Manufacturers must also ensure that residual host cell components are sufficiently removed during the purification of recombinant-derived vaccine components.

Characterization of the recombinant monomeric antigens follows the typical requirements for recombinant proteins and would include peptide mapping coupled with mass spectrometry to assess the integrity of the primary structure, as well as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and other appropriate assays. Characterization of the intact VLPs will focus on assessing the size distribution of the particles using techniques such as transmission electron microscopy (TEM) or cryo-electron microscopy (cryoEM) and dynamic light scattering (DLS).

In-Process Controls

Manufacturers identify critical process parameters and perform appropriate tests to monitor the purification process. Among the latter are filter integrity tests, microbial enumeration tests (see *Microbial Enumeration Tests* ⟨61⟩ and *Bacterial Endotoxins Test* ⟨85⟩), and other suitable tests for known process residues of concern. Process residues include raw materials, culture medium components (e.g., serum and antibiotics), cell substrates (e.g., proteins and DNA), buffer components (e.g., EDTA), and other materials (endonucleases, protamine sulfate, detergents/surfactants) used during purification. In addition to in-process control tests, manufacturers may perform in-process monitoring tests to monitor total protein, specific antigens, or ancillary materials at various steps in the purification process of intermediates. These in-process monitoring tests are carried out to gather product and process knowledge to evaluate consistency in the manufacturing process.

To demonstrate process performance and reliability, manufacturers should characterize inherent process-related impurities (e.g., protein, DNA, and lipids). Process validation studies may be carried out to demonstrate sufficient and consistent removal of certain residuals to an acceptable level such that routine testing is no longer required. If material must be sterile, analysts can perform *Sterility Tests* ⟨71⟩.

INTERMEDIATES

Intermediates are defined as the unformulated active drug substances that are further processed before final formulation and can potentially be stored for long periods of time (see ⟨1235⟩). If intermediates are to be stored, hold times should be validated through a formal stability study or program. Stored intermediates are often suitable for more detailed characterization studies than are harvest bulks or final products (see, for example, *Table 4*). Some release tests are routinely performed before the intermediates are converted to the final bulk; this varies depending on the individual vaccines.

Tests for Intermediates

In general, viral safety lot release testing is done at the bulk harvest stage before any purification. This is true regardless of whether the assay detects the infectious virus or viral components. Typically, the prefiltered harvest stage is the most concentrated due to the limited processing that has occurred; therefore, it is likely to be the best stage of production for testing for adventitious agents. Depending on the specific vaccine, different intermediates than the unprocessed harvest may be

preferred for viral safety, for example, in cases where cytotoxicity of the unprocessed bulk harvest may be a concern. The presence of host cell DNA may need to be assessed in the case of biologics manufactured in animal cells. A hold step may be introduced at this processing step, for example, to combine two or more single virus harvests for further processing.

Table 4. Example list of generic tests used for unprocessed bulk harvest (not required for all vaccine products). A risk-based approach for control should be performed and agreed to with the competent authority.

Assay	Purpose	Method
Adventitious agent	Verify absence of viral contamination	Cell culture with cytopathic effect (CPE) and hemadsorption endpoints
Mycoplasma	Detect mycoplasma	Cell culture, PCR, growth, or fluorescent staining as endpoints (*Mycoplasma Tests* ⟨63⟩)
Microbiological purity	Find evidence of microbial or fungal contamination	Bioburden/absence of bacterial and fungal contamination, other as appropriate aligned with manufacturing conditions
Potency	Measure quantity of infectious virus or antigen	Cell culture with plaque or tissue culture infective dose ($TCID_{50}$) endpoints; RNA quantification, egg infective dose (EID_{50}), plaque assay, single radial immunodiffusion (SRID), immunochemical method

In-process adventitious agent inactivation steps are not included for live viral vaccines, because these steps could compromise the live nature of the vaccine itself. As a result, it may not be possible to validate clearance of any adventitious agents. Therefore, comprehensive testing for adventitious agents and qualification and control of the vaccine source materials (including cell substrate) are essential as part of vaccine safety control. Bioburden is monitored using appropriate microbiological methods based on technical principles described in, for example, ⟨61⟩ or ⟨71⟩.

