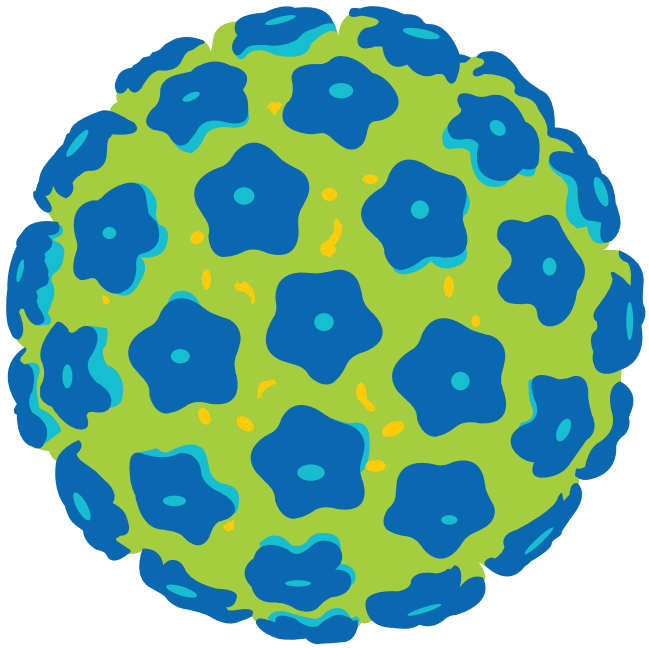
Application Note

Generic Process of Virus-Like Particle (VLP) Based Vaccine Manufacturing

A Virus-Like Particle (VLP) is a biological nanoparticle which consists of the protective protein shell of a virus. VLPs are a specific class of viral subunit vaccines that mimics the overall structure of virus particles without the infectious genetic material. VLPs provides a high immunogenic response due to high density display of epitopes, the capacity to present multiple proteins to the immune system and their size (typically around 40 nm, influenza VLP however 80–120 nm) which seems to be the optimal for uptake by dendritic cells. VLPs don't contain the viral genetic material and so are non-infectious and safe to use as they cannot replicate. Furthermore, VLPs are unlikely to need adjuvants to be highly immunogenic.



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

Table 1 shows examples of VLPs which are currently on the market or under clinical evaluations or development (non-exhaustive list).

Table 1

Disease	VLP Composition	Size	Expression System	Company	Latest Phase*	Source
Hepatitis (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	Paavonen 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	Phase 2	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: drug manufacturers websites on June 2016.

Generic Virus-Like Particle (VLP) Based Vaccine Process

The manufacturing process for VLP-based vaccine is complex. A general outline of the process that we determined in collaboration with Instituto de Biologia Experimental e Tecnológica, Portugal (iBET) is presented in **Figure 1**. There are several methods to produce VLP-based vaccines. For simplification purposes, this tech note will explain production of Baculovirus mediated insect cell expressed virus-like particles. Downstream processing of this can be very well correlated to VLPs produced in other cell culture expression systems.

