⟨1238⟩ VACCINES FOR HUMAN USE—BACTERIAL VACCINES

Add the following:

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INTRODUCTION

An overview of vaccines for human use is presented in *Vaccines for Human Use—General Considerations* ⟨1235⟩, ▲which includes a glossary of terms.▲ (USP 1-May-2021) Bacterial vaccines can be derived from whole cells, either killed or attenuated in their ability to cause disease, or from some component(s) of the intact cell that is important for virulence or damage to the host ▲(see *Table 1* for examples). In some cases, the antigen may be recombinant and produced in a non-pathogenic organism.▲ (USP 1-May-2021) Another subset of bacterial vaccines, derived from toxins, is the toxoids. Bacterial vaccine products can be mixtures of components from different species, from different strains or different serotypes of the same species, or from different components from cells of the same species.

▲Table 1. Bacterial Vaccines Currently Licensed in the US

Polysaccharide-based vaccines • *Haemophilus influenzae* type b (Hib) glycoconjugate[a, b] • Pneumococcal polysaccharides[a] • Pneumococcal glycoconjugates[a] • Meningococcal polysaccharides[a] • Meningococcal glycoconjugates[a] • *S. enterica* ser. Typhi Vi polysaccharide[a]	Bacterial toxoids[b] • Tetanus toxoid[b] • Diphtheria toxoid[b]
Protein subunit vaccines • Acellular pertussis[b] • Anthrax • Meningococcal protein-containing vesicles in combination with recombinant antigens	Live attenuated bacterial vaccines[c] • *S. enterica* ser. Typhi Ty21a • Bacillus Calmette–Guérin (BCG) • *Vibrio cholerae* serogroup O1 ▲ (USP 1-May-2021)

[a] See *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines* ⟨1234⟩ for further information.
[b] Frequently administered with other antigens in a combination vaccine.
[c] Currently there are no inactivated bacterial vaccines licensed in the US.

The simplest bacterial vaccines consist of the purified cell-surface capsular polysaccharides (CPS) from organisms such as *Salmonella enterica* serovar Typhi, various meningococcal serogroups, or pneumococcal serotypes that cause meningitis, otitis media, acute respiratory infections, and pneumonia. Although the typhoid vaccine consists of a single polysaccharide, the meningococcal vaccines contain as many as four serogroup-specific CPS, and the pneumococcal vaccine contains 23 serotypes.

The immunological response to meningococcal and pneumococcal polysaccharides, and to the CPS from *Haemophilus influenzae* type b (Hib), is improved by covalent attachment of the CPS or an oligosaccharide derived from it to a suitable carrier

protein, ▲often a bacterial toxoid or naturally occurring non-toxic mutant toxin.▲ (USP 1-May-2021) The immunological response to these glycoconjugate vaccines is elicited via immunologic pathways different from those induced by purified polysaccharides, creates a T-cell-dependent response, and establishes immunological memory. ▲These vaccines are highly effective in infants. Detailed information on polysaccharide and glycoconjugate vaccines is provided in *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines* ⟨1234⟩.▲ (USP 1-May-2021)

Many bacterial pathogens, including those that cause diphtheria and tetanus, produce toxins that kill tissue. Immunological neutralization of these toxins is sufficient to prevent disease. These subunit vaccines consist of chemically detoxified toxins (toxoids) purified from culture supernatant and are capable of eliciting neutralizing antibodies against the native toxin. ▲▲ (USP 1-May-2021)

Although earlier pertussis vaccines consisted of myriad chemically inactivated whole-cell and toxin components, current acellular products contain various combinations of specific purified proteins, sometimes toxoided (e.g., with fimbriae and other cell-surface protein components). Compared to older products, these vaccines apparently produce protection by a different mode of action but have a lower incidence of adverse events. A combination of diphtheria and tetanus toxoids and an acellular pertussis vaccine form the core components of many polyvalent pediatric and adult combination vaccines. Hib glycoconjugate, hepatitis B, and/or inactivated poliovirus immunogens may also be added to these vaccines. ▲No inactivated whole-cell bacterial vaccines are currently licensed in the US.

Another type of meningococcal bacterial subunit vaccine is based upon pathogen-derived lipid vesicles containing a wide range of immunogenic outer membrane proteins. These vaccines may also contain individually produced recombinant immunogens.▲ (USP 1-May-2021)

Live attenuated bacterial vaccines are currently limited to Bacillus Calmette–Guérin (BCG), which protects against tuberculosis when administered through the skin, and the *S. enterica* ser. Typhi Ty21a construct, which is an oral vaccine against typhoid fever.

