

## Application Note

# Generic process of cell culture based influenza vaccine

## Background

Influenza virus is an enveloped single-stranded RNA virus that causes influenza (flu). There are three serotypes of seasonal influenza virus – A, B, 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. The A type viruses are the most virulent pathogens among the three types, and cause the most severe disease in humans.

Different types of influenza viruses (A, B, and C) 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. Sub-typing of the virus is done by the antigenic variations in the surface glycoproteins, HA (hemagglutinin) and NA (neuraminidase). About 14 subtypes of HA (H1 – H14) and nine subtypes of NA (N1 – N9) have been recovered so far in different combinations, from birds, animals and humans. Three HA subtypes (H1 – H3) and two NA subtypes (N1, N2) have been recovered from humans.

Hemagglutinin (HA, MW ~77kDa) is an immunogenic protein located at the surface of the virus envelope. Neuraminidase (NA, MW ~220kDa) is a surface enzyme protein. Both proteins are the antigens that define the particular strain of influenza and play critical roles in mediating 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 avoid immune response without affecting its ability to bind to the receptor.

Influenza virus undergoes frequent minor genetic mutations known as "antigenic drift" (subtle changes in the antigenic proteins on the virus surface), which allow the viruses to evade host immunity and cause disease despite previous infection or immunization. Larger changes in the type A virus antigens ("antigenic shift") happen about every 10 years, and result in larger epidemics, or pandemics. While seasonal influenza vaccines are usually trivalent, more recently tetravalent, pandemic vaccines are monovalent.

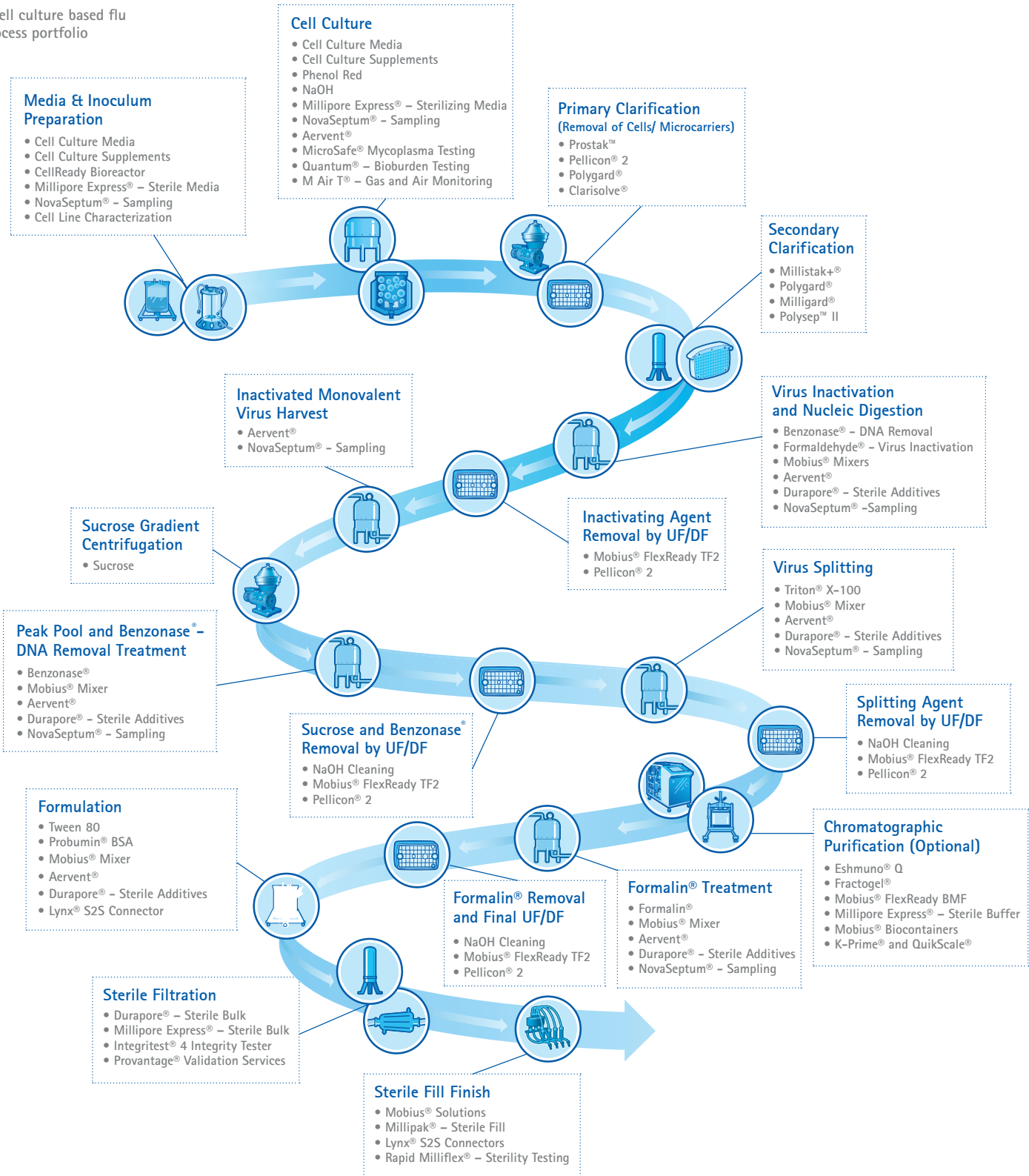
Vaccine manufacturers typically require months for the development and production of a seasonal influenza vaccine each year. There is a long lead time to secure chicken eggs every year for the manufacturing of influenza vaccine. Use of cell culture based influenza vaccine manufacturing eliminates this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