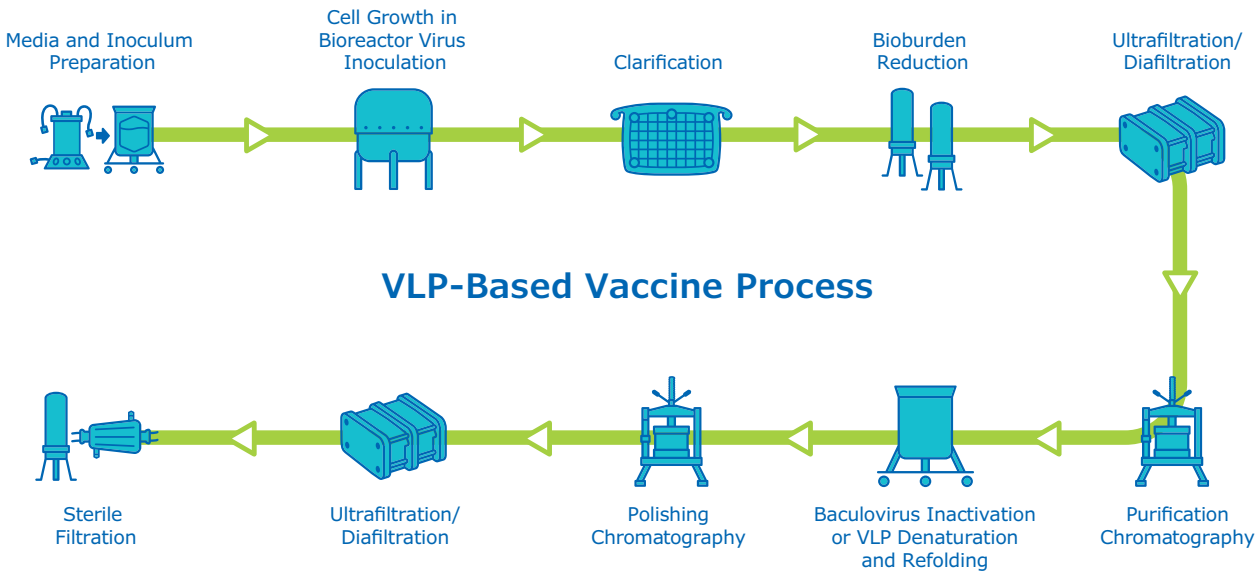


Figure 1. Generic process of VLP-based vaccine production.

Cell Culture

Generally the insect cell line (Sf9, *Trichoplusia ni*) 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 x 10^6 was obtained. The culture is then infected with baculovirus at multiplicity of infection (MOI) of 0.5–1.0. Cell culture is grown at 26° to 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 x g for 30 minutes after which the fluid is decanted and the cell paste stored at -60° to -80 °C.

According to a collaborative work carried out with iBET, the single-use Mobius® bioreactor can be used for production of VLPs in insect cell culture. The Mobius® bioreactor has been used to produce Hepatitis C VLP-based vaccine using Sf9 insect cells and Sf900II cell culture media (see **Figure 2**). Increased agitation rate, baculovirus inoculation at high cell density and use of open-pipe sparger in the Mobius® bioreactor results in better VLP productivity and shown to offer similar productivity as multi-use bioreactors.

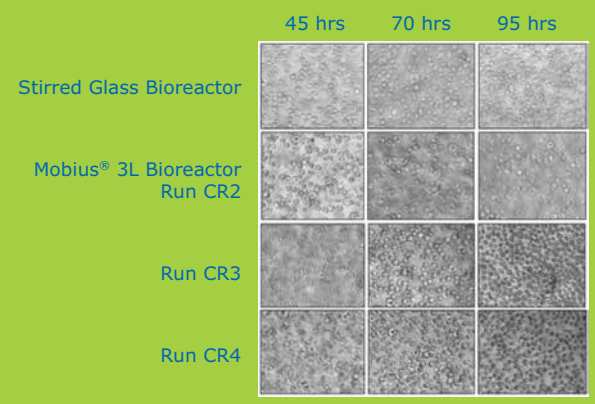


Figure 2. Comparative microscopic evaluation of insects cells (Sf9) grown in 3 and 50 L single-use Mobius® bioreactors.

Production of VLP in yeast is an attractive, low-cost alternative to insect and bacterial systems. *S. cerevisiae* batch cultures producing VLPs could be performed in a complete synthetic medium (CSM) with the addition of Glucose (20 g/L) and Yeast Nitrogen Base (6.7 g/L). A generic composition of CSM is shown in **Appendix 1**. 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. Feeding medium for fed-batch cultures is used as 10 X CSM without supplements.

Insect cells producing VLPs are mostly grown in commercially available serum-free insect media, a variations of IPL-41 basal medium. Serum-free insect cell media are supplemented with Fetal Bovine Serum (FBS), protein hydrolysates and a lipid/surfactant (Pluronic® F-68) emulsion. Supplementation with Heparin is also helpful for reducing the aggregation of cells. Generic composition of cell culture media used in insect cell culture is shown in **Appendix 2**.

Luria Broth (LB) is a medium that traditionally has been used to culture *E. coli* for both research and industrial purposes. LB medium used in shaker flasks at research scale does not differ very much from the standard media used in industrial scale fermenters, although it is generally of a simpler composition.

