vaccine bioprocessing handbook

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Table of Contents

Vaccine Process Overview

The History of Vaccine

The history of vaccine begins with the fight against smallpox, an ancient infectious, disfiguring, and often deadly disease. Edward Jenner, an English physician, introduced the first smallpox vaccine in 1798. He noticed that milkmaids who contracted cowpox—a similar contagious but not deadly disease—were immune to smallpox. Jenner injected pus from cowpox lesions into a young boy. Two weeks later, he infected the boy with smallpox, and the boy survived. This was the first scientific attempt to control an infectious disease on a large scale by means other than transmitting the disease itself. Jenner's friend, Richard Dunning, first used the word vaccination in 1800. Today, smallpox has been eliminated worldwide.

A vaccine acts like a "fire drill" in the immune system. The vaccine triggers an immune response in the body. The body produces "memory" T-lymphocytes and B-lymphocytes that "remember" how to fight the target disease in the future. Unlike natural infections, vaccines never cause illness, but they can cause minor symptoms such as fever. It typically takes a few weeks for the body to produce T-lymphocytes and B-lymphocytes after vaccination.

Generating vaccine-mediated protection is a complex challenge. Currently available vaccines have largely been developed empirically, with little or no understanding of how they activate the immune system. Different vaccine antigens exert different immune responses. This highlights the importance of antigen design. An adjuvant can modify the effect of other agents in the vaccine, which can increase the stimulation of the immune system. Early protection ability of a vaccine is mainly provided by induction of antigen-specific antibodies. Long-term production requires the persistence of vaccine antibodies and/or maintenance of quick and effective reaction of immune memory cells. B cells play a predominant role. T cells are also essential to the induction of high-affinity antibodies and immune memory.

Immunization is our shield against serious diseases. In 2020, vaccines prevent an estimated two to three million deaths every year. Vaccines protect against 26 diseases and help limit the spread of antibiotic resistance by preventing such diseases in the first place. An increase in immunization globally could save an additional 1.5 million people every year. When immunization rates are high, the wider community is protected. This includes infants who are too young to receive vaccines, older adults who are at risk for serious disease, and people who take medication that compromises their immune systems.

Types of Vaccine

There is a wide variety of vaccines. Depending on the type of antigen, they can be classified as one of following:

- **Modified live vaccines** are living pathogens with reduced virulence to prevent them from causing diseases. One dose or a double dose provides strong cellular and antibody response for lifelong immunity. Safety concern: possible reversion to virulence.
- **Killed vaccines** are disease-causing microbes that are inactivated, typically by chemicals. They generate a weak immune response and require additional doses, known as "booster shots." Safety concern: possible failure of virus inactivation procedures.
- **Subunit vaccines** include only the antigens that stimulate the immune system, normally in protein format. These are usually a safer choice because they reduce the chances of adverse reactions; however, they provide only weak immune responses.
- **Toxoid vaccines** are inactivated forms of toxins produced by certain bacteria. They are used as antigens to induce immunity.
- **Polysaccharide vaccines** are composed with capsular polysaccharide. They fail to induce significant and sustained amounts of antibodies in children younger than 18 months. They provide only short-lived immunogenicity. Additional doses cannot be given on repeated exposure.
- **Polysaccharide conjugated vaccines** are composed with capsular polysaccharide conjugated to carrier protein. They can trigger the immune response in the very young (six weeks old). The generation of immunological memory ensures long-lived immunity.
- **Virus-like particle vaccines** contain repetitive high-density displays of viral surface proteins that elicit strong T cell and B cell immune responses. They are noninfectious because they do not contain genetic material. They cannot replicate, which makes them safer. Because they are 40–120 nm in diameter, they are optimal for uptake by dendritic cells. They can be produced in a variety of cell culture systems and can self-assemble *in vivo*.

Types of Vaccine Summary

Type	Description	Antigen	Examples
Modified live vaccines (MLV) [live attenuated vaccine (LAV)]	Vaccines created by altering infectious pathogens to make them harmless while still maintaining their immunogenic profile	**Whole virus or bacteria**	**Rotavirus vaccine**
Killed vaccines (KV) (inactivated vaccine)	Vaccines consisting of virus, bacteria, or other pathogens killed or inactivated using a chemical method (e.g., formaldehyde)	**Whole virus or bacteria**	**Influenza vaccine**
Subunit and toxoid vaccines	Vaccines that contain only a specific protein subunit of a virus or toxoid of bacteria	**Protein or toxoid**	**Acellular pertussis vaccine**
Polysaccharide vaccine	Vaccines that contain polysaccharide derived from bacteria capsular	**Polysaccharide**	**Pneumococcal polysaccharide vaccine**
Polysaccharide conjugated vaccine (PCV)	Vaccines that contain polysaccharide covalently attaching to a carrier protein through a conjugation process	**Polysaccharide conjugated to protein**	**Pneumococcal conjugated vaccine**
Virus-like particle (VLP)	Vaccines that resemble a virus but contains no genetic materials and are therefore noninfectious	**Protein**	**Human papillomavirus vaccine**

Vaccine Process

Even though antigen types vary among different vaccines, the vaccine manufacturing process, in general, is similar for all types.

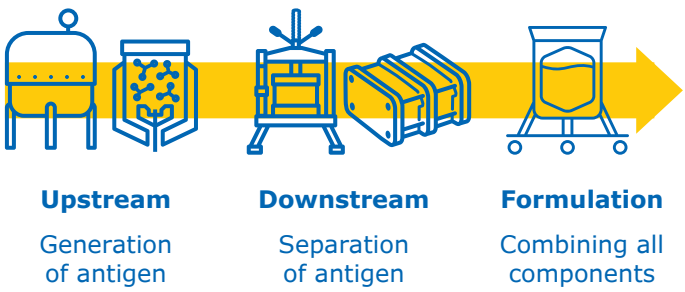

Figure 1: Typical vaccine manufacturing process

Upstream

An antigen is generated in the the upstream process. It can be the pathogen itself or it can be an antigen generated from a recombinant protein. Antigen induces an immune response. There are three types of antigen:

- Virus is generated by primary cells (e.g., chicken fibroblasts) or continuous cell line (e.g., Vero).
- Bacteria are grown in fermenters.
- Recombinant protein is generated by bacteria, yeast, or cell culture.

Downstream

The antigen is separated from impurities in the downstream process. This improves product safety and stability.

- The antigen is released from the substrate (e.g., cell lysis) if necessary and isolated from bulk environment.
- Depending on the characteristics of the antigen and the impurity, purification technologies may be employed: chromatography, ultrafiltration, precipitation, enzyme digest, or other processes.

Formulation

All components that constitute the final vaccine are combined in the formulation process.

- The formulation is designed to maximize the stability while creating a format that enables efficient distribution and preferred clinical delivery method.
- The formulated vaccine may include an adjuvant to enhance the immune response, stabilizers to prolong shelf life, and/or preservatives that ensure that multidose vials can be delivered.

Summary

Vaccines have been protecting against disease since the late 1700s. They function by triggering the immune system to generate short-term and long-term responses.

Because there are many pathogens, there are also many vaccines. They can be classified by antigen character, but the manufacturing flows used to produce them are relatively similar.

Viral Vaccines

Virus (from the Latin virus meaning “toxin” or “poison”) is a microscopic infectious agent that can reproduce only inside a host cell. Viruses consist of two parts: nucleic acid and capsid. Some viruses have a viral envelope. The diameter of most viruses is between 10 and 300 nm.

Viruses are generally attenuated via passage—growing several times in unrelated or foreign hosts such as tissue culture, embryonated eggs, or live animals. Likely, one of these will possess a mutation that enables the virus to infect the new host. However, this mutant normally has a lower virulence than the virus that was in the original host. The genetic information for interacting with the host does not change, enabling it to infect the host, but it causes less damage and so acts as a vaccine. Some of the modern vaccines use genetic engineering to precisely induce attenuation by selective mutation, gene deletion, or substitution. Examples are dengue vaccine and Japanese encephalitis (JE) vaccine.

Attenuated vaccines offer quick immunity, activate all phases of the immune system, and provide more durable long-term immunity. However, secondary mutation can cause a reversion to virulence. This means the vaccine may be able to cause disease in immunocompromised patients (those with AIDS, for example). Additionally, they can be difficult to transport because they must be maintained under certain conditions, such as temperature, to guarantee the survival of the virus.

The live attenuated viral vaccine manufacturing follows a complex, multi-step process. It is not a templated process. The manufacturing process for each viral vaccine is different and is dictated by shape, size, nature, physico-chemical behavior, stability, and host specificity. Though different manufacturers follow different process flows, a general outline of the process is summarized in Figure 2. An important manufacturing challenge is to keep the attenuated virus live and maintain the infective potential of the viral vaccine throughout the downstream processing and formulation until it is administered to healthy individuals. The end objective is to elicit sufficient protective immune response (neutralizing type antibody) against the designated virus upon immunization.

Virus Culture

Viruses are propagated in cell culture, grown either in roller bottles (as a monolayer) or suspension cultures, or bound to microcarriers. A typical pooled roller bottle batch volume is 500–700 L, and suspension culture is 1,000–2,000 L. There are several types of cells used for growing viruses for vaccine application: human diploid, Vero, Per.C6, MDCK, MRC 5, WI38, and 293P cells. Vero cells (developed from African green monkey kidney cells) are most commonly used for viral vaccine manufacturing. Most cell cultures for viral vaccine applications are grown in low-oxygen tension in the presence of ~5% CO_2. Virus inoculation is done aseptically to cell culture that has grown for five to seven days. Virus harvesting is done after 24 to 72 hours of virus inoculation. Depending on the virus type, they either bud out of cells or lyse the cells and emerge in the extracellular culture fluid. In some cases, the cells need to be lysed by the addition of detergents or surfactants (for example, Tween® 20 nonionic detergent) to release the viruses.