The immune response against these ▲▲ (USP 1-May-2021) antigens can be increased by the inclusion of adjuvants. ▲Adjuvants included in▲ (USP 1-May-2021) licensed ▲bacterial vaccines▲ (USP 1-May-2021) in the US are based on aluminum salts such as aluminum hydroxide and aluminum phosphate, although the development and characterization of new adjuvants is an active area of research.

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RAW MATERIALS ▲AND MATERIALS OF ANIMAL ORIGIN▲ (USP 1-MAY-2021)

Raw materials can directly affect the identity, strength, purity, and quality of bacterial vaccines. ▲To achieve▲ (USP 1-May-2021) a consistent manufacturing process, it is critical to use consistent raw materials (e.g., during seed banking, fermentation, harvest, and purification). ▲▲ (USP 1-May-2021) Raw materials for bacterial growth media typically consist of both well-defined chemical entities (e.g., amino acids, carbohydrates, vitamins, minerals) and more complex components (e.g., protein hydrolysates, yeast extracts, peptones). ▲Expectations for the sourcing, quality, and control of raw materials are highlighted in ⟨1235⟩ and in *Cell Banking* ⟨1042⟩.▲ (USP 1-May-2021)

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CELL BANKS

Source and History

▲The development, characterization, stability testing, and storage of master cell banks (MCBs) and working cell banks (WCBs) is a unit operation and is described in detail in ⟨1042⟩.▲ (USP 1-May-2021)The source should generate a sufficient ▲quantity▲ (USP 1-May-2021) of antigen(s) to meet the medical need.▲▲ (USP 1-May-2021) For microbial expression systems such as *E. coli*, ▲▲ (USP 1-May-2021) the manufacturer should describe the method used to prepare the DNA coding for the protein, including the cell and origin of the source nucleic acid.

▲▲ (USP 1-May-2021) The steps in the assembly of the expression construct must be described in detail. This description should include the source and function of the component parts of the expression construct (e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether the protein is synthesized as a fusion protein). Manufacturers should provide restriction endonuclease digestion maps that illustrate the sites used in preparing the expression construct and sites used in the identification of DNA fragments.

A complete nucleotide sequence analysis of the expression construct's coding region for the protein of interest should be performed. The sequence analysis should be provided and should include a complete annotation designating all of the important sequence features. The copy number and physical state of the expression construct should be determined.

▲Requirements for defining and reporting the origins and genealogy of the cells used to construct the cell bank are provided in ⟨1042⟩.▲ (USP 1-May-2021)

Cell Bank Manufacture

▲▲ (USP 1-May-2021)

For microbial expression systems, a single host cell that contains the expression construct is propagated to generate the MCB. Manufacturers should document the cell cloning history and method of transferring the expression construct into the host cell. They also should describe completely the methods and criteria used to amplify the expression construct and to select the cell clone for production. ▲See ⟨1042⟩ for further details.▲ (USP 1-May-2021)

Cell Bank Testing

▲▲ (USP 1-May-2021)

Additionally, for recombinant bacterial expression cell lines (e.g., *E. coli*), molecular characterization testing can include DNA sequencing of the target gene sequence along with the flanking regions, expression construct retention, and plasmid copy number. Analysis of the expression construct at the nucleic acid level should be performed with consideration that this verifies only the coding sequence of a recombinant gene. Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for insertions or deletions and for the number of integration sites. For extra-chromosomal expression systems, the percent of host cells that retain the expression construct should be determined under selected and nonselected growth conditions. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies.

▲▲ (USP 1-May-2021)

Characteristics of the recombinant protein product can also be applied (see below) as another means of defining the ultimate output of the cell line.

▲▲ (USP 1-May-2021) If a new MCB is to be generated by expression construct transfer into host cells followed by clonal selection, then acceptance criteria for both the new clone and the protein produced by the clone should be described and justified.

▲Requirements for validation, testing, storage, and stability assessment of the cell banks are provided in ⟨1042⟩.▲ (USP 1-May-2021)

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▲PROPAGATION AND HARVEST▲ (USP 1-MAY-2021)

Production of the drug substance for a bacterial vaccine requires a fermentation process that is consistent and is ▲sufficient▲ (USP 1-May-2021) to support commercial production. The approach to achieving this has become fairly standardized and provides a relatively high probability of success for early batch production to support a development program. It is still a significant challenge to achieve sufficient productivity to support commercial manufacture of a licensed product. Directly after any fermentation process is the harvest process, which serves as a transition step between biomass expansion and downstream process steps. For the purposes of this chapter, harvest will be considered as an extension of the fermentation process.