Prior to fermentation, the cell suspension from the cell bank vial is used to inoculate a small volume of medium in a small shake flask; this culture is then used to inoculate a larger shake flask. When the culture reaches the desired OD_{600}, the cells are harvested (by centrifugation and re-suspended at a high density in fresh medium containing 20% v/v Glycerol as a cryo-protectant. The cell bank preparation and seed culture preparation are followed by the fermentation. There are three phases of the fermentation process: A batch phase, an exponential fed-batch phase, and an induction fed-batch phase. The fermentation batch phase uses yeast extract — an enriched medium added with Glycerol as the primary carbon source. For the fermentation fed-batch phase, two different fermentation feed media are used; both are similar in composition to the batch medium, but contain either a high concentration of Glycerol alone or Glycerol and Lactose. Lactose is used as a carbon source and as the inducer. Complex media used in recombinant *E. coli* fermentation is shown in **Appendix 3**.

Cell Lysis

VLPs are purified by resuspending cell paste in a tris buffer with 1.6 µM leupeptin and microfluidized to prepare a cell lysate. Most of the VLPs are expressed in the cell and need to be recovered by cell lysis, depending on whether the VLPs are secreted to the extracellular medium. There are reported cases influenza VLP produced in insect cell culture where cell lysis is not required. In some cases, to increase product yield cell lysis are required. Cell lysis releases HCP and hcDNA that must be removed. Sometimes Benzonase® endonuclease treatment is used to digest hcDNA which is later removed by the subsequent downstream process. Therefore, the current trend is to design a clone compatible with an efficient secretory pathway. Freeze-thaw, use of detergents, homogenization or sonication are typically used as methods for cell lysis, with high-pressure lysis being the most common. The harvest solution forced through a small fixed orifice at a high pressure is in rapid transfer of the sample from a region of high pressure to one of low pressure causing 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. 1% Triton® X-100 is used as one of the cell lysis method by detergent. Use of 25 mM Na_2HCO_3, pH 8.3 also is also reported. At the process for Gardasil® (Merck & Co)* (HPV VLPs), the cell slurry is passed twice through a homogenizer, which achieves a cellular disruption of greater than 95%. The mixture is incubated at 4 °C for 12–20 h for complete lysis.[3]

Chemical induced cell lysis can be carried out in Mobius® mixing systems. Handling of buffers used in cell lysis and storage of lysate can be combined using single-use Mobius® bags, Lynx® CDR connector and NovaSeptum® 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 due to the robustness and scalability. 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 (Prostak™ filtration module or hollow fibers 0.22–0.65 µm) or NFF or centrifugation. Polygard® CN cartridge filters and Clarigard® filters had worked well for this application (yields >70%). In some cases Millistak+® HC depth filters and Clarisolve® filters can be used, but it requires proper optimization. Positive charge in such depth filters can result in product loss due to adsorption. However, adding salt to feed (100 mM NaCl) or performing a pre- and post-buffer flush with salt (0.5 M NaCl) can enhance the product recovery.

*Cook (2003). Process for purifying human papillomavirus virus-like particles. US Patent US 6602697 B1.

Benzonase® Endonuclease 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/dose for parenteral vaccines, and 100 µg DNA/dose for oral vaccines.[20] 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 which 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 will vary 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 1–4 hours. In cases with reduced Benzonase® endonuclease concentration, or for processes which require incubation at low temperature or neutral pH, incubation times of 8–12 hours may be required. For each process it is recommended to perform low volume scouting experiments to identify optimal Benzonase® endonuclease incubation conditions for adequate nucleic acid digestion. DNA digestion can be monitored with assays such as agarose gel electrophoresis, Q-PCR, or the Threshold Immunoassay.

After the incubation step is completed, the Benzonase® endonuclease enzyme must be removed in subsequent processing steps. In cases where Benzonase® endonuclease enzyme, a 60 kDa dimer, is sufficiently smaller than the VLP product, hence TFF can be utilized to achieve separation. Typically, a 300 Kda Pellicon® 2 device with a Biomax® membrane is recommended to allow Benzonase® endonuclease passage while retaining the product molecule. For processes where the molecular weight difference between VLP and Benzonase® endonuclease enzyme does not allow for TFF separation, either anion or cation exchange chromatography resins (Fractogel® TMAE, Fractogel® SO_3, Fractogel® DMAE, and Eshmuno® Q resins, etc.) can be used. The Benzonase® endonuclease isoelectric point is 6.85. Benzonase® endonuclease removal can be monitored using the Benzonase® ELISA Kit II, 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 Mobius® mixer integrated with buffer bags and a sterile filter to introduce Benzonase® endonuclease enzyme and NovaSeptum® sampling system for QC testing.