Clarification

Clarification removes the cells or cell debris and harvests viruses. Zonal centrifugation is commonly used for primary clarification. Some manufacturers also use tangential flow filtration (TFF) under low shear conditions or normal flow filtration (NFF), in most case depth filtration, for clarification of viral vaccine. Attenuated viruses are fragile and shear sensitive. Microfiltration (MF) TFF devices (without screen) are preferred to minimize shear. Solid content in viral

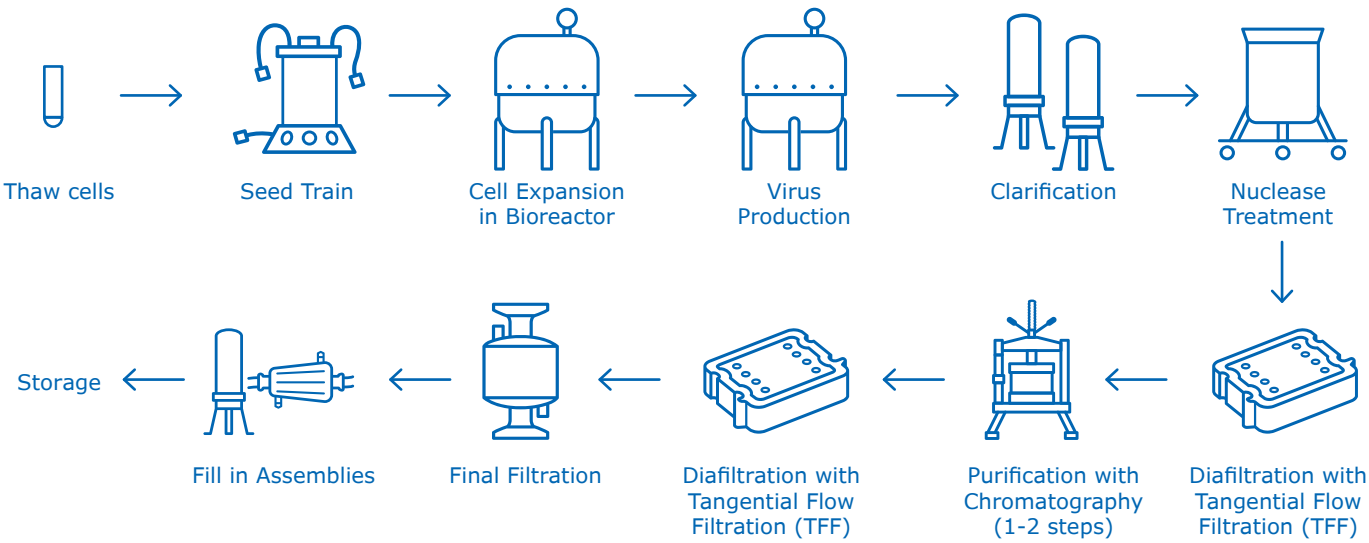

Figure 2: Generic attenuated viral vaccine process

vaccine harvest is low, so normal flow filters also work well for such applications. Some attenuated live viruses tend to bind to cell surfaces or get trapped in lysed cell debris. This leads to their removal during clarification resulting in poor virus recovery. Because viruses are negatively charged, it is important to be aware of adsorptive effects on filter media.

Nuclease Treatment

Nucleic acids are negatively charged large molecular components that interfere in virus purification. Carryover nucleic acid from lysed cells is a key contaminant in viral vaccine processes. Viruses propagated in human diploid cells or non-human cells (for example, viruses grown in dog kidney cell lines [MDCK]) pose a greater risk of nucleic acid carryover. Regulations require that carryover host cell nucleic acid content should be below 10 ng/dose of attenuated viral vaccine.

Benzonase® endonuclease is commonly used to degrade the nucleic acids such as RNA and DNA of the host cells to as low as three to eight base pairs (<6 kDa). The virus harvest is treated with ~0.9 to ~1.1 units/mL of Benzonase® endonuclease at 30–34 °C for four to eight hours. After Benzonase® endonuclease treatments, the harvest is diafiltered using TFF (100–300 kDa ultrafiltration devices) operating at low crossflow to remove Benzonase® endonuclease and degraded nucleic acid components. The typical flux (for 300 kDa Biomax® ultrafiltration membrane) is 25 LMH at 1.5–3.0 psi transmebrane pressure (TMP) and 4-5 L/min/m² feed flow rate.

Chromatography

Benzonase® endonuclease treatment is sufficient to bring most attenuated viral vaccines—measles, mumps, rubela, polio, rota, and yellow fever among others—to the desired level of purity during the concentration and diafiltration step. However, chromatography is normally required to bring new generation of viral vaccines like Japanese encephalitis virus (JEV) and dengue virus (DENV) to the desired level of purity. For example, sulfate ester covalently linked to a cellulose matrix can be used to purify JEV. The virus binds to matrix based on mixed-mode interaction with virus surface receptors or heparin-binding domain present on a few enveloped viruses. As viruses are negatively charged, anion exchange chromatography (Q or DEAE) works well in bind and elute mode or flow through mode. These operations run in mild conditions with low salt. Post chromatography, the eluted virus is concentrated by using 100–300 kDa TFF devices. Purity of the live viral vaccines is determined by measuring the removed contaminants (bovine serum albumin, ovalbumin, residual DNA, host cell protein, etc). Quality and quantity of virus in the purified bulk is determined by estimation of virus concentration based on HA titre, neutralizing antibodies, and CCID50 infectivity assay.

Sterile Filtration, Formulation, and Fill Finish

Final virus vaccine bulk is comparable to that of water. During final filter, vaccine is filter sterilized using 0.22 µm sterile-grade filtration.

Many of the attenuated viral vaccines are finally formulated with different strains. These multivalent vaccines include rota, polio, and dengue. They are aseptically blended after sterile filtration. Most of the live attenuated viral vaccines do not need any adjuvants because they are naturally potent immunogens. Most of them are lyophilized (freeze-dried). Examples are measles, mumps, and rubella. The final formulation of attenuated viral vaccines contains a small amount of antibiotics (neomycin), excipient (human serum albumin, HAS), stabilizer (hydrolyzed gelatin, egg protein, sorbitol, sucrose) and buffering agents (NaCl, other salts). Most of these vaccines are administered subcutaneously except for rota virus and polio virus vaccines, which are isotonic solutions that are administered orally.

Spotlight: Influenza Vaccines

Influenza virus is an enveloped single-stranded RNA virus that causes influenza (flu). There are three serotypes of seasonal influenza virus—A, B, and C. While type A affects humans and non-humans, type B affects only humans, and type C occurs much less frequently and affects humans very rarely. Type A viruses are the most virulent pathogens among the three types; they cause the most severe disease in humans.

The differences in these types of influenza viruses are based on the antigenic differences of the two internal structural proteins, nucleocapsid (NP) and matrix (M) proteins. These proteins have no cross reactivity among the three types. Subtyping of the virus is done by the antigenic variations in the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). About fourteen subtypes of HA (H1–H14) and nine subtypes of NA (N1–N9) have been recovered so far, in different combinations, from birds and mammals, including humans. Three HA subtypes (H1–H3) and two NA subtypes (N1, N2) have been recovered from humans.

Hemagglutinin (HA, MW ~77 kDa) is an immunogenic protein located at the surface of the virus envelope. Neuraminidase (NA, MW ~220 kDa) is a surface enzyme protein. These proteins are the antigens that define the particular strain of influenza and play critical roles in mediating the entry of the virus into the target cell. The HA protein is involved in attachment and membrane fusion in the endosome of the infected cell. The antigenic domains are on the surface and can be altered. The virus can thus prevent the triggering of an immune response and still maintain the ability to bind to the receptor.

Influenza virus undergoes frequent minor genetic mutations known as “antigenic drift.” This is defined as subtle changes in the antigenic proteins on the virus surface that allow the viruses to evade host immunity and cause disease despite previous infection or immunization. Large antigenic shift changes in the type A virus antigens happen about every ten years, resulting in larger epidemics, or pandemics. Seasonal influenza vaccines are usually trivalent, and more recently tetravalent, pandemic vaccines are monovalent.

Vaccine manufacturers typically require months for the development and production of a seasonal influenza vaccine each year. There is a long lead time to secure chicken eggs every year for the manufacturing of influenza vaccine. Use of cell culture–based influenza vaccine manufacturing eliminates this bottleneck and the possibility of contamination with the avian flu virus, which can originate from eggs. The cell culture–based manufacturing process is also more reproducible as it is less affected by the growth rate of the virus because different influenza strains grow at different rates in eggs, which leads to variability in yield. Cell culture–derived viruses are also of higher initial purity, and the absence of egg-based proteins (collagens and albumins) presents advantages in purification of the inactivated harvest.

1. Cell-Based Influenza Vaccine

There is no template process for cell culture–based influenza vaccine, and manufacturers follow different methods of manufacturing and select various technologies for their own process philosophies. A typical cell culture–based flu vaccine process is shown in Figure 3. Typical commercial bioreactor sizes range from 2,500–5,000 L. Typical total process yield is approximately 35% and is highly dependent on the virus strain. Based on this yield and recovery, a total of 50–100 doses of vaccine can be produced per liter of cell culture.