Fermentation Starting Materials: Cell Inoculum

The cell inoculum for the fermentation process is the single most important component for establishing a reproducible fermentation process. In early development before finalizing the fermentation conditions, manufacturers typically must generate an interim source of this inoculum, a process development cell bank (PDCB). The origin of the PDCB should be a clonal isolate of the original transfected or isolated strain that demonstrates suitable growth properties and produces the antigen of interest in sufficient quantity and quality for the intended purpose. The use of a clonal isolate ensures that the genetic starting point for each batch is the same and that subtle variations in process conditions will not inadvertently allow one population versus another to dominate the culture. That is, the PDCB is used for fermentation development to ensure that variations in the fermentation conditions can be interpreted without the overlay of competition between populations of transfectants.

Initial development of the fermentation process, preferably with the PDCB, typically precedes production of the MCB and WCB. Best practice is to derive these cell banks from the same clonal isolate as the PDCB to reduce the need for a second cycle of fermentation development when the WCB is deployed. Substitution of a WCB for the PDCB at the final stages of fermentation development is common practice, but care must be taken to constrain such experiments to optimization of the fermentation process. More detail is found in *Cell Banks* above.

Fermentation Hardware

The biomass production process typically begins with a small-volume inoculum in an initial fermentation volume that is 20- to 100-fold larger than the initial inoculum volume. This initial passage is often followed by one or more intermediate fermentations that expand the production volume by 20- to 100-fold at each step until the production fermentation volume (typically 500–3000 L) is reached. Routine manufacture at these scales requires well-controlled fermentation conditions and physical facilities that meet the economic and current good manufacturing practices (cGMP) ▲requirements▲ (USP 1-May-2021) for a successful product.

Bacterial fermentations have traditionally been carried out in glass, glass-lined, or passivated stainless steel fermenters that comply with cGMP requirements, particularly when using large fermenters (e.g., those with >1000 L working volume) because of containment issues with such large volumes of liquid. Traditional fermentation systems require hard-piped control systems that meet the need for clean-in-place and steam-in-place capability. The bioburden and complexity of the facility will increase if the fermentation operations must also accommodate multiple product lines.

Smaller fermentation batches are increasingly performed in disposable bioreactors such as single-use bags with completely disposable product contact surfaces, including sensors and probes. These systems are becoming readily available, are less expensive, and are more flexible than fixed equipment and they meet the needs of the competitive business and evolving cGMP expectations and requirements. A note of caution is warranted, however, because this disposable technology can lead to changes in the material of product contact surfaces. Such changes then require re-evaluation and sometimes revalidation of the manufacturing process for late-stage development and commercial products. Thus, the reduced cleaning burden may ▲be accompanied by▲ (USP 1-May-2021) an increased need for extractability and leachability studies.

Harvest Hardware

Harvest of the fermentation product can focus on recovery of either the wet cell mass from which the product will be extracted or the fermentation broth from which the product will be directly purified. In the former case, centrifugal separation is typically employed. Production-scale centrifuges can be either closed operations with a fixed volume of input and manual recovery of the pellet, or continuous-flow operations that automatically eject and recover the clarified supernatant and/or the accumulated pellet. Although centrifuges are efficient in harvesting a fermentation product, shear forces can have significant effects on the product stream (e.g., lysed cells, sheared molecules in solution). Alternatively, and particularly when the product is secreted into the solution rather than retained in the cells, membrane filtration systems may be used to clarify the product stream for subsequent purification. Tangential-flow and depth filtration systems can be effective for recovering soluble product with less concern about shear forces.

In all cases, monitoring the processing of the fermentation output and solids removal from the liquid matrix can be a simple yet effective approach to monitor process consistency and comparability. Off-line tools such as HPLC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), or Western blot analyses can track product integrity issues such as aggregation or proteolysis.

Process Development

A productive, robust fermentation process is the result of careful consideration of a broad collection of variables, considered alone as well as in conjunction with product design and downstream processing. Fermentation process variables include chemical inputs (e.g., carbon sources, minerals, vitamins, trace elements, antifoam, and gases), physical inputs (e.g., temperature, mixing, and pH), and biological processes [e.g., nutrient utilization rates, metabolite levels, and inductors (quantity/type/addition duration)].

Product Requirements

A critical consideration in process development is anticipating how much of the product will be needed. Too little product caused by operating at too small a scale imposes supply constraints and often post-licensure urgency to scale up the process. In contrast, too much product results in excess inventory, expiring lots, infrequent manufacturing, ▲▲(USP 1-May-2021) and generally poor economics. A clear market evaluation is needed before designing the manufacturing process or committing to a process for scale-up or -down.