Concentration and Buffer Exchange

Depending on type of VLPs, expression system and VLP titre, a 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 will need this step. For example, the production of HPV L1 may not include this UF/DF step. Alternatively, influenza VLPs may need use of TFF devices with 300–1000 kDa membranes (Pellicon® 2 with Biomax® membrane and C screen). During concentration and buffer exchange the host cell proteins (HCP) will be 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)

Typically, an anion exchange column (packed with Fractogel® TMAE or Fractogel® DEAE resin) is used to reduce DNA and endotoxin levels. Due to the size of the particles, there are inherent mass transfer limitations that play a significant role during chromatography. The large particles reduce mass diffusion during column loading, therefore reducing effectiveness of the step and limiting the column's overall dynamic binding capacity. Use of packed bed as a flow-through mode may offer an alternative.

Before the chromatography step, the material may be diluted with 20 mM Tris-HCl to reduce salt concentration. Fractogel® DEAE ion exchange resin works well for this application. After washing with Tris-HCl buffer, the product is eluted with a phosphate buffer and the pH is adjusted for final polishing step.

Some VLPs are also purified by ultracentrifugation CsCl or Sucrose gradient. Typically 20% to 60% discontinuous sucrose gradient in Phosphate buffer with 0.5 M NaCl, pH 7.2 is used for centrifugation at 6,500 xg for 18 hours at about 4–10 °C. VLPs form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that are collected from the gradient and stored. This product is diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. At this step this product may contain intact baculovirus particles.

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

The sucrose ultracentrifugation works by layering different and discontinuous concentrations of sucrose (20 to 60%). Each concentration is then collected for analysis after centrifugation. Given the differential density of each sucrose layer, the VLPs will migrate until reaching a zone of similar density, separating them from other contaminants that could not be previously removed. Typically the discontinuous sucrose gradient is prepared in 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 xg for 18 hours at about 4–10 °C or 37,000 xg for 3 hours or even 100,000 xg for 1 hour at 4 °C. VLPs form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that are collected from the gradient and stored. Sucrose can be removed by dialysis against PBS and diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process.[6,14]

While ultracentrifugation techniques are well established and convenient for small-scale production, they can prove to be time consuming and poorly scalable. As a result, other purification methods are being used, such as ion-exchange chromatography.[8,15] Examples of buffers that can be used in the ion exchange chromatographic step include phosphate (±citrate), Tris-HCl, MOPS, HEPES and sometimes incorporating a stabilizing agent, such as sucrose.[9]

Fractogel® DEAE resin can for example be chosen to purify the VLPs. In this case, the material is first diluted with 20 mM Tris-HCl to reduce salt concentration. After washing with Tris-HCl buffer, the product is eluted with a phosphate buffer and the pH is adjusted for final polishing step. 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.[5,12] Other anion-exchange resins have also been reported, such as Fractogel® TMAE, DMAE and Eshmuno® 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).[1,11,13] Alternatively, the Fractogel® 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 (here 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. Chromabolt® prepacked columns containing Fractogel® and Eshmuno® ion exchange resins can be employed for this application. These columns can be easily connected to the Mobius® FlexReady system with Smart Flexware® assemblies or a K-Prime® stainless steel chromatography system.

In certain processes, the use of membrane adsorption and monolith technology is reported to provide better dynamic binding capacity (DBC) than particle-based resins. Although in theory, monolith allows a straight-forward scalability due to their flow-independent DBC, the actual scale-up of these columns can be challenging since their polymerization process is highly exothermic, resulting in the possibility of an inhomogeneous structure.[2,7,10] Multimodal resin that employ both size exclusion and binding based separation also demonstrates VLPs purification in flow-through mode. In this case, large molecular entity like VLPs are excluded from entering and interacting with the bead while small contaminants are trapped in the adsorptive core.[8,16]

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). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods (bind & elute or flow through). Methods of inactivation comprise of incubating the sample containing the VLPs in 0.2% of BPL for 3 hours at about 25–27 °C. The baculovirus can also be inactivated by incubating the sample containing the VLPs at 0.05% BPL at 4 °C for 3 days, then at 37 °C for one hour. Some manufacturers use Triton® X-100 and tributyl phosphate (TBP) at concentrations of 1% and 0.3%, respectively, for 30 min at room temperature (25 °C) for inactivation of baculovirus. The chromatography steps used for purification of VLPs are also strictly monitored for it's ability to separate VLPs from baculovirus. Ion exchange steps has been shown to remove $10^{2\text{-}5}$ baculovirus particles during purification of VLPs.