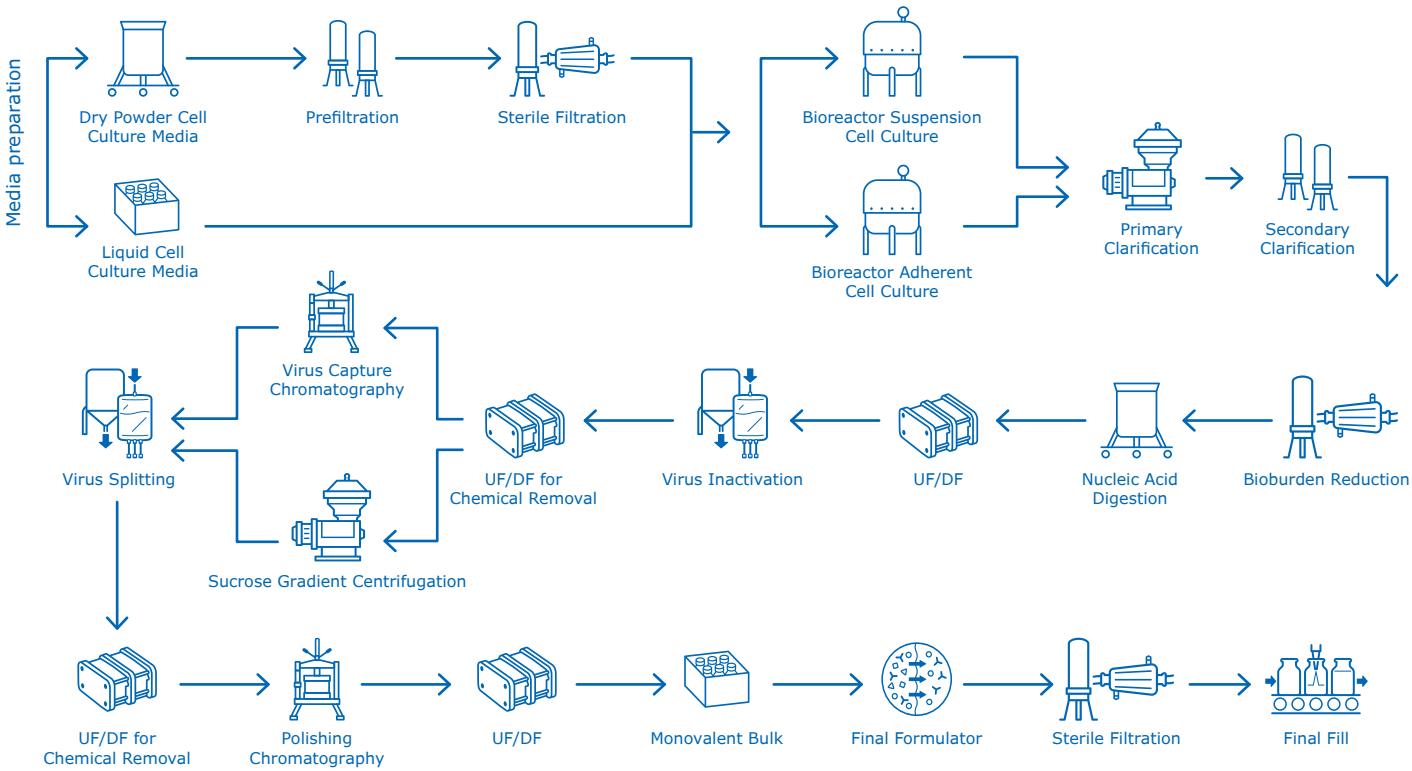

Figure 3: Generic cell culture-based flu vaccine process

Clarification

1. Centrifuge → Pre filtration → Final filtration

2. Depth filtration → Final filtration

3. Tangential flow filtration → Final filtration

A prerequisite for a successful infection is the addition of proteases to the medium, preferably trypsin or a similar serine proteases. These proteases extracellularly cleave the precursor protein of hemagglutinin (HA_0) into active hemagglutinin (HA_1 and HA_2). Only cleaved hemagglutinin leads to the adsorption of the influenza viruses on cells with subsequent virus assimilation into the cell, which leads to further replication. Passaging of the MDCK cells requires trypsinization with trypsin enzyme, which is stopped using an equimolar volume of trypsin inhibitor solution. Cells from roller bottles are used to inoculate 3-liter bioreactors and single-use bioreactors (SUBs).

MDCK cell culture can produce up to $1x10^9$ pfu/mL of influenza virus upon infection and incubation for three to five days. Parameters such as multiplicity of infection (MOI), incubation time, and temperature must be optimized for each cell line and each strain of virus. Assuming 45 µg of HA per dose (0.5 mL), it is likely that a 1,000-L to 2,000-L bioreactor (with microcarrier-based MDCK suspension culture) would produce 20 million doses per season. It has been estimated that production from an optimized 1,000-L bioreactor using solid microcarriers and MDCK cells would be comparable to production from approximately 31,000 eggs.

It is important to consider virus yield and contaminant removal level for optimization of the clarification step. As influenza virus and the host cell DNA are negatively charged, positive-charged filters with filtration aids sometimes produce high adsorption of virus along with good DNA removal. It is essential to optimize this adsorption tradeoff using filtration flux and recovery by carefully selecting buffer conditions (salt, pH, etc.). Centrifugation is sometimes used for primary clarification. TFF (0.45 µm or 0.65 µm) is also used for primary clarification in which flu virus is recovered in the permeate. Typical yield is ~50% with TFF as the primary clarification step. At the secondary clarification step, 0.22 µm final filtration is preferred to 0.45 µm.

Virus Inactivation

Formaldehyde is the most frequently used inactivating agent in vaccine manufacturing. Formaldehyde inactivates a virus by irreversibly crosslinking primary amine groups in surface proteins with nearby nitrogen groups in DNA or proteins. These crosslinking bonds can associate with non-viral proteins; as a result, initial partial purification of live infectious virus is required to prevent irreversible chemical bridging between viral proteins and impurities. Inactivation is carried out at 32 °C for twenty-four hours with formalin concentrations of 0.1%. An alternative for inactivation is UV radiation at a wavelength of 254 nm. The effective dose depends on various factors such as size and diameter of the UV lamp, distance between the UV source and virus-containing medium, UV light intensity, and the exposure time for the virus-containing medium. The general dose is 5-200 m_J/cm^2.

Ultrafiltration/Diafiltration (UF/DF)

At this stage, whole virus particles are concentrated in order to reduce the process volume downstream. UF/DF is used to remove low-molecular-weight impurities and for buffer exchange. There are multiple UF/DF steps in a cell culture flu vaccine process. UF/DF is implemented to remove DNA, host cell protein (HCP), and Benzonase® endonuclease, and at the post-ultracentrifugation step, to remove sucrose. A 300 kDa device works well for volumetric concentration. Typical conditions are 10–15 psi transmembrane pressure (TMP), 5–6 L/min/m^2 feed flow, and 20–50X concentration factor. The average flux is about 50 LMH. Due to a high concentration factor, some processes are performed in a fed batch concentration mode.

500 kDa with flux control operation is used to remove host cell DNA and proteins. A TFF process results in good low-molecular-weight DNA removal but is less efficient to remove high-MW molecules. In some cases, a 1000 kDa filter results in good virus retention, depending on the size of the virus strain. In permeate controlled two-pump-based TFF, typical average filtration flux is 20–35 LMH at feed flow rate of 5–6 L/in/m^2 and TMP of 4–5 psi.

Density Gradient Centrifugation

Either density gradient (zonal) centrifugation or chromatography is commonly used to further purify the virus. Zonal centrifugation is done in two steps: pelleting and fractionation. The advantage of zonal centrifugation is a product of high purity. However, yield is low, and operation is cumbersome.

Chromatography

In the cell culture–based process, size exclusion and anion exchange chromatography are performed on inactivated virus to remover DNA and HCP. Due to the large size of the virus, the anion exchange chromatography step is operated in flow-through mode. In some cases, size exclusion chromatography (SEC) is followed by anion exchange chromatography (AEX). SEC is generally used to remove small solutes and proteins, but separation from nucleic acid is difficult to achieve. AEX resin is used with NaCl at sufficient concentration such that the influenza virus does not bind to the resin while nucleic acid and other impurities do bind to the resin. Although the size of hcDNA and the influenza virions is similar, the random coil of DNA promotes stronger binding to the resin compared with the binding of rigid sphere of virions.

Adding detergent to prevent aggregation of virions can improve purity (lower hcDNA) and product yield. The average product yield in the SEC step is 85% with 30–35% reduction in total protein content and nucleic acid. An average product yield from the anion exchange step is more than 80% with an approximately 60–70-fold reduction of nucleic acid. The overall product yield from chromatographic purification is 50–55% with a 15–20-fold reduction in total protein and more than a 500-fold reduction in nucleic acid.

DNA Removal with Nuclease Treatment

The U.S. Food and Drug Administration (FDA) requires that a parenterally administered dose is limited to 100 pg of residual host DNA. The European Medicines Agency (EMA) and World Health Organization (WHO) allow 10 ng per parental dose and 100 µg per dose for orally administered vaccine. Benzonase® endonuclease, a genetically engineered endonuclease, cleaves all forms of DNA and RNA. One unit of Benzonase® endonuclease degrades approximately 37 µg DNA in thirty minutes to as low as 3–8 base pairs (<6 kDa). If not present in the original buffer system, 1–2 mM of $MgCl_2$ is needed for optimal Benzonase® endonuclease performance. DNA presence in feed material depends on the cell/virus types as well as the methods and techniques used at the harvest step. After the Benzonase® endonuclease treatment, a quantitative removal of Benzonase® endonuclease from the process stream is required in the subsequent purification steps. Therefore, it is better to use Benzonase® endonuclease treatment sufficiently upstream. Several methods are used to remove Benzonase® endonuclease from the process: TFF (MW cut-off 300 kDa), anion-exchange chromatography (AEX), and zonal centrifugation. There are reports indicating the use of ~0.9 -1.1 U/mL of Benzonase® endonuclease for treatment of harvest to degrade host cell nucleic acid at 30 to 37 °C in four to eight hours. Due to regulatory requirements, residual Benzonase® endonuclease must be measured and detected in the process. ELISA-based methods are used for this detection.

Splitting Process

Most influenza vaccines are “split” vaccines, which means they are produced by detergent treatment. During this step, the structure of the influenza virus is dissociated by breaking down the envelope and releasing the internal antigenic components of the virus such as viral RNA–associated capsid nucleoprotein and envelope inner protein matrix (M protein). This splitting process removes some of the viral components, which results in a less-reactogenic vaccine.

Prior to fragmentation, the concentrated monovalent (single strain) viral suspension is diluted with a sterile buffer. Viral fragmentation takes place when amphiphilic non-ionic detergent such as Triton® X-100 solution (0.5 %) and/or anionic sodium deoxycholate (DOC) is added to the suspension of the purified influenza. Polysorbates (Tween® 80 polyethylene sorbitol ester) and cetyltrimethyl ammonium bromide (CTAB) are also suitable for the virus splitting step. Fragmentation requires continuous stirring to mix the process fluid with the detergent for at least one hour at room temperature. The length of the fragmentation step can be extended up to twenty-four hours if necessary. A secondary virus inactivation is employed after the virus splitting process as an additional safety measure to ensure complete inactivation of every component.

Sterile Filtration

Some cell culture–based influenza vaccines, such as the vaccine for H5N1, are whole virus vaccines. For these viruses, sterile filtration of the final bulk is done at the end of the process. For split virus vaccines, the final sterile filtration is done after the splitting step. Depending on the influenza strain, the capacity of the final sterile filters is occasionally low (around 20–50 L/m^2). In these cases, 0.45 µm or 0.65 µm prefilters are used to protect the sterilizing grade filter and increase its capacity. The capacity of the sterilizing filter is in the range of 200–400 L/m^2 depending on the feed quality and prefilter, if one is used. A 0.45/0.22 µm sterilizing filter is also often used as a terminal sterile filter (if prefiltration is deemed necessary) to trim the process from two to one filtrations.