# Generic process of cell based flu vaccine

There is no template process for cell culture based influenza vaccine, and manufacturers follow different methods of manufacturing and select various technologies with their own process philosophy. A typical cell culture based flu vaccine process is shown in Figure 1. Typical commercial

bioreactor sizes range from 2500 – 5000 L. Typical total process yield is approximately 35% and is highly dependent on virus strain. Based on the above yield and recovery, a total of 50-100 doses of vaccine can be produced per liter of cell culture.

Figure 1. Cell culture based flu vaccine process portfolio



## 1. Serum free media type and sterile filtration

Cell culture media is used for propagation of cells. The media components are sterile filtered. During the growth phase, most of the cell culture undergoes media exchange, either in continuous or batch mode. After cell propagation, cell growth media is exchanged to virus propagation media. Serum free media is preferable. Millipore Express® SHC or SHR membrane is commonly used for sterile filtration of cell culture media. Based on available data, capacity of SHR and SHC for filtration of media seems to be 1500 L/m<sup>2</sup> at 20-25 psi. Capacities of filters for different media are presented in Table 1.

Milligard® and Polysep™ II pre-filter have demonstrated good protection of sterile filter resulting in increase in sterile filter capacity up to > 5000 L/m<sup>2</sup> at 20-25 psi. Millipore Express® HPF is also used when prefiltration is necessary prior to Millipore Express® SHR. Combination of other prefilters and Durapore® can also perform well for similar application.