Polishing

When microbial systems are used, especially with *E. coli*, lipopolysaccharide (LPS) or endotoxin needs to be removed from the feed stream. Ion exchange chromatography in bind & elute mode or membrane adsorption technology in flow through mode works at this step. Hydrophobic nature of VLPs which makes endotoxins interact with the particles themselves cause issues in separation of LPS. Bound endotoxins can be released by treatment with solvents, mild detergents or combination of both. To avoid this problem, yeast (*Pichia pastoris*) or insect cell (Sf9) based expression systems are preferred.

Fractogel® TMAE ion-exchange chromatography resin has 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 contains VLPs. However, the VLP and some VLP-derived impurities can have similar electrostatic properties. When the difference in size is significantly different, some manufacturers have found success in using one or more size-exclusion chromatography (SEC) steps as an alternative. Stabilizing agents and compounds preventing ion interactions between SEC resin and the VLPs. For example, a SPG buffer made of 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4 and 0.0049 K-glutamate pH 8.0±0.2 has been reported to offer best VLP recovery when used with Fractogel® BioSEC size exclusion resin. In some cases, interaction of VLP with the resins can lead to retarded elution.

It should be noted that UF/DF constitutes an interesting option at this stage to remove lower molecular weight impurities. In addition, UF/DF 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® excipients. Some final formulations may contain amino acids, amorphous aluminum hydroxyl phosphate sulfate, carbohydrates, L-Histidine, mineral Salts, Polysorbate 80, sodium borate, etc. Final product is sterile filtered using 0.22 µm filter (Durapore® CVGL filter).

Gardasil®, 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 mcg of polysorbate 80, 35 mcg of sodium borate (borax), residual traces (<7 µg/dose) of yeast protein and water for injection. The product does not contain a preservative or antibiotics.[17]

Cervarix® (GSK), 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 a preservative.[18]

Hecolin™, Hepatitis E vaccine (VLP expressed in *E. coli*), approved in China, many other Hepatitis B vaccines (VLP expressed in Yeast), and recent publications and patents indicated use of different excipients for stabilization of VLP-based vaccine formulation.[19] A list of excipient reported to be used in VLP-based vaccine formulations is indicated in **Appendix 4**.

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 Durapore® 0.22 µm sterilizing grade filter. Mixing of different antigens, excipients (buffering agents, preservatives, stabilizer, detergent, etc.) and adjuvant can be done in Mobius® mixer in fully-closed operation. Single-use bags containing formulation reagents can be connected to a Mobius® Mixer through Lynx® CDR connector. After compounding and formulation, the product can be aseptically transferred to single-use filling systems for final filling and vialing.

Analytical Assays

Table 2 describes analytical tests performed for VLP containing HA, NA and M1 antigen of influenza virus produced in a baculovirus/insect cell culture system.

Table 2

Assay	Method*
Characteristics	
Appearance (visual)	Appearance
Particle size	Malven Zetasizer nano series
pH at 24 ºC	pH
Identity	
Western blot (HA, NA, M1, BV, Sf9 proteins)	Western immunoblot analysis
Purity	
SDS-PAGE scanning densitometry (HA + NA + M1)	SDS-PAGE and scanning densitometry
M1 and HA protein %	SDS-PAGE and scanning densitometry
Total protein (bicinchoninic acid, or BCA)	BCA protein assay
Potency	
Single radial immunodiffusion (SRID)	SRID potency assay
Hermagglutination assay	Hermagglutination titer
NA assay	Neruamindidase assay
Impurities	
Infectious baculovirus quantitation	Baculovirus quantitation by plaque assay
Baculovirus protein ELISA	Baculovirus protein ELISA
Sf9 protein ELISA	Sf9 protein ELISA
Total DNA (predominantly baculovirus)	DNA/RNA concentration
Sf9 DNA	DNA quantitation by QPCR
Residual β-Propiolactone (BPL)	BPL concentration by gas chromatography
Excipients	
Osmolality	Osmolality using microosmometer
PS80	PS80 concentration by SPEHPLC-EL SD
Safety	
Endotoxin LAL	Chromagenic LAL assay
Mycoplasma	Mycoplasma detection
Spiroplasma	Spiroplasma detection

* All methods are qualified standard operating procedures.