Formulation

Only a few split influenza virus vaccines in the cell culture–based influenza vaccine market are formulated with adjuvant. Adjuvants based on oil-in-water emulsions used in commercial influenza vaccine formulation are MF59 and AS03. Because formulated vaccine cannot be filter sterilized, adjuvant and purified split virus antigens are filter sterilized separately and then aseptically blended. Typically, 0.22 µm filters are used to sterile filter emulsion-based adjuvants. Due to the nature of oil-in-water adjuvants, it is important to use a filter that will prevent breakthrough in necessary bacterial challenge retention testing. The final formulation often contains a buffering agent (such as sodium citrate dehydrate, citric acid monohydrate, potassium chloride, potassium dihydrogen phosphate, and/or disodium phosphate dehydrate), an isotonic aid (sodium chloride), and a stabilizer (magnesium chloride hexahydrate or calcium chloride dehydrate). For example, Optaflu® vaccine[1], a cell culture–based trivalent seasonal influenza vaccine (split vaccine, inactivated HA and NA), is formulated with adjuvant MF59. A dose of 0.5 mL of Optaflu® vaccine contains total 45 µg HA (15 µg x 3) and 0.25 mL MF59 adjuvant.

Summary: Cell-based influnza vaccine

Remarkable progress has been made in the production of cell-based influenza vaccine over the past ten to twenty years. Cell-based production provides an innovative method for solving the production bottleneck that often occurs with traditional egg-based influenza production. This makes it not only a valuable alternative method for seasonal influenza vaccine, but also an essential technology as we prepare for the possibility of a pandemic influenza event. There are unique challenges in cell-base influenza vaccine, however, over the years, solutions have been developed to overcome these challenges.

2. Egg-Based Influnza Vaccine

Figure 4 illustrates the generic process for the production of inactivated split-virion type of influenza vaccine produced in embryonated chicken eggs. This represents the majority of influenza vaccines currently offered on the market. With more than thirty years of a commercial scale application, it is a well-characterized process.

Virus Propagation and Harvest

Fertilized chicken eggs are placed into an incubator and kept there at ~37 ºC for nine to twelve days for

1. Manufactured by Novartis.

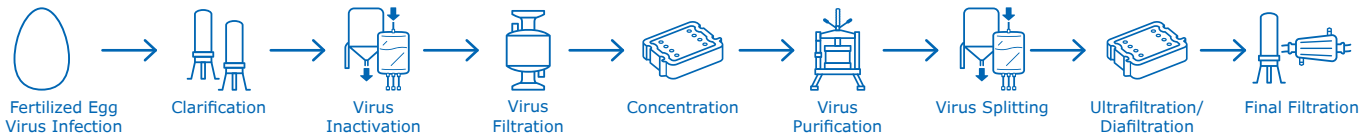

Figure 4: Generic process for the production of inactivated split-virion type of influenza vaccine produced in embryonated chicken eggs

embryonic development. The allantoises of viable embryos are inoculated with the influenza virus. Antibiotics (typically a mixture of polymyxin and neomycin) are added into the inoculum to suppress potential bacterial growth. The infected eggs are then kept for two to three days at ~33–35 ºC in a humidity-controlled incubator to assure propagation of the viruses at optimal conditions. The propagation process is stopped by rapid refrigeration of the incubated eggs at ~2–8 ºC. The allantoic fluid rich in viral particles is collected from the infected eggs. To speed up the process and increase capacity, this step of the upstream part of the process is often automated with the use of automatic inoculators, incubators, and harvesters. One egg produces 3 to 10 mL of allantoic fluid. The yield of HA can vary significantly between 0.7 to 3 eggs required per dose of trivalent vaccine, depending on the strain and manufacturer. A traditional egg-based influenza vaccine manufacturing facility at full capacity is capable of processing up to 600K eggs per day. Eggs account for ~50% of bulk vaccine cost. Typical full-scale commercial batches are based on ~250–350K eggs per day (~1,500–2,000 L of allantoic fluid).

Clarification of Allantoic Fluid

A multiple-step clarification is required for the removal of large contaminants from the allantoic fluid, especially rudimentary tissue compounds such as feather, beak, blood vessel, and blood cells). A low-speed continuous centrifugation at 4,000–5,000 g is commonly applied during the primary clarification step. Larger particles are separated into the pellet, and the influenza virus stays in the supernatant. The secondary clarification step is accomplished through normal flow filtration (NFF). An alternative option for secondary clarification step is tangential flow filtration (TFF) with a 0.65 µm or 0.45 µm microfiltration membrane device operated with permeate flux control. Because influenza virus is negatively charged at operational pH and can potentially bind to positively charged depth filters containing diatomaceous earth, an optimized high-salt buffer flush, normally as 1-2 M NaCl, can be used to increase process yield.

Virus Inactivation

This chemical treatment step ensures that no active virus (influenza virus or any contaminating virus) proceeds through the downstream process. It is preferably performed as soon as possible in the process to limit the possibility of contamination. However, as inactivation should be performed in a homogenous suspension that is free from particles that may not be penetrated by the inactivating agent, some manufacturers choose to inactivate the virus later in the process. Sometimes, for safety reasons, two inactivation steps are performed.

β-propiolactone (BPL), binary ethylenimine (BEI) and formaldehyde are commonly used. As cross-linking agents, they react with viral proteins, eliminating influenza virus infectivity while retaining immunogenicity. Classically, inactivation with BPL is done at 4 °C for 16 hours at a BPL final concentration of 0.1% by volume. (This concentration should not be exceeded at any time during manufacturing.) Formalin is typically used at 0.02% for 18 to 72 hours at 37 °C. 2 mM binary ethyleneimine (BEI) has been proved to inactivate the influenza virus in 48 hours at 37 °C. To prevent aggregation or precipitation from occurring during the inactivation, glycerol and sucrose are often added.

Virus Concentration

At this stage, whole virus particles are concentrated using TFF in order to reduce the volume to be processed in the downstream ultracentrifugation step. Diafiltration also partially removes egg proteins (45 kDa ovalbumin, 76 kDa ovotransferrin, 49 kDa ovoglobulins, 14 kDa lysozyme, and others), DNA, and inactivation agents. Ultrafiltration membranes with 300–1000 kDa can be used in a permeate flux–controlled system; the device and operational parameters must be carefully selected depending on the viscosity and the fouling properties of the influenza fluid. Typical volumetric concentration at this step ranges from 5–40 X. Typical flux is 20–50 LMH at 1.5–3 psi TMP and 5–6 L/min/m² crossflow.

Virus Separation Using Ultracentrifugation

Sucrose density gradient is created by overlaying a lower concentration of sucrose on higher concentrations in a zonal centrifuge chamber. A sucrose gradient could consist of layers extending from a high concentration of sucrose of up to 70% w/v to a low of below 15%. Increments vary depending on the product to be purified. The product fluid is pumped into the centrifuge rotor/gradient and processed at G-forces in the range of ~40–150K. The particles travel through the gradient of sucrose concentrations until they reach the layer with a density that matches their own. The fraction(s) of interest containing the influenza virus are collected for further downstream processing. The fractions are strain dependant. The virus yield on this step can vary from ~60 to ~90% (The HA assay sensitivity could have ~30% inherent variability.).

Fragmentation of Influenza Virus

Before fragmentation (splitting), the concentrated monovalent (single strain) viral suspension is typically diluted with a sterile buffer (phosphate-buffered saline [PBS], for example) to the standardized optical density (OD) value correlated to the viral protein content in

the process fluid. Fragmentation of viruses is typically executed by adding amphiphilic nonionic detergent such as Triton™ X-100 aqueous solution and/or anionic sodium deoxycholate (DOC) to the suspension of the purified influenza. Polysorbates (Tween® family of products) and cetyltrimethyl ammonium bromide (CTAB) are also suitable for the virus-splitting step. Fragmentation is normally done with continuous mixing of the process fluid and the detergent(s) for at least one hour at room temperature. The length of the fragmentation step may be extended up to twenty-four hours if necessary. During this step, the viral structure of influenza is dissociated as the envelope is broken down and the internal antigenic components of the virus such as viral RNA-associated capsid nucleoprotein and envelope inner protein matrix (M protein) are released.

Note: The virus inactivation step (in addition to or instead of the one in the upstream part of the process) may be employed on this stage of the process.

Ultrafiltration/Diafiltration

After splitting, the product fluid is subjected to a buffer diafiltration using TFF. This removes the detergent components, and the product is placed in its final buffer. As the virus particles are split, tighter membranes must be used to retain the viral fragments. TFF membranes provide full retention of the viral components of interest while allowing the removal of detergent and sucrose (along with inactivating agents). An average expected flux with 50 kDa is around 30–40 LMH at 5–7 psi TMP and 4–5 L/min/m² cross flow.

Sterile Filtration of Final Purified Bulk

Each separately produced monovalent (single-strain) influenza bulk undergoes a sterile filtration step before it is moved into final formulation and a fill facility. Both PES and PVDF types of sterile-grade membranes may be successfully used in the step. The finished bulk is then formulated, filled, and packaged for administration.

Existing vaccines typically contain 15 mg of each of the three components. The majority of seasonal inactivated split vaccines are not adjuvanted.

The use of squalene-based oil-in-water emulsion-type proprietary adjuvant systems AS03 (Pandemrix®[3]) and MF59 (Focetria®[2]) in H1N1 pandemic vaccines enabled the eliciting of protective antibody levels with a lower amount of the viral antigen—7.5 mg per a dose. This so-called dose-sparing effect helps to increase the number of available vaccine doses, which is particularly important in a pandemic when supply cannot meet demand because of limited manufacturing capacity.

Summary: Egg-Based Influnza Vaccine

The manufacturing of egg-based influenza vaccine is an established, well-characterized process with over thirty years of commercial scale application. Even so, it presents numerous unique challenges. The ongoing genetic mutations of the virus require annual production of a seasonal vaccine incorporating components of selected viral strains. Yields of the strains are variable and often could not be known until an actual full-scale production occurs. A vaccine license must be issued for each new seasonal vaccine for the length of one year only.

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2. Manufactured by Novartis. 3. Manufactured by GlaxoSmithKline.

Virus-Like Particle (VLP) Vaccines

A virus-like particle (VLP) is a biological nanoparticle that consists of the protective protein shell of a virus without its viral genome. VLPs are a specific class of viral subunit; they mimic the overall structure of virus particles but do not contain the infectious genetic material. VLPs provide a high immunogenic response because of their high-density display of epitopes, the capacity to present multiple proteins to the immune system, and their size (typically around 40 nm; influenza VLP is 80–120 nm), which seems to be optimal for uptake by dendritic cells. Because VLPs don't contain viral genetic material and cannot replicate, they are noninfectious and safe. Furthermore, VLPs are unlikely to need adjuvants to be highly immunogenic.