Process Design

If one has a reasonably defined production need and an initial estimate of product yield, it is possible to extrapolate the scale of the fermentation from the volume (yield), production frequency, and expected productivity. Commercial fermentation of bacterial cultures is routinely carried out in volumes as large as 3000 L, but larger volumes are also used. A few large lots per year can be advantageous for a very robust process but may be limited by downstream process capabilities and/or the stability of the fermentation product as a production intermediate. An additional consideration may be the difficulty in generating enough lots to ensure that the fermentation process is indeed robust. Failure of a large lot carries important financial and inventory risks.

A large number of lots can impose logistical problems if the turnaround time is too tight or the coordination of downstream events becomes too complex. The logistics include quality control testing, which depends on the number rather than the size of lots. Production that involves a large number of smaller lots can require blending of multiple intermediate lots in order to produce a final drug product lot. This can cause challenges if product-related problems occur and may entail root-cause investigations. In general, appropriate fermentation sizing results in a process that has a turnaround time of less than 1 week, that can be accommodated with one or a few purification runs, and that results in one to several fills of final product after each purification cycle.

Early Development Considerations

During early development of a biological product, the most important fermentation considerations are an appropriate, well-defined MCB and a fermentation process that is reasonably productive, reproducible, and scalable. The fermentation process is often underestimated when one considers the physical, chemical, and biological control of the process as process volumes change by orders of magnitude.

The mechanics of the fermentation process are an important consideration. Fermentations are typically studied in shake flask experiments or even microscale reactors that can readily accommodate many experiments conducted in parallel. Although this is attractive for the initial identification of process conditions, the ultimate culture vessel should be a controlled fermenter where growth conditions can be controlled and monitored in a more rigorous and comprehensive manner. Manufacturers should begin work in small-scale fermenters as early as possible to ensure that robust, controlled experiments can be run to refine the initial fermentation conditions.

Fermentation harvest processes also should be scalable. Although it is possible to scale centrifugation conditions, it is a challenge to maintain equivalent centrifugal conditions, particularly in a flow-through mode. Filtration processes can usually be scaled more predictably, provided that the membrane manufacturer is anticipating the needs of the process development scientist.

When manufacturers define a process, they should evaluate its robustness by purposeful deviations such as changes in the sources of raw materials and the time and temperature limits of unit operations. Such evaluations better define the rationale

for setting process limits and for identifying which ▲parameters▲ (USP 1-May-2021) are most critical to the success of the manufacturing process.

Process Monitoring

On the basis of early development process characterization data, manufacturers should be able to identify key analytical measures that, if applied to all lots, can either verify the correct progression of the process or serve as a sentinel to determine whether a specific batch is showing signs of deviation from the typical profile. In the absence of such data, an aberrant process may go unnoticed or may not be detected until testing of a process intermediate shows either an out-of-trend or out-of-specification result.

For a fermentation process, many critical variables (e.g., optical density, pH, and specific nutrient levels) can be measured online and in real time to potentially allow the intervention to bring a given process back into the normal range, or at least to identify the point in the process at which the deviation occurred. Such data can be valuable in identifying potential process improvements. Conversely, in the absence of such data, troubleshooting can be a challenging and protracted process.

Scale-Up

Just as early development requires a focus on small-scale operations, scale-up becomes essential at some point to ensure that sufficiently large lots can be made to meet program needs. As these needs become increasingly complex, larger lots are essential to ensure that multiple experiments and observations can be tied to the same lot of product, which in turn is ▲essential▲ (USP 1-May-2021) to understanding critical process and product variables. If proper process engineering considerations were taken into account at the smaller production scale, scale-up can usually be done in 10-fold increments in volume with a reasonable expectation that significant process performance or product changes will not be seen. This approach may require adjustments at an intermediate scale if the initial fermentation was based on too small a volume or if the final production scale is very large. Again, process monitoring data can be very helpful in evaluating the success of the scaled-up process.

If clinical development studies are performed at less than full manufacturing scale, as they usually are, manufacturers will be obliged to relate the comparability of the process performance and the product characteristics at the different scales. Analytical data can be compelling, but in their absence or ▲when scale-related differences are present,▲ (USP 1-May-2021) manufacturers must demonstrate that these differences are not clinically significant. However, the use of comparability protocols for scale changes will ▲need▲ (USP 1-May-2021) to be approved by the local regulatory authority. ▲▲ (USP 1-May-2021) Analysts must take care to ▲separate▲ (USP 1-May-2021) differences caused by fermentation scale-up from changes caused by harvest or purification scale-up. One way to accomplish this is to compare process intermediates obtained, when possible, during the fermentation and harvest processes. As an example, online monitoring of fermentation conditions such as pH or glucose level can be used to demonstrate similarity during the time course of the fermentation. Similarly, measurements ▲obtained▲ (USP 1-May-2021) at the end of the fermentation process (e.g., final cell density, cell viability) and intermediate measurements during harvest (e.g., turbidity of clarified broth, wet cell mass in the pellet) provide useful information for evaluating the similarity or differences during scale-up.