SPE = solid-phase extraction; ELSD = evaporative light-scattering detection

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Appendix 1

Yeast (*Saccharomyces cerevisiae*) Media Composition

Chemical Name	Concentration	Cat. No.
Nitrogen Source		
Ammonium Sulphate – Suitable for the biopharmaceutical production EMPROVE® bio ACS, NF	5 g/L	**101816**
Carbon Source		
Glucose – Anhydrous suitable for biopharmaceutical production Ph Eur, BP, USP, ACS	20 g/L	**137048**
Vitamins		
(D)+-Biotin	2 µg/L	
Ca-Panthotenate	400 µg/L	
Folic Acid – for biochemistry	2 µg/L	**103984**
Inositol	2000 µg/L	**104731**
Nicotinic Acid – Extrapure. Ph Eur, BP, USP	400 µg/L	**500005**
p-Aminobenzoic Acid – 4-Aminobenzoic acid, suitable for use as excipient EMPROVE® exp USP	200 µg/L	**100102**
Pyridoxine – Pyridoxol® (GSK) (pyridoxine) Vitamin B_6 base	400 µg/L	**501260**
Riboflavin	200 µg/L	**500257**
Thiamine HCl – Thiamine chloride hydrochloride (Vitamin B_1-hydrochloride) Ph Eur, BP, USP, FCC	400 µg/L	**500923**
Trace Elements		
Boric Acid – powder, suitable for use as excipient EMPROVE® exp Ph Eur, BP, NF	500 µg/L	**100162**
Copper Sulphate – Copper(II) sulfate pentahydrate powder, suitable for use as excipient EMPROVE® exp Ph Eur, BP, USP	40 µg/L	**102788**
Potassium Iodide – Sodium iodide, suitable for use as excipient EMPROVE® exp Ph Eur, BP, JP, USP	100 µg/L	**106520**
Ferric Chloride – Iron(III)-chloride-hexahydrate, suitable for use as excipient EMPROVE® exp Ph Eur, JP	200 µg/L	**103814**
Manganese Sulphate – Manganese(II) sulfate monohydrate spray-dried, suitable for use as excipient EMPROVE® exp Ph Eur, USP, FCC	400 µg/L	**105999**
Sodium Molybdate – Reagent grade	200 µg/L	**843899**
Zinc Sulphate – Heptahydrate suitable for use as excipient EMPROVE® exp Ph Eur, BP, JP, USP, FCC	400 µg/L	**108881**

Chemical Name	Concentration	Cat. No.
Minerals		
Potassium Dihydrogen Phosphate (KH_2PO_4) – Potassium dihydrogen phosphate cryst., suitable for use as excipient EMPROVE® exp Ph Eur, BP, NF, E 340	1 g/L	**104871**
Magnesium Sulphate – for analysis EMSURE®	0.5 g/L	**106067**
Sodium Chloride	0.1 g/L	**137017**
Calcium Chloride – anhydrous powder Reag. Ph Eur	0.1 g/L	**102378**