Manufacturing of VLPs involves cell-based expression of the virus-shell protein. VLPs can be expressed in several heterologus expression systems. There are many VLP-based vaccines in commercial distribution and clinical trials that are produced in mammalian cell culture, baculovirus/insect cell culture system, microbial fermentation (yeast, *E. coli*, etc.) and plants (tobacco, etc). VLPs are either assembled *in vivo* followed by purification from cell lysate, or the partially assembled protein is recovered from cell lysate and assembled into VLPs *in vitro*.

Figure 5 is a non-exhaustive list of VLPs that are currently on the market or under clinical evaluation or development.

Figure 5: Types of Vaccine Summary

Disease	VLP Composition	Size	Expression System	Company	Latest Phase*	Source
Hepatitis B (Hep B)	HBsAg	22 nm	CHO, yeast, E. coli, fungi, transgenic plants or plant cells	Sanofi Pasteur Biologics Co., Merck & Co./MSD, GSK	Licensed	Cregg et al (1987) Smith et al (2002)
Cervical Cancer (HPV)	L1, major capsid protein	40-50 nm	Yeast, insect cells, E. coli, Tobacco	Merck & Co./MSD, GSK	Licensed	Reavonen et al (2007
Influenza	HA, NA, M1	80-180 nm	Insect cells, plant cells	Novavax, Medicago, Inc.	Phase 3	Krammer et al (2010)
Norwalk virus	Capsid proteins VP6, VP7	38 nm, 70 nm, 75 nm	Insect cells, transgenic plants, plant cells, mammalian cells, yeast	Takeda Vaccines	Phase2	Jiang et al (1992) Jian et al (1998) Vierira et al (2005)
Alzheimer's Disease	Amyloid β + L1 of Baculovirus	~50-55 nm	Insect cells	Novartis/GSK & Cytos Biotechnology	Phase 1	Chackerian, 2010
Hepatitis E (HEV)	Capsid protein	23-40 nm	E. coli, insect cells, transgenic plants	Xiamen Innovax Biotech, Genelabs, GSK	Licensed	Maloney et al (2005)
Respiratory Syncytial Virus (RSV)	G+F proteins + Newcastle disease virus	120 nm	Insect cell culture	Novavax	Phase 3	Smith et al (2012) Raghunandan et al (2014)
Ebola	VP40 and glycoprotein	30-40 nm	Insect cells HEK293	Novavax	Phase 1	Sun et al (2009) Hahn et al (2015), Bioprocessing Journal
Malaria	Pfs25 protein VAR2CSA protein	~19 nm	Yeast, E. coli, plant cells, insect cells	University of Copenhagen Fraunhofer, Center for Molecular Biotechnology	Phase 1	Thrane et al (2015)

*Source: Vaccine manufacturers websites on June 2016

Generic VLP-Based Vaccine Process

The manufacturing process for VLP-based vaccine is often complex. There are several methods of production. For the purpose of simplification, we will explain the production of baculovirus-mediated insect cell expressed VLPs. The downstream processing steps outlined in Figure 6 can be extrapolated to VLPs produced in other cell culture expression systems.

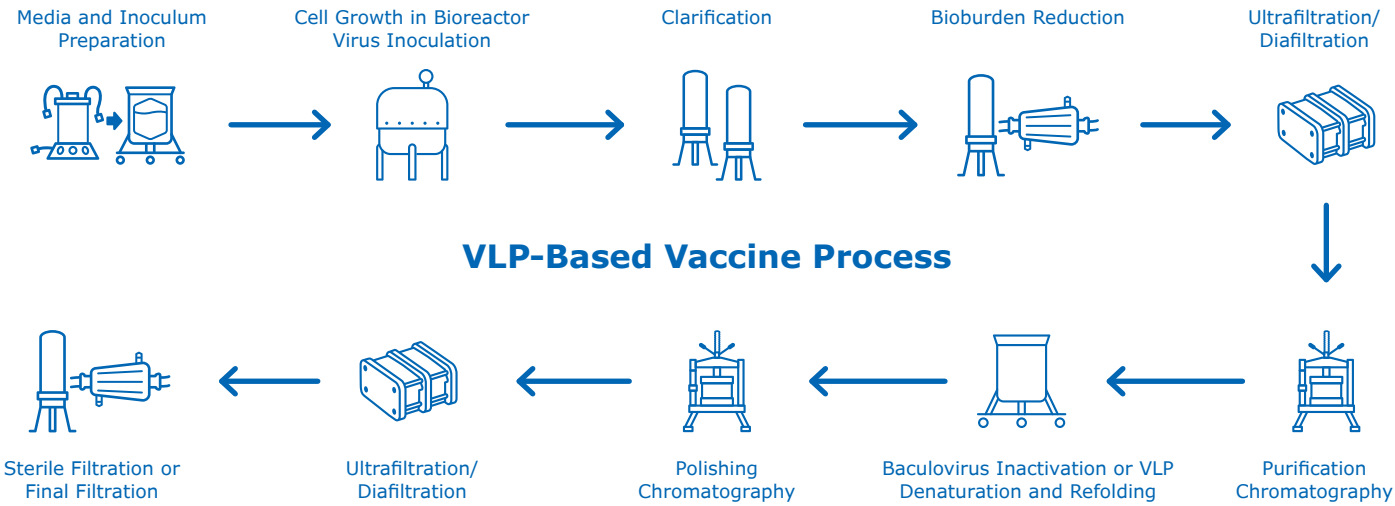

Figure 6: A general outline of the VLP-based vaccine manufacturing process

Cell Culture

Generally, the insect cell line (Sf9, Hi-5) working cell bank is expanded through a series of shake flask cultures and transferred to a bioreactor at 1.0–1.2 x 10^6 cells/mL and allowed to expand until a defined culture volume at a cell density of 1.5 to 2.0 x 10^6 cells/mL is obtained. The culture is then infected with baculovirus at a multiplicity of infection (MOI) of 0.5–1.0. The cells are grown at 26–28 °C. VLPs are harvested approximately 48 to 96 hours post infection. The Sf9 cell density and viability at the time of harvest can be about 1.5 x 10^6 cells/mL with at least 20% viability. Some manufacturers harvest when the cell viability drops below 50%. Typically, culture is harvested by centrifugation at 1000 g for thirty minutes after which the fluid is decanted, and the cell paste stored at -60° to -80 °C.

Insect cells for the production of VLPs are mostly grown in commercially available serum-free insect media, a variation of IPL-41 basal medium. Serum-free insect cell media are supplemented with fetal bovine serum (FBS), protein hydrolysates, and a lipid/surfactant emulsion. Supplementation with heparin is also helpful to reduce the aggregation of cells.

According to a collaborative work carried out with Instituto de Biologia Experimental e Tecnológica", our collaborator in Portugal (iBET), single-use bioreactors can be used for production of VLPs in insect cell culture. Typically, bioreactors have been used to produce hepatitis C VLP-based vaccine using Sf9 insect cells and Sf900II cell culture media. Agitation and sparging rates as well as inoculation at high cell density are key process parameters in a bioreactor that contributes toward more efficient VLP productivity. For more information, watch our webinar by clicking here.

As an alternative, production of VLP in yeast is an attractive, low-cost alternative to insect and bacterial systems. *S. cerevisiae* batch cultures producing VLPs can be performed in a complete synthetic medium (CSM) with the addition of glucose (20 g/L) and yeast nitrogen base (6.7 g/L). For optimal performance, culture medium could be supplemented with leucine 1.8 mM, glutamate 20 mM, and succinate 50 mM. Potassium hydroxide (2N) is used for pH adjustments. The feeding medium for fed-batch cultures is 10 X CSM without supplements.

Cell Lysis

VLPs are purified by resuspending cell paste in a tris buffer with 1.6 µm leupeptin. They are microfluidized to produce a cell lysate. Most VLPs are expressed in the cell and must be recovered by cell lysis, depending on whether the VLPs are secreted to the extracellular medium. There are reported cases of influenza VLPs produced in insect cell culture without cell lysis. In some cases, cell lysis is required to increase product yield. Cell lysis releases host cell proteins (HCP) and host cell DNA (hcDNA) that must be removed. Sometimes Benzonase® endonuclease treatment is used to digest hcDNA, which is later removed by the downstream process. Therefore, the current trend is to design a clone compatible with an efficient secretory pathway. Freeze-thaw, detergents, homogenization, or sonication are typically used for cell lysis, with high-pressure lysis being the most common. The harvest solution is forced through a small fixed orifice at a high pressure. The rapid transfer of the sample from a region of high pressure to one of low pressure causes cell disruption. Some manufacturers add NaCl to the medium to a concentration of about 0.4 to 1.0 M (preferably about 0.5 M) to avoid VLP aggregation. If chemicals are used, 1% Triton® X-100 solution is the most common detergent used. Sometimes 25 mM Na_2HCO_3, pH 8.3, is also also used. In the process for Gardasil®[4], the cell slurry is passed twice through a homogenizer to achieve a cellular disruption of greater than 95%. The mixture is incubated at 4 °C for 12 to 20 hours for complete lysis.

Chemically induced cell lysis can be carried out in appropriate mixing systems. Handling of buffers used in cell lysis and storage of lysate can be combined using a single-use bag, connector, and sampling system for an integrated operation.

Clarification

Centrifugation, depth filtration, or microfiltration tangential flow filtration (TFF) can be used for clarifying the cells or cell lysate. Generally, microfiltration is preferred because it is robust and scalable. High shear during lysis micronizes the cellular debris. This, coupled with the large size of the VLPs, can make clarification problematic. The removal of cell and cellular debris from the cell culture medium containing VLPs is accomplished by either TFF, normal flow "dead-end" filtration (NFF), or centrifugation. Dead-end 0.2 µm filters work well for this application (yields >70%). In some cases, depth filters can be used, but proper optimization is required. Positive charge in depth filters can sometimes result in product loss due to adsorption. However, adding salt to the feed (100 mM NaCl) or performing pre- and post-buffer flushes with salt (0.5 M NaCl) can enhance the product recovery.

Nuclease Applications in VLP Processes

Benzonase® endonuclease is employed in the VLP purification process to degrade residual nucleic acids in order to meet regulatory purity requirements.