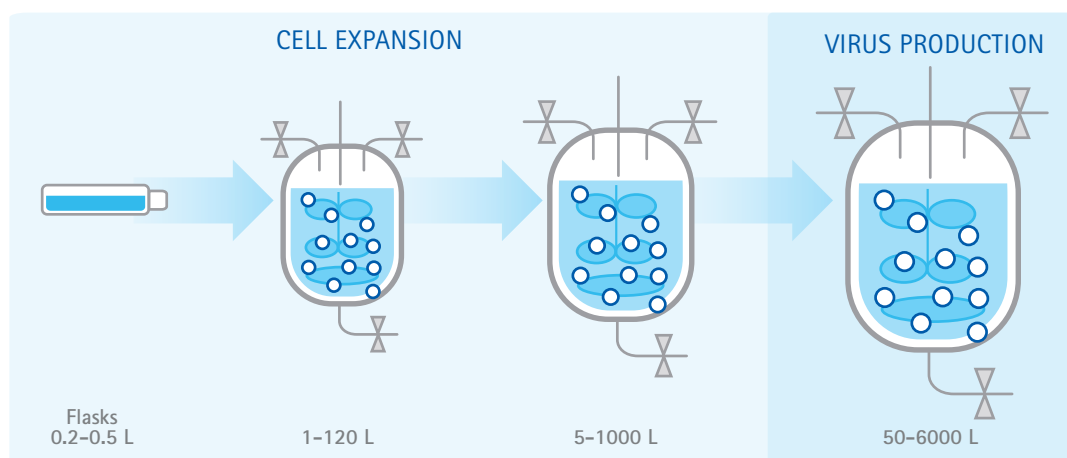
Medium	Filter		Vmax	
	1 stage	SHC or SHR (with prefilter)	Pre filter	Final filter
MDCK Cell Propagation Medium	1 stage	SHC or SHR (with prefilter)	1500 L/m <sup>2</sup>	
	2 stage	Pre filter	Final Filter	Pre filter
Milligard® or Polysep™ II		Millipore Express® SHC or Millipore Express® SHR	1000 L/m <sup>2</sup>	> 5000 L/m <sup>2</sup>
MDCK Virus Propagation Medium	Millipore Express® SHC or Millipore Express® SHR		> 5000 L/m <sup>2</sup>	
Vero Cell Propagation Medium	Durapore® 0.1 µm		~1000 L/m <sup>2</sup>	
Vero Virus Propagation Medium	Durapore® 0.1 µm		~4000 L/m <sup>2</sup>	

**Table 1.** Typical Vmax for serum free media used to grow MDCK and Vero cells

## 2. Cell types, cell culture conditions and viral Infection

Many types of cell lines have been used for cell culture based flu vaccine development, including EBx™, VERO, and MDCK. Cell culture is typically conducted in continuous or batch mode. On occasion, microcarriers (such as Cytodex1 or Cytodex3) are used for adherent

cell cultivation. Cells concentration before virus infection reaches about 1x10<sup>6</sup> cells/mL. Typical bioreactor size and scale up along with general upstream processes is shown in the Figure 2.



**Figure 2.** Schematic upstream process of cell based influenza vaccine

Chicken embryo fibroblasts, and other avian cells, have been used for influenza virus replication. Several continuous mammalian cell lines have been adequately tested, including diploid cell lines (MRC-5, WI-38, and FRhI-2) and continuous cell lines (PER.C6, NIH-3T3, BHK21, CHO, Vero and MDCK). Only Vero, PER.C6 and MDCK have consistently yielded influenza virus titers high enough to be considered commercially viable. MDCK cell expansion is generally initiated by using frozen stock of the cell bank stored in a liquid nitrogen tank and thawing the cells directly in a T-75 flask which contains pre-warmed serum free proprietary medium. During cell expansion, these cells are sub-cultured every 3–4 days in successively larger flasks until final transfer to roller bottles at 37°C in a 5% CO<sub>2</sub> incubator.

MDCK cells are generally grown on Cytodex3 microcarrier cultures at 2–3 g/L at 37°C. Stirred-tank 3L and 50L bioreactors can also be used for cell propagation. Cells density at inoculation is generally 10<sup>4</sup>–10<sup>6</sup> cells/mL and pH approximately 7.4 is maintained by sparging CO<sub>2</sub> and by addition of 1M NaOH as needed. Dissolved oxygen is generally maintained close to 50% of air saturation by sparging pure oxygen. Cultures are generally infected with influenza strains 72–96 h post-seeding. Prior to virus addition, approximately 60–80% of spent growth medium is removed and replaced with the same amount of proprietary infection serum free medium (I-SFM). A virus stock is added to achieve the desired pfu/mL in culture. Temperature during infection is generally maintained at 33°C.