Change to read:

PURIFICATION

A general overview of purification for ▲▲ (USP 1-May-2021) vaccines is presented in ⟨1235⟩. In addition to a description of critical processing equipment, reagents, and processing steps, manufacturers should provide the rationale for the purification process. ▲▲ (USP 1-May-2021) As with the other processes, analysts should consider the source of all raw materials and ensure that they come from reliable vendors who adhere to cGMP and can ensure a long-term supply. The cGMPs will apply to late-stage clinical supplies and commercial materials. The removal of non-product-related impurities (e.g., processing reagents, endotoxins, contaminating cell proteins or nucleic acids, and other residual contaminants) should be verified.

The drug substance can be one of several types of compounds, e.g., polysaccharides (wild type or modified), proteins (wild type, mutant, toxoids, or recombinant), products of conjugation of polysaccharides and proteins, or products of conjugation of peptides and proteins.

To define and control purification processes for drug substances and drug products, the manufacturer should establish targets for process parameters and tolerances for all critical process steps including yields, activity, and purity to ensure the efficacy, safety, and consistency of the final product. Requirements for pooling, if applicable, should be established. The requirements and conditions for storage of intermediates, bulks, and final containers must be established by an official stability program. The use, reuse, regeneration, and cleaning of all drug product/drug substance contact equipment (e.g., filters, chromatographic columns and resins, tanks, and process lines) should be validated. In addition, extractable/leachable studies should be performed for all product contact equipment (e.g., disposable bag systems, chromatographic column resins, and process lines).

Polysaccharide Purification

▲Methods for the purification and in-process control of the quality of polysaccharide antigens used as vaccines or for the manufacture of glycoconjugate vaccines are presented in ⟨1234⟩.▲ (USP 1-May-2021)

Protein Purification

The classes of bacterial protein vaccines include toxoids, nontoxoid ▲antigens▲ (USP 1-May-2021) (e.g., pertussis antigens), engineered recombinant products, and naturally occurring ▲variants▲ (USP 1-May-2021) (e.g., CRM197) used as carrier proteins ▲in glycoconjugates.▲ (USP 1-May-2021)

TOXOIDS

At the end of fermentation, the toxin-containing culture medium should be separated aseptically from the bacterial mass as soon as possible or placed in a cold room until separation can be effected. The toxin content, ▲expressed as limit of flocculation▲ (USP 1-May-2021) (Lf/mL), is checked using a flocculation assay with the appropriate antitoxin standard to monitor production consistency (the culture should contain NLT 40 Lf/mL). The toxin is purified first to remove any components that could cause adverse reactions in humans. A typical process includes depth filtration followed by 0.2-µm filtration to assist in the removal of cellular debris. Following preliminary purification, the toxin is then detoxified with formaldehyde or glutaraldehyde or any suitable chemical reagent by a method that avoids both destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly upon exposure to heat. Some toxoids require a single addition of formaldehyde, but others can require multiple additions. Alternatively, the toxin could be detoxified and then purified or partially purified by depth filtration, detoxified by the addition of an appropriate aldehyde, filtered using 0.2-µm filtration, and then pooled. The pooled toxoid solution is further purified by clarification with activated carbon, followed by multiple ammonium sulfate precipitation steps that further fractionate and concentrate the toxoid. Typical additional purification steps include concentration, diafiltration, and/or chromatography. Purification before detoxification results in a purer product and can be advantageous if the toxoid is to be used as the protein component of a protein–carbohydrate conjugate (because copurifying high-molecular-weight glycans will be removed before detoxification).

During detoxification and purification, ▲testing must be performed to control and ensure the consistency of the purification process, including▲ (USP 1-May-2021) endotoxin testing according to *Bacterial Endotoxins Test* ⟨85⟩, ▲testing for▲ (USP 1-May-2021) formaldehyde and protein ▲content, and testing of the▲ (USP 1-May-2021) irreversibility ▲of toxoiding.▲ (USP 1-May-2021) If the material must be sterile, ▲the sterility test described in▲ (USP 1-May-2021) *Sterility Tests* ⟨71⟩ can be performed.

PROTEINS/RECOMBINANT PROTEINS

Proteins used to make vaccines can be recombinant (in their native state or engineered to modify certain amino acids) or they can be naturally occurring ▲variants▲ (USP 1-May-2021) that have no wild-type activity but are capable of inducing the appropriate immune response. Proteins are harvested from the fermenter and are extracted (for example, by mechanical and chemical disruption) and then purified by suitable methods, typically consisting of filtration–concentration steps (e.g., ultrafiltration, tangential-flow filtration, diafiltration, centrifugation, selective precipitation, and even direct capture using expanded-bed chromatography or big-bead technologies). The enriched protein solution can be further purified using appropriate filtration and chromatographic steps. For all equipment that contact drug substances (e.g., chromatographic resins, membranes, disposable bag systems, or process lines), manufacturers should assess extractables and leachables. Analysts should determine the column resin life for all chromatographic systems used in the purification (including number of uses, reconditioning requirements, and storage conditions).