The European Medicines Agency (EMA) and World Health Organization (WHO) allow 10 ng DNA per dose for parenteral vaccines and 100 µg DNA per dose for oral vaccines. Additionally, in order to minimize the risk of host cell nucleic acid oncogenicity, DNA size must be reduced to 100–200 base pairs in length. Benzonase® endonuclease is typically applied as a batch incubation step that occurs either before or after lysate clarification. For adequate DNA digestion, 10–50 U/mL Benzonase® endonuclease is typically required, although the optimal Benzonase® endonuclease concentration varies with DNA and RNA concentration, incubation temperature, pH, time, and magnesium concentration. Maximum Benzonase® endonuclease activity occurs at 37 °C, pH 8. A concentration of 1–2 mM Mg^2+ is essential to maintain Benzonase® endonuclease activity. Applying excess Benzonase® endonuclease under optimal conditions can result in sufficient nucleic acid digestion in one to four hours.

4. Human Papillomavirus 9-valent vaccine manufactured by Merck.

In cases with reduced Benzonase® endonuclease concentration, or for processes that require incubation at low temperature or neutral pH, incubation times of 8 to 12 hours may be required. For each process, it is recommended that low-volume scouting experiments are performed to identify optimal Benzonase® endonuclease incubation conditions for adequate nucleic acid digestion. DNA digestion can be monitored with assays such as agarose gel electrophoresis, qPCR, or the threshold immunoassay.

After the incubation step, the Benzonase® endonuclease enzyme must be cleared in subsequent processing steps. In cases where Benzonase® endonuclease enzyme, a 60 kDa dimer, is sufficiently smaller than the VLP product, TFF can be used to achieve separation. Typically, a 300 kDa membrane is recommended to allow the passage of Benzonase® endonuclease while retaining the product molecules. For processes in which the molecular weight difference between VLP and Benzonase® endonuclease enzyme is not sufficient for TFF separation, either anion or cation exchange chromatography resins can be used. The Benzonase® endonuclease isoelectric point is 6.85. Benzonase® endonuclease removal can be monitored using an ELISA kit, which allows for specific quantification of residual active and non-active endonuclease. DNA digestion using Benzonase® endonuclease enzyme can be carried out in a single-use mixer integrated with buffer bags and a sterile filter to introduce Benzonase® endonuclease enzyme and appropriate sampling system for quality control (QC) testing.

Concentration and Buffer Exchange

Depending on the type of VLPs, expression system, and VLP titer, an ultrafiltration/ diafiltration (UF/DF) step may be included in the process to concentrate and buffer exchange the product and make it ready for next step. Not every process requires this step. For example, the production of HPV L1 may not include this UFDF step. Alternatively, influenza VLPs may require TFF with 300–1000 kDa membranes. During concentration and buffer exchange, the HCPs are further reduced. Typically, the retentate is buffer exchanged with diafiltration (DF) to 25 mM Tris HCl, pH 8.0 for subsequent purification by ion-exchange chromatography.

Primary Purification (Ion-Exchange Chromatography/Ultracentrifugation)

VLPs are commonly purified by ultracentrifugation over a CsCl, Sucrose or Iodixanol gradient. The use of CsCl for the purification step should however be avoided. Indeed, in some cases, CsCl-purified VLP can appear to be heterogeneous in size because of broken particles and may introduce impurities into the downstream process. Aggregation during storage and functionality reduction can also cause complications in downstream process.

In sucrose ultracentrifugation, different and discontinuous concentrations of sucrose (20–60%) are layered. Each concentration is then collected for analysis after centrifugation. Given the differential density of each sucrose layer, the VLPs migrate until they reach a zone of similar density. This separates them from other contaminants that could not be previously removed. Typically, the discontinuous sucrose gradient is prepared in phosphate-buffered saline (PBS) buffer with 0.5 M NaCl, pH 7.2- or 20-mM Tris HCl, pH 7.5. This solution is then centrifuged either at 6,500 g for 18 hours at about 4–10 °C, or 37,000 g for three hours, or even 100,000 g for one hour at 4 °C. VLPs form a distinctive visible band between about 30% to 40% sucrose or at the interface (in a 20% and 60% step gradient) are collected from the gradient and stored. Sucrose can be removed by dialysis against PBS and diluted to 200 mM of NaCl in preparation for the next step in the purification process.

While ultracentrifugation techniques are well established and convenient for small-scale production, they can prove to be time consuming and poorly scalable. Alternatively, other purification methods such as ion-exchange chromatography can be used. Buffers that can be used in the ion exchange chromatographic step include phosphate (±citrate), Tris HCl, MOPS, HEPES, and sometimes stabilizing agents such as sucrose.

Weak ion-exchangers such as DEAE resins can be used to purify the VLPs. The material is first diluted with 20 mM Tris HCl to reduce salt concentration and then loaded in the column. After washing with Tris HCl buffer, the product is eluted with a phosphate buffer. In the final polishing step, the the pH is adjusted. Alternatively, phosphate buffer at 20 mM with pH 7.5 and 150 mM NaCl has been used in diafiltration, column equilibration, and 1:2 dilution of the product prior to purification. Elution is performed using stepwise NaCl concentrations]. Other anion-exchange resins have also been used—TMAE, DMAE, and Q resins—with significant reduction of DNA and endotoxin levels. These resins were tested using phosphate or HEPES buffer, pH 7.2 at varying NaCl concentration (150–1000 mM). Alternatively, TMAE resin can be loaded in 20 mM sodium phosphate buffer at pH 7. A pre-wash step with 20 mM sodium phosphate +0.4 M NaCl (to remove *E. coli* fragments) can be performed, and VLP elution is done at higher NaCl concentration. Rinsing with 1–2 M NaCl helps DNA removal. Prepacked columns containing ion exchange resins can be employed for this application.

In certain processes, membrane adsorption and monolith technology can provide better dynamic binding capacity (DBC) than particle-based resins. Although, in theory, monolith technology allows a straightforward scalability due to their flow-independent DBC, the actual scale-up of these columns can be challenging since their polymerization process is highly exothermic, which results in the possibility of an inhomogeneous structure. Multimodal resins that employ both size exclusion and binding-based separation also demonstrate VLP purification in flow-through mode. In this case, large molecular entities like VLPs are excluded from entering and interacting with the bead while small contaminants are trapped in the adsorptive core.

Baculovirus Inactivation

Insect cell–based expression system can result in $10^{10\text{-}12}$ baculovirus particles in the process. As a regulatory requirement, baculovirus must be removed from the

final product through orthogonal downstream steps during purification of VLPs. As a safety measure, some manufacturers perform inactivation of baculoviruses prior to removal. Inactivation is done by chemical methods using formalin or β-Propiolactone (BPL).

Selective precipitation and chromatographic methods (bind-and-elute or flow-through modes) can be used for removal and/or inactivation of intact baculovirus. Inactivation of baculovirus can be accomplished in multiple ways:

- Incubating in 0.2% BPL for three hours at about 25–27 °C.
- Incubating at 0.05% BPL at 4 °C for three days and then at 37 °C for one hour.
- Incubating in Triton® X-100 solution and tributyl phosphate (TBP) at concentrations of 1% and 0.3% respectively for thirty minutes at room temperature (25 °C).

The chromatography steps used for purification of VLPs are strictly monitored for their ability to separate VLPs from baculovirus. For example, ion-exchange chromatography can remove 10^2 to 10^5 baculovirus particles during purification of VLPs.

Polishing

When microbial systems are used, especially with *E. coli*, lipopolysaccharide (LPS) or endotoxins must be removed from the feed stream. Ion-exchange chromatography in bind-and-elute mode or membrane adsorption technology in flow-through mode works at this step. The hydrophobic nature of VLPs makes endotoxins interact with the particles themselves. This causes issues in the separation of LPS. Bound endotoxins can be released by treatment with solvents, mild detergents, or a combination of both. To avoid this problem, yeast (*Pichia pastoris*) or expression systems based on insect cells (Sf9) are preferred.

Weak ion-exchange chromatography resins have been used successfully for final polishing steps. In this step, VLPs pass through the column while residual baculovirus and DNA binds to the column. The flow-through fractions contain VLPs. However, the VLPs and some VLP-derived impurities can have similar electrostatic properties. When the difference in size is significant, one or more size-exclusion chromatography (SEC) steps may work as an alternative. Stabilizing agents and compounds prevent ion interactions between SEC resin and VLPs. For example, a Sucrose-Phosphate-Glutamic Acid (SPG) buffer made of 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M KH_2PO_4, and 0.0049 K-glutamate, pH 8.0±0.2 may result in good VLP recovery when used with size-exclusion resin. It should be noted that UFDF constitutes an interesting option at this stage to remove lower-molecular-weight impurities. In addition, UFDF and SEC both efficiently allow buffer exchange for the final formulation with superior and easier scalability for tangential flow filtration.

Sterile Filtration and Formulation

VLP-based vaccines are typically formulated in sucrose and Tween® detergent. Some final formulations may contain amino acids, amorphous aluminum hydroxyl phosphate sulfate, carbohydrates, L-histidine, mineral salts, polysorbate 80, sodium borate, etc. The final product is sterile filtered using a 0.22 µm filter.

Gardasil®[5] is the quadrivalent Human papillomavirus virus-like particle vaccine produced in yeast cells that contains purified VLPs adsorbed on aluminium containing adjuvant (amorphous aluminium hydroxyphosphate sulfate). Along with the antigen, each 0.5 mL dose of the vaccine contains approximately 225 µg of aluminium (as amorphous aluminium hydroxyphosphate sulfate adjuvant), 9.56 mg of sodium chloride, 0.78 mg of L-histidine, 50 µg of polysorbate 80, 35 µg of sodium borate (borax), residual traces (<7 µg/dose) of yeast protein, and water for injection. The product does not contain any preservative or antibiotics.

Cervarix®[6] is the bivalent Human papillomavirus virus-like particle vaccine produced in insect cells that contains purified VLPs formulated with AS04 adjuvant. Along with the antigen, each 0.5-mL vaccine dose contains adjuvant (50 µg of the 3-O-desacyl-4’- monophosphoryl lipid A [MPL], and 0.5 mg of aluminum hydroxide), 4.4 mg of sodium chloride, and 0.624 mg of sodium dihydrogen phosphate dihydrate. Each dose may also contain residual amounts of insect cell and viral protein (<40 ng) and bacterial cell protein (<150 ng) from the manufacturing process. Cervarix® does not contain any preservatives.