### 3. Clarification

When using a bioreactor with microcarriers, the first step in the clarification process is microcarrier removal. In this step, a salt solution is added to the culture to remove the cell from the surface of the microcarrier. Some manufacturers use dextran sulfate, others just use standard PBS. Trypsin is often added after the addition of the salt solution and the culture is then usually mixed for 10–15 minutes. The microcarriers are then removed from the cell culture. Polygard® CR99 (100mm) and Clarisolve® 60HX have been successfully used to remove microcarriers. The cell culture is then further clarified to remove the cells and cellular debris.

For suspension cultures to remove cells and cell debris, typically, three clarification sequences can be applied, depending on the primary clarification step:

1. Centrifuge → Pre filtration (Polysep™ II, Polygard® CR, CN, Clarigard®) → Final filtration (Durapore® 0.45 µm or 0.22 µm)

A prerequisite for a successful infection is the addition of proteases to the medium, preferably trypsin or similar serine proteases. These proteases extracellularly cleave the precursor protein of hemagglutinin (HA<sub>0</sub>) into active hemagglutinin (HA<sub>1</sub> and HA<sub>2</sub>). 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 uses trypsinization with trypsin enzyme, which is stopped using an equimolar volume of trypsin inhibitor solution. Cells from roller bottles are used to inoculate 3L bioreactors and single use bioreactors (SUB).

MDCK cell culture can produce up to 1x10<sup>9</sup> pfu/mL of influenza virus upon infection and incubation for 3–5 days. Parameters such as multiplicity of infection (MOI), incubation time and temperature need to be optimized for each cell line and each strain of virus. Assuming 45 microgram of HA per dose (0.5 mL), it is likely that a 1,000 – 2,000 L sized bioreactor (with microcarrier-based MDCK suspension culture) would suffice to produce 20 million doses per season. It has been estimated that an optimized 1,000 L bioreactor using solid microcarriers and MDCK cells would be comparable with approximately 31,000 eggs.

2. Depth filtration (Millistak™ DOHC or COHC, Polysep™ II, Polygard® CR, CN) → Final filtration (Durapore® 0.45 µm or 0.22 µm)
3. Tangential Flow Filtration (Durapore® 0.45 µm or 0.65 µm) → Final filtration (Durapore® 0.45 µm or 0.22 µm)

It is important to consider virus yield and contaminant removal level for optimization of the clarification step. As influenza virus and host cell DNA are negatively charged, Millistak+® and Polysep™ II filters sometimes show 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 wherein 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.

## 4. Virus inactivation

Formalin® is the most frequently used inactivating agent in vaccine manufacturing. Formaldehyde inactivates a virus by irreversibly cross-linking primary amine groups in surface proteins with close by nitrogen groups in DNA or proteins. These cross-linking bonds can associate with non-viral proteins; as a result, initial partial purification of live infectious virus is required to avoid irreversible chemical bridging between viral proteins and impurities. Inactivation is carried out at 32°C for 24 hr with Formalin® concentrations of 0.1%. Another 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 mJ/cm².

## 5. Ultrafiltration / Diafiltration (UF/DF)

At this stage, whole virus particles are concentrated in order to reduce the process volume downstream. UF/DF is performed 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, HCP, and Benzonase®, and at the post-ultracentrifugation step to remove sucrose. Pellicon® 2 Biomax® 300kD C-screen device works well for volumetric concentration. Typical conditions are 10-15 psi transmembrane pressure (TMP), 5-6 L/min/m² 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.

Biomax® 500 kDa V-screen with flux control operation is used to remove host cell DNA and proteins. TFF operation shows good low molecular weight (MW) DNA removal, but is less efficient to remove high MW molecules. In some cases a 100 kDa filter shows good virus retention, depending on the size of the virus strain. In permeate controlled 2-pump based TFF, typical average filtration flux is 20-35 LMH at feed flow rate of 5-6 L/min/m² and TMP of 4 - 5 psi.

## 6. Density Gradient Centrifugation

Either density gradient (zonal) centrifugation or chromatography is commonly used to further purify the virus. Zonal centrifugation has two steps: pelleting and fractionation (Figure 3). The advantage using zonal is high purity. However, yield is low and operation is cumbersome (Table 2).