The type of chromatography used to purify proteins depends on the physical/chemical properties of the desired protein as well as those of other molecular entities in the harvest culture. As an example, CRM_{197} can be purified using a multi-step chromatographic process: production material is first diafiltered and then separated by ion-exchange chromatography [diethylaminoethyl (DEAE-Sepharose)] in order to purify the target protein from other molecular entities present in the purification stream. The peak of interest is collected and ammonium sulfate is added, followed by 0.22-µm filtration to condition the material before loading on the hydrophobic-interaction chromatography column (phenyl-Sepharose) for purification of the target protein based on its surface hydrophobicity. The peak fraction is then diluted with Water for Injection and is separated onto a ceramic hydroxyapatite column to further purify the target protein based on its surface charge. The eluted peak is then buffer exchanged into the storage buffer by ultrafiltration/diafiltration using cross-flow membrane filtration, followed by 0.22-µm filtration to yield the sterile purified concentrate.

In-process control of protein purification includes monitoring specific protein content and critical process steps as well as monitoring the removal of unwanted fermentation and purification components. The pH is critical for ion-exchange chromatography, and therefore pH should be monitored. For steps designed to remove endotoxin, procedures in ⟨85⟩ are used to monitor column eluants. Bioburden is monitored according to *Microbial Enumeration Tests* ⟨61⟩ after filtration and chromatography steps. If the material must be sterile, the sterility test in ⟨71⟩ should be performed.

POLYSACCHARIDE–PROTEIN CONJUGATES

▲An overview of the production, purification, and in-process testing of glycoconjugate vaccines is presented in ⟨1234⟩.▲ (USP 1-May-2021)

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INTERMEDIATES

An intermediate or process intermediate in vaccine manufacture is the reaction product of each step in the process except the last one, which forms the final product. Examples of intermediates are bulk-purified polysaccharides, proteins, and activated polysaccharides that conjugate to protein.

Most vaccine production processes are stepwise and take more than one elementary step to complete. An intermediate is produced from raw materials at one or more process steps (e.g., bacterial growth, extraction and purification, and▲/or▲ (USP 1-May-2021) chemical modification), eventually resulting in the drug substance. The identification of the key intermediates, their production, and sampling for analytical tests must be defined in controlled documents (e.g., batch records, analytical protocols).

Intermediates, which can ▲sometimes▲ (USP 1-May-2021) be stored for considerable periods of time before further processing, ▲must▲ (USP 1-May-2021) be included in a formal stability program. ▲▲ (USP 1-May-2021) Stability studies in normal or accelerated conditions should be performed to define the maximal hold time for intermediates and ▲also should be performed▲ (USP 1-May-2021) when significant process changes are implemented.

From raw material to finished drug substance, testing throughout the process ensures a quality product. Testing of intermediates is a key quality control step to ensure their identity and purity. The quality attributes of the intermediate are commonly tested in conjunction with further processing, and their release testing should be considered. Standard operating procedures (SOPs) must be properly defined for the analytical control tests. Because of their critical role in the production process, some key intermediates could be included in formal release testing, in addition to the intermediates identified for in-process testing.

▲▲ (USP 1-May-2021)

Examples of tests for the structural characterization and purity estimation of protein-based intermediates include the following:

- Identity and molecular size [(SDS–PAGE), Western blot, size-exclusion chromatography–ultraviolet (SEC–UV), SEC–fluorescence, size-exclusion chromatography–multi-angle laser light scattering (SEC-MALLS), reversed-phase chromatography]
- Endotoxin content (see ⟨85⟩)
- Residual nucleic acids (colorimetric assay; see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩)
- Bioburden (total viable aerobic count of microbial contamination; see ⟨61⟩)
- Other proteins as impurities (SDS–PAGE, Western blot, SEC–UV, SEC–fluorescence, reversed-phase chromatography)

The tests previously ▲described▲ (USP 1-May-2021) for protein-based intermediates are also applicable when the protein-based product is defined as the drug substance. In addition to the examples above, many other methodologies can be applied for the identity and purity evaluation. ▲Appropriate tests for saccharide and glycoconjugate intermediates are discussed in ⟨1234⟩.▲ (USP 1-May-2021)

Stability tests for intermediates can include physicochemical methods (see *Intermediates* above), which are formally included within an analytical panel for the stability study. In addition, biological and immunochemical tests [e.g., enzyme-linked immunosorbent assay (ELISA)] can be included. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided that testing is performed at a sufficient number of steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing. If intermediates must be stored and/or subsequently shipped to a different location for further processing, the stability of these materials must be demonstrated.