Hecolin™[7] is a hepatitis E vaccine (VLP expressed in *E. coli*) approved in China. There are multiple hepatitis B vaccines (VLP expressed in yeast), and recent publications and patents indicate the use of different excipients for stabilization of VLP-based vaccine formulation.

Formulation of VLP-based vaccine can be achieved using single-use components. Individual components can be prepared in solution and filter sterilized to a 2D or 3D bag using 0.22 µm sterilizing grade filter. Mixing of different antigens, excipients (buffering agents, preservatives, stabilizers, detergents, etc.) and adjuvant can be done in a closed operation. Single-use bags containing formulation reagents can be connected to a any mixer through sterile quick-connects. After compounding and formulation, the product can be aseptically transferred to single-use filling systems for final filling and vialing.

Summary

VLPs have gained continued interest because of their advantages over traditional vaccines. The absence of viral genomic material enhances safety of VLP-based vaccines. The choice of production platforms described in this section depends on several factors including cost and the need for post-translational modifications (PTMs), which can be essential in generating an optimal immune response.

5. Manufactured by Merck. 6. Manufactured by GlaxoSmithKline. 7. Jointly developed by the China National Institute of Diagnostics and Vaccine Development in Infectious Diseases (NIDVD) and Xiamen Innovax Biotech Co., Ltd

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Polysaccharide Conjugate Vaccines

Polysaccharides are relatively complex carbohydrates. Because they are polymers made up of many monosaccharides joined together by glycosidic bonds, they are very large, often branched, macromolecules.

Bacterial polysaccharides represent a diverse range of macromolecules that include peptidoglycan, lipopolysaccharides, capsules, and exopolysaccharides. The functions of these compounds include structural cell-wall components (e.g., *peptidoglycan*) and important virulence factors (e.g., *Streptoccus pneumoniae, Neisseria meningitidis, Haemophilus influenzae*).

Polysaccharide-based infections of bacterial origin such as meningitis, pneumonia, and influenza continue to be widespread worldwide. Because they are a particular threat to young children, childhood vaccination is necessary.

Typical Polysaccharide Conjugated Vaccines

Disease	Microorganism	Strains used in vaccine	Antigen
Pneumonia	Streptococcus pneumoniae	**23 different strain**	**Polysaccharide capsule**
Meningitis	Neisseria meningitidis	**A, C, Y, W-135**	**Polysaccharide capsule**
Haemophilus Influenza	Haemophilus influenzae	**B**	**Polysaccharide capsule, polyribosyl phosphate (PRP)**

Free polysaccharides are T-cell-independent antigens because they are not capable of binding to the major histocompatibility complex molecules, which means they cannot stimulate T-helper lymphocytes. However, polysaccharides are only able to elicit antibody responses by stimulating B cells, and only weak responses in the immature immune system of infants and young children are elicited. Free polysaccharide antigens elicit effective T-independent responses in adults, but only weak responses in the immature immune system of infants and young children.

Covalently attaching a carrier protein to the haptenic polysaccharide molecule transforms it into a T-dependent antigen that has a high efficacy in eliciting an immune response in infants and young children. These vaccines are called *polysaccharide conjugate* vaccines. Vaccines against *Streptoccus pneumoniae, Neisseria meningitidis,* and *Haemophilus influenzae* are produced by this method.

Polysaccharide conjugate vaccines are designed to provide protective immunity against the different epidemiologically pathogenic serotypes of each bacterium.

- *Neisseria meningitidis* vaccine is quadrivalent against serotypes A, C, Y, and W-135.
- Type B is the most prevalent serogroup for *Haemophilus influenzae*.
- There are twenty-three strains of *Streptococcus pneumoniae* that are known to cause infections. The conjugate vaccine available against pneumococcus consists of polysaccharides from seven strains that cover 80% of pediatric cases in the USA.

The polysaccharides are generally conjugated to non-toxic, non-reactogenic carrier proteins tetanus toxoid (a 150 kDa protein from the gram-positive anaerobic bacteria Clostridium tetani) or CRM 197 (a 68 kDa protein from *Cornybacterium diphtheriae*, which is expressed as a single-point mutated recombinant protein in *E. coli* or *Pseudomonas*).

Polysaccharide conjugate vaccine manufacturing follows a complex multi-step process which doesn't have a template. Figure 7 shows a general outline.

Fermentation

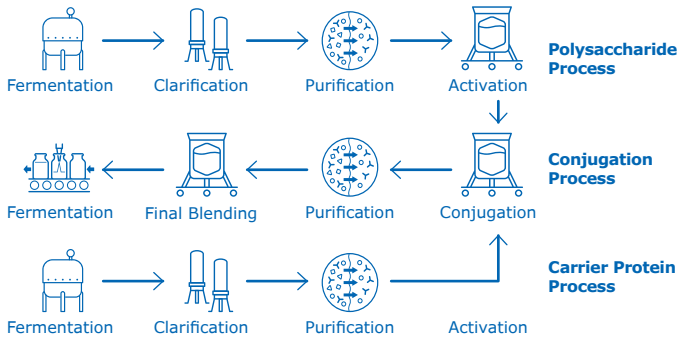

Figure 7: Generic polysaccharide conjugated vaccine (PCV) process

The fermentation media is sterilized *in situ* inside the fermentor for a period of sixteen to twenty hours. The process requires a high-mass transfer coefficient and dissolved oxygen. Silicone-based antifoams are also added. The polysaccharides (PS) are extracellular, secreted into the fermentation media during the growth of the bacteria. To facilitate the secretion of the PS into the media, in some cases at the stationary phase of the fermentation cycle, sDOC, a mild detergent, is added. Maximum OD600 of post fermentation broth is 5–8.

Clarification

The organisms used for PCV, such as *Haemophilus, Pneumococcus* and *Neisseria* (meningitidis), are highly pathogenic. All workers involved in culture clarification are vaccinated for protection. Post fermentation, the cells are immediately removed by an online centrifuge operating at 15,000 g. Secondary clarification, in which the centrate has an Nephelometric Turbidity Units (NTU) of around 60, is performed using depth filtration with a filter of adequate capacity. Post clarification, the harvest is passed through sterilizing-grade filter (used for bioburden reduction).

TFF Concentration

The initial polysaccharide secreted in the case of *H. influenzae* is around 1000 kDa. It is concentrated 5X to a concentration of 3–5 g/L of PS by 100 kDa Biomax® ultrafiltration membrane. Since the polysaccharides are

viscous, membrane polarization is very rapid. V-screen cassettes with a permeate control using secondary pump are recommended. Also, because polysaccharides are shear sensitive, operations are carried out at low crossflow and low TMP (6 psi).

Precipitation and Purification

A three-step precipitation procedure improves the polysaccharide purity profile:

1. Small molecular weight impurities are removed by precipitation with 0.4% v/v of ethanol in 5% Na-acetate, pH 6.7. The precipitate is removed by centrifugation at 12000 g for thirty minutes, and the supernatant is collected for further processing
2. The polysaccharide antigen is precipitated by 0.75% v/v of ethanol in 7.2% Na-acetate, pH 6.7. The supernatant discarded.
3. The precipitate is dissolved in water and subjected to detergent precipitation by the addition of 1.5% v/v of a 10% solution of hexadecyltrimethyl ammonium bromoide, also known as cetyltrimethylammonium bromide (CTAB). CTAB precipiates the PS and removes 95% of nucleic acid as well as 90% of the protein contaminants in the supernantant. The precipiated polysacchharide is pelleted and collected. The supernatant is discarded by centrifugation

The precipitated PS is dissolved in 0.25 M NaCl solution to a turbid solution. Clarification using an appropriate depth filter is performed. Further precipitation of PS using 0.75% v/v ethanol is performed, and the precipitate is dissolved in water.

Anion-Exchange Chromatography

Polysaccharides of certain *Pneumococcus* strains (7F, 14, and 33F) are not precipitated by CTAB. Anion-exchange chromatography is used to separate the nucleic acid and protein components from the PS. A 0.5% v/v solution of potassium iodide is added to the PS fraction of the anion-exchange eluate to form a KI-detergent complex and further purify the polysaccharide by precipitation, which is redissolved in NaCl solution. The solution at times is subjected to filtration through activated carbon filters to remove nucleotide and peptide impurities.

Ultrafiltration/Diafiltration

In the case of *H. influenzae*, the purified polysaccharide is 1000 kDa and is called polyribosylribitol phosphate (PRP), which is treated with carbonate/bi-carbonate buffer, pH 11.0 (incubation at 4 °C, two to six hours) to degrade PRP to 250±100 kDa. The PRP cannot be conjugated directly to the carrier protein tetanus toxoid (TT). It must be activated by attaching a linker arm, adipic acid dihydrazide (ADH). Cyanogen bromide is added at 4 °C in 5% molar excess (i.e., 2.7 mL/mg of PRP, 3M ADH solution [linker arm]) at 3% wt/vol and held at 4 °C for sixteen hours. UFDF is performed with 10 kDa membrane to remove unreacted components with 5 volumes diafiltration with water. Post 10 kDa UFDF, the PS-ADH complex is filtered through a 0.22 µm filter. The PS-ADH complex is conjugated either to tetanus toxoid (TT) or diphtheria toxin (CRM 197).

Activation of the Carrier Protein and Ultrafiltration/Diafiltration

Tetanus toxoid (150 kDa) is prepared from the anaerobic bacteria *Clostridium tetani*. After toxoidation, activation is required before conjugation. The toxoids are derived from tetanus toxin by detoxification with formaldehyde, which combines with the amino groups of the toxin leaving a limited number of amino groups available for conjugation. Toxoid isoelectric point, which is close to 6.8 pH, is lowered by MES buffer to 5.5–6.5 to offer positive charge to the tetanus toxoid (TT). Hydrazine hydride/dihydride buffer is added and held for four to five hours for conversion of amino groups. The process is deactivated by the addition of NaOH (2%). Finally, 20–30X diafiltration is performed to remove chemicals using a 30 kDa TFF membrane.

Conjugation with Carrier Protein

The conjugation of PRP-ADH complex to carrier protein TT follows an aldehyde conjugation process. Sodium meta para hydride is added to PRP-ADH for activation in conjugation buffer (HEPES with EDTA, pH 7.0–8.0). The PRP-ADH and activated TT are mixed in a 1:1–1.3 ratio. The conjugation is completed in two to three days.