Other product-specific testing may be appropriate. For example, for live influenza vaccines, each lot of viral harvest is tested for the cold-adapted phenotype (can grow at 25°) and the temperature-sensitive phenotype (restricted replication at 37°–39°). [NOTE—The attenuation phenotype (restricted replication in the upper and lower respiratory tract of ferrets) is tested at viral seed stage.] Each lot of viral harvest is also tested extensively by in vitro methods for contaminating agents. Viral harvests from the A and B strains are subsequently blended and diluted as required to the desired potency to produce bulk vaccine. If intermediates are stored prior to drug substance manufacturing, stability over the hold time should be established.

DRUG SUBSTANCE

The drug substance is normally the bulk antigen stored in an appropriate container and may be partially formulated in anticipation of subsequent steps. In some cases, the drug substance might already have been combined with adjuvants and excipients to be exceptionally agreed with the National competent authority. The drug substance may have been filtered, sterile filtered, or aseptically processed and then tested respectively for bioburden with adequate acceptance limits or for sterility.

Drug substances can be stored in containers at temperatures that ensure their stability before further processing. Storage times for drug substances should be validated through a formal stability study or program. Purity testing may occur with the drug substance in lieu of drug product. Product- and process-related impurities are monitored to confirm that their levels meet the requested specifications. Because viral vaccine drug substances generally have high potency, and subsequent dilution to the drug product potency may be significant, measurements of process impurities are best obtained from the drug substance rather than from the final container.

Examples of release tests for the drug substance may include:

- Appearance
- Identity of antigen
- Potency and activity, or quantity of epitope or antigen
- Infectious titer (live, attenuated viral vaccines)
- Protein integrity and purity [e.g., by SDS–PAGE, size exclusion–high-performance liquid chromatography (SEC–HPLC), cation exchange (CEX)]
- Monomer content in multimeric VLP vaccines
- Impurities
- Endotoxins content
- Bioburden or sterility
- Mycobacteria
- Mycoplasma and spiroplasma

In addition to the examples listed above, other methodologies can be applied for identity and purity evaluation. For instance, specific impurities that must be measured are agreed upon between manufacturers and the licensing authority during the licensure process.

DRUG PRODUCT

General guidelines for vaccine products are provided in ⟨1235⟩. This includes information regarding labeling and assessing the stability of vaccines.

Mechanically, the processes of formulation and filling for viral vaccines have changed little over time. Formulation is usually a simple dilution of high-potency drug substance to the appropriate strength for filling that takes into account yield losses expected through subsequent temperature exposures (including freeze–thaw cycles) and, where applicable, losses due to lyophilization. Some nonreplicating, subunit, and recombinant VLP vaccines are produced as formulations that are adjuvanted, alum adsorbed, or both. It is important to note that production of drug product lots involves filling containers into the tens or hundreds of thousands, and special attention must be paid to ensuring homogeneity of product over so many containers with volumes ranging from 0.1 to 10 mL. For live products, thermal degradation can occur over the course of the filling operation, which should be considered in process design. For products containing aluminum suspensions, homogeneity of the suspension is a significant concern. Generally, upon agreement with the relevant licensing authority, liquid product is "overfilled", both with respect to volume (to ensure the ability to withdraw a full dose) and with respect to potency (to ensure a statistically acceptable probability that each container receives the declared potency at the end of expiry), given the variability of the process and assays used.

LOT RELEASE TESTS

General requirements for drug product lot release testing of vaccines are detailed in ⟨1235⟩.

Potency Assays

General requirements for potency tests are described in ⟨1235⟩. Many types of potency-indicating tests exist for viral vaccines and the selected potency test depends on the type of vaccine. Examples of viral vaccine potency assays include:

- Specific virus neutralization assay
- Quantitative PCR
- Viral antigen quantitation
- Viral titre
- Quantitative plaque formation assay
- Immunogenicity assay

Common Tests

Tests for sterility, purity, residual moisture (lyophilized product), bacterial endotoxins, identity, excipients, and manufacturing residuals are described in ⟨1235⟩, except as described below. Viral and rickettsial vaccines and antigens are generally exempted from a requirement for a pyrogenicity test.