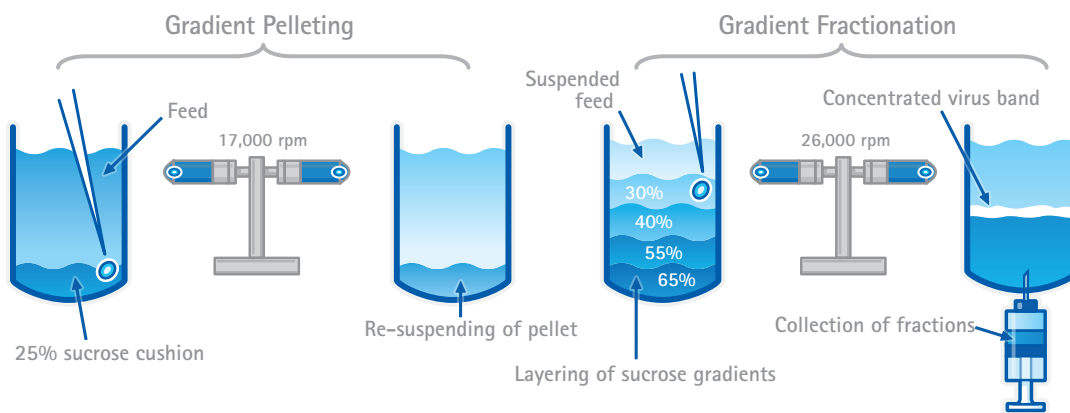


Figure 3. Schematic process of zonal centrifugation

Steps	% Overall Flu Recovery	% HCP removal	% DNA removal
Step -1: Gradient pelleting	60	99.9%	67-75%
Step-2: Gradient fractionation	30	Below detection limit	Below detection limit

Table 2. Typical yield, HCP and DNA removal using zonal centrifugation

## 7. Chromatography

In the cell culture based process, size exclusion and anion exchange chromatography are performed on inactivated virus for DNA and HCP removal. 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 leads to its adsorption to the resin as compared to the 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 anion exchange step is more than 80% with approximately 60-70 fold reduction of nucleic acid. The overall product yield from chromatographic purification is 50-55 % with 15-20 fold reduction in total protein and more than 500 fold reductions in nucleic acid. Fractogel® TMAE resin (anion-exchange, bind and elute type) can be used for influenza virus purification.

## 8. DNA removal with Benzonase® treatment

According to requirements published by the FDA, a parenterally administered dose is limited to 100pg of residual host DNA, while the EMEA and WHO (World Health Organization) allow 10ng per parental dose and 100µg/dose for orally administered vaccine. Benzonase®, a genetically engineered endonuclease, cleaves all forms of DNA and RNA. One unit of Benzonase® degrades approximately 37µg DNA in 30 min to as low as 3-8 base pairs (<6 kDa). 1-2mM of MgCl<sub>2</sub> (if not present in the original buffer system) is needed for optimal Benzonase® 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® treatment, a quantitative removal of Benzonase® from the process stream is required in the subsequent purification steps. Therefore it is better to use Benzonase® treatment sufficiently upstream. TFF (Biomax® 300kDa), chromatography (AEX) and zonal centrifugation are commonly used to remove Benzonase® from the process. There are reports indicating the use of

~0.9 - 1.1 U/mL of Benzonase® for treatment of harvest to degrade host cell nucleic acid at 30-37°C in 4-8 hr. Due to regulatory requirements, residual Benzonase® need to be measured and detected in the process. The method for residual Benzonase® detection is by using ELISA-based methods (Benzonase® ELISA kit II).