Purification of Polysaccharide Conjugate

Because of the increase in molecular weight caused by the conjugation of PRP-ADH-TT, a 300 kDa membrane is used for concentration. This also facilitates the removal of unreacted low-molecular-weight impurities to a certain extent. To separate the unreacted PS from the conjugated complex, butyl and octyl chemistries in a hydrophobic interaction chromatography (HIC) mode is used. In many cases, gel filtration chromatography is also used. Post chromatography UFDF into the formulation buffer 20 mM Tris with 0.005% NaCl is performed, and the product is sterile filtered.

Formulation

Polysaccharide vaccines are either lyophilized or formulated with adjuvant (aluminim phosphate). *Haemophilus influenzae* type B vaccine (Hib vaccine) is commonly used for tetravalent/pentavalent (D, T, P/aP, HepB, Hib) vaccine preparation formulated with aluminium-based adjuvant. The final formulation cannot be filter sterilized. The antigen is filter sterilized separately and aseptically blended with sterile adjuvant. The fermentation and downstream processing of individual strains is done separately and finally blended together in defined ratio prior to formulation.

Summary

Conjugated vaccines are necessary for the protection of infants and young children because their immune systems are immature and do not respond to polysaccharide vaccines. The success of approved vaccines has already been proven; countless lives have been saved. A step-by-step approach to upstream and downstream processing of polysaccharide conjugated vaccines can be beneficial.

Viral Vector Vaccines

A live vector vaccine is a vaccine that uses a weakened or harmless microorganism to transport pieces of the antigen in order to stimulate an immune response. Vectored vaccines show promise in their reliably to induce potent cell-mediated immunity, which is essential for complex disease like AIDS, malaria, and cancer among others.

Viruses and bacteria can both be used as vectors. Attenuated *Salmonella typhi* (Ty21a) and *Lactobacillus acidophilus* are two common bacterial vectors. Bacterial vector vaccines are administered orally for mucosal immunity. Common viral vectors are adenovirus, canarypox, lentivirus, and alphaviruses. They transfect their own DNA into the host cell, which is later expressed to produced new viral particles.

A Japanese encephalitis (JE) vaccine uses an attenuated yellow fever virus (YFV-17D) encoding the JE preM-Env protein. It is the first human viral vectored vaccine on market. There are also twelve viral vector vaccines currently in use for veterinary diseases. The approved vaccines include adenovirus, fowlpox virus, attenuated yellow fever (YFV-17D), and vaccinia virus vectors, all of which are relevant as potential human viral vectored vaccines.

Typical Viral Vectors in Vaccine

Vector Name	Size (nm)	Cell Line	Used in vaccine
Adenovirus (Ad-5)	70–90	**HEK293, PER. C6**	**Malaria, HIV, Hep. B**
Canarypox (ALVAC)	200–300	**Chicken embryo fibroblasts**	**HIV**
Alphavirus (Sindbis, Semliki Forest, etc)	70	**Chicken embryo fibroblasts, vero**	**SARS-Cov, Ebola**
Lentiviral vector	80–100	**HEK293**	**HIV**
Modified Vaccinia Virus Ankara (MVA)	230	**Chicken embryo fibroblasts, EB66**	**HIV, smallpox**
Attenuated yellow fever virus (YFV-17D)	50–90	**Embryonated chicken egg, Vero**	**West Nile, Dengue**

Vectored vaccine elicits strong humoral and cell-mediated immune responses that result in immunological memory. They can be targeted by viral tropisms for particular cells such as intestine cells, brain cells, etc., inducing desired immunity. Vectored vaccines can encode for several antigens from different pathogens, introducing the possibility of a single vaccine for several diseases. They are relatively inexpensive, and some are easily transportable.

Since the live virus being used is an attenuated form of a human pathogen, there is always a risk of reversion to virulence. Some of the vectors under consideration, such as adenovirus, have the capability of transforming cells to a cancerous phenotype. Many large vectors (canarypox and vaccinia, for example) cannot be sterile filtered because they are too large to pass through 0.22 µm membrane. Completely closed or aseptic manufacturing practices must be followed for these large vectors.

There are several advanced molecular biology methods available for the design of the unique genetic make-up of vectors. The core of vectored vaccine innovation is in the molecular architecture and design of the vector. We will focus on the manufacturing process of adenovirus-based vaccine as a typical representative of vectored vaccine, but we will not cover vector design and development.

Adenoviruses are non-enveloped viruses with a ds-DNA, 70–90 nm in size. They are efficient at transducing target cells and can be produced at high titres (>10^{11}/mL). Adenovirus (AV) in its normal form is a pathogen that causes respiratory ailments such as conjunctivitis and the common cold. It is also capable of infecting neurons, damaging cells, and invoking strong immune responses. However, adenoviruses used as vectors are specially designed not to cause any disease and are generally regarded as safe.

The manufacturing process for adenovirus vectors is straightforward and fairly templated; a general outline is shown in Figure 8. Typical pilot scale bioreactor size for adenovirus production is 20 L cell culture, which becomes ~4 L after UFDF and ~500 mL post chromatography and final sterile filtration. Full-scale process volumes range from 100 to 200 L, and overall process yield is typically 65%.

Cell Culture

Efficient manufacture of adenovirus vectors can be accomplished using genetically engineered human cell lines that complement the deleted adenoviral genes required for replication (e.g., 293-ORF6 cells, HEK293, PER.C6). These cells have a well-characterized safety profile and can be adapted for growth in serum-free suspension. Production cells are grown in stirred-tank bioreactors with serum-free culture medium.

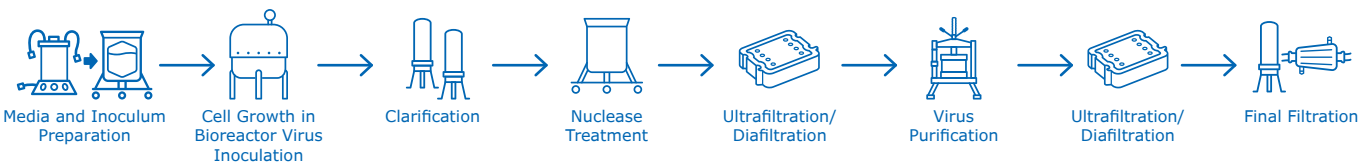

Figure 8: Generic adenovirus-based vectored vaccine process

During the adenovirus infection phase, the metabolic processes of the production cell line are significantly increased to support vector manufacture. In order to facilitate successful vector production, a medium exchange step is performed to remove spent medium containing metabolites such as lactic acid, which can be detrimental to virus production. The virus yield drops significantly when the media pH < 7. A 50–100% typical cell density during adenovirus infection is 0.5–9.0 x 10^6 cells/mL. The adenovirus titer during harvest generally ranges from 10^9 to 10^{11} pfu/mL.

Cell Lysis and Clarification

Cells are lysed either mechanically or by a chemical lysis agent (e.g., non-ionic detergent) for the harvesting of adenoviruses. Lysis with Triton™ X-100 solution is most common. Clarification is performed to remove the cells or cell debris and harvest adenoviruses. A depth filter is commonly used for primary clarification. Some manufacturers also use tangential flow filtration (TFF) at low shear conditions depth filtration or normal flow filtration (NFF) for clarification of adenoviral vaccine harvests. Filter capacities depend on cell density at harvest, the degree of lysis, and the particle size distribution. Typical lysate turbidity is >200 NTU. Similar to viral vaccine process, depth filter followed by bioburden reduction filter is commonly used for this application. TFF may also be an option in addition to the NFF options. Typical loading for TFF is 20-30 L/m^2. Some manufacturers also use centrifugation for primary clarification. Filtrate from secondary clarification has been, in some instances, filtered through 0.45 µm for bioburden reduction (~250–500 L/m²). Post clarification turbidity is in the range of 5–10 NTU. This unit operation is conducted at room temperature.

Nuclease Treatment

Carryover nucleic acid from lysed cells is a key contaminant in adenovirus vaccine processes. Viruses propagated in nonhuman cells (i.e., HEK293, PER.C6) pose a greater risk of nucleic acid carryover. Regulations require that carryover host cell nucleic acid content should be below 10 ng/dose of attenuated viral vaccine. Nucleic acids are negatively charged; they are large molecular components that can interfere with virus purification. Virus harvest is treated with about 0.9 to about 1.1 U/mL of Benzonase®endonuclease at 30-34 °C for four to eight hours.

Ultrafiltration/Diafiltration

After Benzonase® endonuclease treatment, the harvest is diafiltered using TFF (100-300 kDa UF devices). The typical flux for 300 kDa is ~25–50 LMH at 5–10 psi TMP at 5–7 L/min/m² feed flow rate. Next, 4–10 X concentration and 5–8 N difiltration are performed. More than 99% retention of adenoviruses is typical. Some manufacturers perform a vector concentration step to reduce overall volume before Benzonase® endonuclease treatment. Diafiltration is then performed to facilitate buffer exchange for further processing, such as downstream chromatographic processing. Sometimes an overnight hold step is employed prior to downstream purification. Consequently, a filtration step is performed to reduce the risk of bioburden and to protect the downstream chromatography columns.

Chromatography

Small-scale clinical lots are typically purified using CsCl-based density gradient ultracentrifugation. However, for large-scale production, column chromatography is employed. Two- or three-step column chromatography purification is normally used for adenovirus production. Purification methods commonly used are ion exchange and size exclusion chromatography (optional). Anion exchange is used to remove HCP, DNA, RNA, and other major contaminants. Size exclusion chromatography is used for trace contaminant removal. Typically, in anion-exchange columns, the adenovirus feed (5×10^{12} virus particles/mL of resin) is loaded at 75 cm/hour flowrate in 50 mM Tris-HCl, pH 8.0 in 5% glycerol and eluted in salt gradient. Adenovirus elutes at ionic strength of 40 mS/cm. Weak ion-exchangers are also proven to work for purification of adenovirus resulting in high purity and yield.

Sterile Filtration

Sterile filtration ensures the sterility of the final formulated product. A filter pore size of 0.22 µm or less is required to eliminate microbial contaminants.

Summary

Viral vectors are vehicles that deliver the genetic payload to target cells. Advancements have been made in the vector design to ensure safety of these types of vaccines. This section describes a template to manufacture of adenovirus-based vaccine used in the bioprocessing industry. Compared to the conventional vaccines, adenovirus vector-based vaccines can express a wide range of antigens from virus, bacteria, or protozoan. They elicit long-term immune responses against infectious diseases.

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