Appendix 2

Insect Cell Media Composition

Chemical Name	Concentration	Cat. No.
Inorganic Salts		
Ammonium Molybdate ($(NH_4)_2MoO_4$, $4H_2O$) – Ammonium heptamolybdate tetrahydrate (ammonium molybdate) powder extra pure	0.04 mg/L	**101181**
Calcium Chloride ($CaCl_2$, anhydrous) – anhydrous powder Reag. Ph Eur	500 mg/L	**102378**
Cobalt Chloride ($CoCl_2$, $6H_2O$) – for analysis EMSURE®	0.05 mg/L	**102539**
Copper Chloride ($CuCl_2$, anhydrous) – for synthesis	0.2 mg/L	**818247**
Ferric Sulfate ($FeSO_4$, $7H_2O$)	0.55 mg/L	**103963**
Manganese Chloride ($MnCl_2$, $4H_2O$) – for analysis EMSURE® ACS	0.02 mg/L	**105927**
Magnesium Sulfate ($MgSO_4$, anhydrous) – for analysis EMSURE®	918 mg/L	**106067**
Potassium Chloride (KCl)	1200 mg/L	**137009**
Sodium Bicarbonate ($NaHCO_3$)	350 mg/L	**137013**
Sodium Chloride (NaCl)	2850 mg/L	**137017**
Sodium Phosphate Monobasic (NaH_2PO_4,H_2O)	1160 mg/L	**106349**
Zinc Chloride ($ZnCl_2$) – for analysis EMSURE® ACS, ISO, Reag. Ph Eur	0.04 mg/L	**108816**
Amino acids		
β-Alanine – for biochemistry	300 mg/L	**101008**
L-Arginine Hydrochloride – L-Arginine monohydrochloride, suitable for use as excipient EMPROVE® exp Ph Eur, BP, USP	800 mg/L	**101544**
L-Asparagine – L-Asparagine monohydrate, suitable for use as excipient EMPROVE® exp Ph Eur	1300 mg/L	**101565**
L-Aspartic acid – extra pure Ph Eur, BP, USP	1300 mg/L	**100129**
L-Cystine • 2Na	119.14 mg/L	
L-Glutamic acid – suitable for use as excipient EMPROVE® exp Ph Eur	1500 mg/L	**101791**
L-Glutamine – suitable for use as excipient EMPROVE® exp DAB, USP	1000 mg/L	**100286**
Glycine – Glycine cryst., suitable for use as excipient EMPROVE® exp Ph Eur, BP, JP, USP	200 mg/L	**100590**
L-Histidine – suitable for use as excipient EMPROVE® exp Ph Eur, USP	200 mg/L	**104352**
L-Hydroxyproline – for biochemistry	800 mg/L	**104506**
L-Isoleucine – suitable for use as excipient EMPROVE® exp Ph Eur, USP	750 mg/L	**105357**
L-Leucine – suitable for use as excipient EMPROVE® exp Ph Eur, USP	250 mg/L	**105020**
L-Lysine Hydrochloride – L-Lysine monohydrochloride, suitable for use as excipient EMPROVE® exp Ph Eur, BP, USP	700 mg/L	**105701**
L-Methionine	1000 mg/L	
L-Phenylalanine – suitable for use as excipient EMPROVE® exp Ph Eur, USP	1000 mg/L	**107267**
L-Proline – suitable for use as excipient EMPROVE® exp Ph Eur, USP	500 mg/L	**107430**
L-Serine – suitable for use as excipient EMPROVE® exp Ph Eur, USP	200 mg/L	**107647**
L-Threonine – for biochemistry	200 mg/L	**108411**
L-Tryptophan – suitable for use as excipient EMPROVE® exp Ph Eur, BP, USP	100 mg/L	**108396**
L-Tyrosine Disodium Salt Dihydrate – for cell culture media	360.4 mg/L	**102413**
L-Valine – for biochemistry	500 mg/L	**108495**

Chemical Name	Concentration	Cat. No.
Vitamins		
Para-Aminobenzoic acid – 4-Aminobenzoic acid, suitable for use as excipient EMPROVE® exp USP	0.32 mg/L	**100102**
D(+)-Biotin	0.16 mg/L	**851209**
Choline chloride – suitable for use as excipient EMPROVE® exp DAB 10, FCC	20 mg/L	**500117**
Folic acid – for biochemistry	0.08 mg/L	**103984**
myo-Inositol – suitable for use as excipient EMPROVE® exp Ph Eur, FCC	0.4 mg/L	**104731**
Nicotinic acid (Niacin) – extra pure Ph Eur, BP, USP	0.16 mg/L	**500005**
D-Calcium Pantothenate	0.008 mg/L	
Pyridoxine Hydrochloride	0.4 mg/L	
Riboflavin – Ph Eur, BP, JP, USP, E 101 (i)	0.08 mg/L	**500257**
Thiamine Hydrochloride – Thiamine chloride hydrochloride (Vitamin B_1-hydrochloride) Ph Eur, BP, USP, FCC	0.08 mg/L	**500923**
Vitamin B_{12} (cyanocobalamin) – Ph Eur, BP, USP	0.24 mg/L	**524950**
Sugars		
D-Glucose (Dextrose) – anhydrous suitable for biopharmaceutical production Ph Eur, BP, USP, ACS	2500 mg/L	**137048**
Maltose – Maltose monohydrate, suitable for use as excipient EMPROVE® exp	1000 mg/L	**105911**
Sucrose	1650 mg/L	**100892**
Others		
Fumaric acid NF	4.4 mg/L	**817073**
Alpha-Ketoglutaric acid/2-Oxoglutaric acid – For biochemistry	29.6 mg/L	**105194**
L(-)-Malic acid	53.6 mg/L	
Succinic acid – Succinic acid cryst., suitable for use as excipient EMPROVE® exp NF, JPE	4.8 mg/L	**100681**