Manufacturing Residuals

Expectations for the control of manufacturing residuals, including surfactants and those chemicals used in the inactivation process(es) are described in ⟨1235⟩. Formaldehyde and other chemicals used to manufacture vaccines must be minimized by purification steps in the manufacturing process. Limits are set on their residual content in the final vaccine, and content specifications are defined in the approved product license application.

Requirements to control residual material from cell substrates are described in ⟨1235⟩, including the steps that should be taken to prevent potential complications for hyper responsive recipients of these vaccines.

Preservatives

Chapter ⟨1235⟩ provides information on the acceptable use of antimicrobial preservatives, the types of preservatives used, the minimization of thimerosal content as far as possible, and the production of thimerosal-free vaccines. Limits and content specifications are set for each vaccine in the product license.

Excipients

Formulation chemicals for viral vaccines are often introduced during drug substance production, during the infection phase, or at harvest to enhance process productivity, particularly for enveloped live virus vaccines. Non-enveloped viruses or vaccines with extensive purification may introduce formulation chemicals only at the end of the purification process. A notable exception is formulation for combination viral vaccines, where the individual viruses may need stabilizers that are different from the global optimum for the mixture. In these cases, the final formulation of chemicals may be introduced late in the process to preserve maximum potency.

Formulation components may be included to minimize oxidation, to compete for other reactions that would damage the product, to provide pH buffering or tonicity, and in the case of lyophilized formulations, to replace water that would otherwise bind to proteins or provide other functions in the lyophilization process (e.g., cake formation/integrity). Chemically defined components are desirable and all components should meet appropriate pharmacopeial standards. In some instances, macromolecules such as albumin or dextran or fragments thereof (such as hydrolyzed gelatin) may be included in formulations to function as highly effective stabilizing agents. For those that originate from animal or plant sources or for new excipients, risk assessment should be conducted on the origin and processing of the materials to ensure freedom from infectious agents, and for other biological safety aspects (see *Excipient Biological Safety Evaluation Guidelines* ⟨1074⟩). Where pharmacopeial monographs exist, the excipients should conform. In addition to stabilizing agents, viral vaccines may contain preservatives if

the final container contains multiple doses. Preservatives should meet antimicrobial effectiveness testing requirements in the formulation (see *Antimicrobial Effectiveness Testing* ⟨51⟩). It is common for preservatives to reduce the sensitivity of drug product sterility testing. This needs to be addressed during sample qualification for release testing to ensure adequate safety (see *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩). In addition, some formulations may be incompatible with other in vitro or in vivo safety testing and may require alterations of conditions or sample pretreatments to achieve valid test results.

Adjuvants

Aluminum compounds including aluminum potassium sulfate (alum), aluminum phosphate, aluminum hydroxide, and combinations of these compounds are currently approved adjuvants for vaccine use. Chapter ⟨1235⟩ outlines sections of the 21 CFR §610.15 regulation governing the use of aluminum and the allowed amounts. Aluminum is quantified using colorimetric, titrimetric, or emission or atomic absorption spectroscopic methods of analysis (see *Aluminum* ⟨206⟩). The degree of adsorption of protein components to the adjuvant should be determined.

The adjuvants MPLA and squalene emulsions (AS03 and MF59 C.1) are licensed for vaccine use. MPL is quantified either through degradation to fatty acid methyl esters (FAMEs) and quantification by gas chromatography, or through quantification of the glucosamine content. Squalene is usually quantitated by HPLC.

OTHER INFORMATION

Additional guidelines for viral vaccines covering product retention, shelf life, expiry dates, storage, container, and labeling are detailed in ⟨1235⟩.

APPENDIX

Selected Regulatory Documents

- 21 CFR §600.3 (Definitions)
- 21 CFR §610.2 (Lot release)
- 21 CFR §610. 9 (Alternative tests implementation)
- 21 CFR §610.10 (Potency)
- 21 CFR §610.12 (Sterility)
- 21 CFR §610.13 (Purity, moisture, pyrogens)
- 21 CFR §610.14 (Identity)
- 21 CFR §610.15 (Constituent materials)
- 21 CFR §610.17 (Combinations)
- 21 CFR 610.18(c)(1)(ii) [Tumorigenicity]
- 21 CFR 610.18 (d) (Egg records)
- 21 CFR §610.30 (Mycoplasma)▲ (USP 1-May-2021)