Change to read:

▲FILTERED BULK▲ (USP 1-MAY-2021) (DRUG SUBSTANCE)

▲The final step in the manufacturing of antigen drug substance is the preparation of filtered bulk or drug substance.▲ (USP 1-May-2021)

The drug substance is the final product of the antigen manufacturing process, before the formulation of the final vaccine dosage. The final bulk may be prepared aseptically or may include a sterilization step. Sampling for analytical tests for release and stability studies ▲▲ (USP 1-May-2021) must be defined in controlled documents (e.g., batch records, analytical protocols).

A drug substance can be stored ▲▲ (USP 1-May-2021) before further processing, but if it is stored the drug substance must be included in a formal stability program. ▲▲ (USP 1-May-2021) Stability studies in normal or accelerated conditions should be performed to define maximal hold times. A stability program is required for formal stability studies, and the studies must be executed according to a protocol that contains detailed information about the types of tests, specifications, ▲storage conditions,▲ (USP 1-May-2021) testing intervals, and time points.

Testing of the drug substance must be performed to ensure its identity and purity. All testing must be done according to established SOPs, and all tests must have specifications (or provisional specifications, where applicable).

▲Many▲ (USP 1-May-2021) methodologies can be applied for identity and purity evaluation. For instance, the specific impurities that must be measured are determined based on negotiations between manufacturers and the national drug regulatory agency during the licensure process. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided that testing is performed at sufficient steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing.

All of the results must be reported in a controlled document. Stability tests can include both physicochemical methods (see stability information, above) and biological/immunochemical tests (e.g., ELISA and serum bactericidal activity; see *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩).

Change to read:

DRUG PRODUCT AND LOT RELEASE

General principles are described in ⟨1235⟩, which outlines the lot release procedure in accordance with 21 Code of Federal Regulations (CFR) §610.1 and 21 CFR §610.2. For products that will be used in the US, samples and protocols containing all appropriate tests must be submitted to the FDA for review and/or testing. If the FDA determines that the lot meets the standards of safety, purity, and potency required for the particular vaccine, the ▲manufacturer distributes and markets the product.▲ (USP 1-May-2021)

Tests required for each lot-release protocol include potency, general safety, sterility, purity, identity, and constituent materials. Potency and potency-related tests are different for each bacterial vaccine. The inclusion of these tests makes each bacterial vaccine lot-release protocol unique.

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established based on either physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific culture tests, or in vivo or in vitro immunological tests. In large part, identity testing is performed to distinguish the vaccine from other materials that are manufactured at the same site (21 CFR §610.14). The same tests that establish identity may also be appropriate for defining the quantity of immunogen present in the final vial. This is especially important for carbohydrate-based vaccines that are dosed by mass and for which physicochemical measures of antigen quality are used.

Immunochemical methods, which include immunoprecipitation methods and immunoelectrophoretic methods, have been useful. Immunoprecipitation methods, such as flocculation and precipitation, can be carried out in solution or in a gel matrix and involve mixing the antigen with an appropriate antibody, leading to the formation of flocculating or precipitating aggregates that can be detected visually or by light scattering (▲e.g.,▲ (USP 1-May-2021) nephelometry). The ratio of reactants must be varied to optimize the detected response. In solution this can be achieved by titrating one reactant with the other, and increased sensitivity can be obtained by using antigen- or antibody-coated particles (e.g., latex) as reactants. In gel systems, a gradient is created as one or more of the reactants diffuse, creating a visible line where precipitation occurs. Immunoelectrophoresis (IE) is a qualitative technique that combines two methods: gel electrophoresis followed by immunodiffusion. Crossed IE is a modification of the IE method that is suitable both for qualitative and quantitative analysis. Visualization and characterization of immunoprecipitation lines can be performed using selective or nonselective stains, fluorescence, enzyme or isotope labeling, or other relevant techniques. Selective staining methods are usually performed to characterize nonprotein substances in the precipitates. In translucent gels, such as agar or agarose, the precipitation line becomes clearly visible in the gel provided that the concentration of each of the reactants is appropriate.

Where multiple active components are present as a result of copurification (e.g., certain acellular pertussis vaccines), the manufacturer must demonstrate that the composition of the product is consistent between batches, unless this has been validated during the development of the manufacturing process.