Appendix 3

E. coli Media Composition

Chemical Name	Concentration	Cat. No.
Inorganic Salts		
Sodium Chloride (NaCl)	5000 mg/L	**137017**
Potassium Dihydrogen Phosphate (KH_2PO_4)	3500 mg/L	**137039**
Potassium Phosphate dibasic (K_2HPO_4)	5000 mg/L	**137010**
Ammonium Phosphate dibasic $(NH_4)_2HPO_4$	3500 mg/L	**101206**
Magnesium Sulfate Heptahydrate/($FeSO_4$, $7H_2O$) – Magnesium sulfate heptahydrate, suitable for use as excipient EMPROVE® exp Ph Eur, BP, USP, JP	500 mg/L	**105882**
Trace Metals Formulation*		
Ferric Chloride* ($FeCl_3$) – Iron(III)-chloride-hexahydrate, suitable for use as excipient EMPROVE® exp Ph Eur, JP	1600 mL/L	**103814**
Cobalt Chloride ($CoCl_2$) – For synthesis	1500 mL/L	**802540**
Copper Chloride ($CuCl_2$)	100 mL/L	**818247**
Zinc Chloride Tetrahydrate* ($ZnCl \bullet 4H_2O$)	200 mL/L	
Sodium Molybdate* ($NaMoO_4$)	200 mL/L	
Boric acid* (H_3BO_4) – powder, suitable for use as excipient EMPROVE® exp Ph Eur, BP, NF	200 mL/L	**100162**
Hydrochloric Acid* (HCl)	10 mL	
Water* (H_2O)	to 1000 mL	
Sugars		
Sucrose	1650 mg/L	**100892**
Others		
Yeast Extract	5000 mg/L	
Tryptone	800 mg/L	
Antifoam – Poloxamer 188 suitable for biopharmaceutical production Ph.Eur., NF	0.5 mL/L	**137065**

Appendix 4

Chemicals used in a VLP-based Vaccine Process

Chemical Name	Cat. No.
Sodium dihydrogen phosphate dihydrate suitable for the biopharmaceutical production EMPROVE® bio Ph Eur, BP, USP, JPE	**137018**
di-Sodium hydrogen phosphate dihydrate suitable for the biopharmaceutical production EMPROVE® bio Ph Eur, BP, USP	**137036**
Polysorbate 80	**817061**
Aluminum hydroxide (no PCS/GMP grade, only Lab grade)	**101091**
Sodium borate	**106303**
L-Histidine	**104352**
D-Sorbitol	**111597**
Mannitol	**105303**
D(+)-Trehalose dehydrate (no PCS/GMP grade, only Bioscience Calbiochem grade)	**625625**
Sucrose	**100892**
di-Potassium hydrogen phosphate (anhydrous)	**137010**
Potassium dihydrogen phosphate	**137039**
Citric acid monohydrate	**137003**
Sodium citrate dihydrate	**137042**
Potassium chloride	**137009**
Sodium chloride	**137017**
Glycerol, Anhydrous (vegetable) suitable for biopharmaceutical production EMPROVE® bio Ph Eur, BP, JP, USP, ACS)	**137028**
Lactose monohydrate powder, Suitable for biopharmaceutical production EMPROVE® bio Ph Eur, BP, NF, JP)	**137045**
Benzonase	**101695**
Tris-HCl, Tris (hydroxymethyl) aminomethane hydrochloride GR For analysis	**108219**
Sucrose, (suitable for density gradient ultracentrifuge)	**107654**

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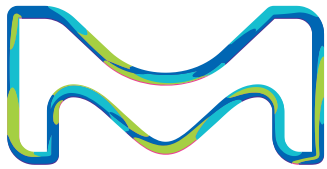

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