▲For toxoid vaccines or toxoid carriers in glycoconjugate vaccines,▲ (USP 1-May-2021) manufacturers should demonstrate that reversion to toxicity has not occurred (and will not occur over the shelf life) for a product derived from or containing a toxoid material, unless the contrary has been validated. This may require the use of a cell line or an in vivo test, although enzymatic approaches are being validated.

An antigenic purity test is an assay that assesses the ▲quality▲ (USP 1-May-2021) of antigen and is used for diphtheria and tetanus toxoid vaccines. The antigen content is determined by a flocculation assay ▲and is typically expressed in Lf/mg.▲ (USP 1-May-2021)

The manufacturer should prove a high and consistent level of immunogen adsorption to any solid-phase adjuvant (such as aluminum phosphate or aluminum hydroxide) that is consistent with the release specification.

For certain vaccines such as the anthrax vaccine and toxoid vaccines, the manufacturer is required to demonstrate that the vaccine is protective against disease or death in animal models challenged with a predefined dose of the target pathogen. This generally requires definition of the animal model, route of administration, vaccine dilutions required, a means to observe effects, and a reference vaccine against which effects are compared. The data should be analyzed appropriately (see *Analysis of Biological Assays* ⟨1034⟩).

Stability-indicating assays are those used to determine the stability of the product. Of primary importance is the potency assay, although glycan degradation may be important ▲when evaluating▲ (USP 1-May-2021) glycoconjugate vaccines.

Other Vaccine Components and Vaccine Properties

Aluminum compounds are the primary adjuvants used in vaccines in the US. Chapter ⟨1235⟩ provides provisions of 21 CFR §610.15 governing the use of aluminum and amounts allowed. The adjuvants widely used in bacterial vaccines include aluminum potassium sulfate (alum), aluminum phosphate, aluminum hydroxide, and combinations of these compounds. Bacterial vaccines formulated with such adjuvants are referred to as adsorbed vaccines, and this term may be included in the official name of the vaccine. Other adjuvant systems may be evaluated. Aluminum is quantitated using colorimetry, titrimetry, emission or atomic absorption spectroscopy, or inductively coupled plasma–mass spectrometry.

For regulations regarding residual manufacturing reagents, see the FDA's 1999 *Guidance for Industry: Content and Format of Chemistry, Manufacturing, and Controls Information and Establishment Description Information for a Vaccine or Related Product*. Manufacturing reagents such as formaldehyde and glutaraldehyde are sometimes used in inactivation, the toxoid-making processes, or elsewhere during manufacture and may be present in residual amounts in the final product. Limits for formaldehyde and other residuals must be minimized in accordance with the approved product license.

Common preservatives used in bacterial vaccines include thimerosal, phenol, 2-phenoxyethanol, and benzalkonium chloride. 21 CFR §610.15 provides additional information about the minimization of thimerosal content and the production of thimerosal-free vaccines. Limits and content specifications are set for each bacterial vaccine in the product license.

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins ▲(when required),▲ (USP 1-May-2021) as indicated in ⟨85⟩.

Each lot of final containers of a vaccine intended for use by injection may be tested for pyrogenic substances as indicated in *Pyrogen Test* ⟨151⟩ and 21 CFR §610.14.

Each lot of dried product ▲must▲ (USP 1-May-2021) be tested for residual moisture [see *Loss on Drying* ⟨731⟩ and FDA's *Guideline for the Determination of Residual Moisture in Dried Biological Products* (January 1990)]. Residual moisture should be determined for lyophilized vaccines.

▲In some cases, a▲ (USP 1-May-2021) general safety test is performed on biological products intended for administration to humans with the purpose of detecting extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR §610.11.

▲Excipients, preservatives, diluents, and adjuvants are tested to meet generally accepted standards of purity and quality per 21 CFR §610.16 and/or applicable guidance documents at an appropriate stage of manufacture.▲ (USP 1-May-2021)

The sterility of each lot of each product is ▲evaluated▲ (USP 1-May-2021) according to procedures described in ⟨71⟩ and 21 CFR §610.12 for both bulk and final container material.

▲Information on retention samples, record keeping, storage conditions, and expiry dates, as well as guidelines for the package insert, is included in ⟨1235⟩.▲ (USP 1-May-2021)

Delete the following:

▲OTHER REQUIREMENTS

Retention samples are held by the manufacturer for at least six months after the expiration date. Enough material of each lot of each product is held for examination and testing for safety and potency (see 21 CFR 600.13).

Records are maintained concurrently with each step in the manufacture and distribution of product such that at any time successive steps of manufacture and distribution may be traced (see 21 CFR 600.12).

For storage conditions, see 21 CFR 610.50 and 53.

For shelf life/expiry date, see 21 CFR 610.50 and 53.▲ (USP 1-May-2021)