## 9. Splitting process

Most influenza vaccines are "split" vaccines, which are produced by detergent treatment. During this step the structure of 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). Virus splitting removes some of the viral components, resulting in less reactogenic vaccine. Prior to fragmentation, the concentrated monovalent (single strain) viral suspension is diluted with a sterile buffer. Viral fragmentation is executed by adding amphiphilic non-ionic detergent such as Triton® X-100 (0.5 %) and/or anionic sodium deoxycholate (DOC) to the suspension of the purified influenza. Polysorbates (Tween 80) and cetyltrimethyl ammonium bromide (CTAB) are also suitable for the virus splitting step. Fragmentation requires continuous mixing/stirring of the process fluid with the detergent(s) for at least one hour at room temperature. The length of the fragmentation step could be extended (up to 24 hr) based on the process needs. A secondary virus inactivation is employed after the virus splitting process as an additional safety measure to ensure complete inactivation of every component.

## 10. Sterile Filtration

Some cell culture based influenza vaccines, such as the vaccine for H5N1, are whole virus vaccine. In such cases sterile filtration of 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, capacity of the final sterile filters (Durapore® 0.22 µm) is occasionally low (around 20 - 50 L/m<sup>2</sup>); in such cases 0.45 µm or 0.65 µm Durapore® is used as pre-filter. Observed capacity of Durapore® filter is in the range from 200-400 L/m<sup>2</sup> depending on the feed quality and pre-filter, if used. 0.45/0.22 µm Durapore® is also used as a terminal sterile filter (if prefiltration is deemed necessary) to trim the process from 1 to 2 filters.

## 11. Formulation

Few split influenza virus vaccines in the cell culture based influenza vaccine market are formulated with adjuvant. Oil in water emulsion based adjuvants used in commercial influenza vaccine formulation are MF59

and AS03. Formulated vaccine cannot be filter sterilized, hence adjuvant and purified split virus antigens are filter sterilized separately and then aseptically blended. Millipore Express® SHF has been used to sterile filter emulsion-based adjuvants. Due to the nature of oil-in-water adjuvants, care must be taken in sizing to prevent break-through 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, calcium chloride dehydrate). For example, Optaflu®, a cell culture based trivalent seasonal influenza vaccine (split vaccine, inactivated HA and NA) from Novartis, is formulated with adjuvant MF59. A dose of 0.5 mL of Optaflu® vaccine contains total 45µg HA (15 µg x 3) and 0.25 mL MF59 adjuvant.

## 12. Flu Vaccine Analytics

The final bulk is subjected to quality control (QC) testing to ensure the biological, chemical and physical stability of the vaccine. The following analytical assays are commonly performed as batch release tests:

- HA content (SRID/Single Radial Immuno-diffusion)
- Total protein content (Bradford)
- Vaccine purity/identity (SDS-PAGE, Western blot, SRID)
- Total NA activity (through enzymatic or antigenic activity) or HA/NA ratio
- Residual host cell protein and DNA (PCR)
- Residuals of formaldehyde, sucrose, Benzonase®
- Sterility/Endotoxin (LAL)

## 13. Process review

The overall process for cell culture based manufacturing of influenza vaccine is summarized in Figure 1. Table 3 provides device/format recommendation by Merck Millipore to process a batch of cell culture based influenza vaccine. Although this is a generic recommendation, there are several other options available that can be used.

**Table 3: Chemicals used in vaccine process that are supplied by Merck Millipore**

Chemical Name	Catalogue No.
Formaldehyde solution min. 37% stabilized with about 10% methanol Ph Eur,BP,USP	104002
Polyethylene glycol 6000 suitable for use as excipient EMPROVE® exp Ph Eur	817007
Polyethylene glycol 20000 suitable for use as excipient EMPROVE® exp Ph Eur	817018
Ammonium sulfate suitable for the biopharmaceutical production EMPROVE® bio ACS,NF	101816
Sodium acetate trihydrate suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,USP	137012
Sodium acetate anhydrous suitable for the biopharmaceutical production EMPROVE® bio USP	137046
Sodium chloride suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,USP	137017
Sodium hydroxide pellets suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,NF,ACS	137020
Sodium dihydrogen phosphate dihydrate suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,USP,JP	137018
Sodium thiosulfate pentahydrate cryst., suitable for use as excipient EMPROVE® exp Ph Eur,BP,USP	106514
di-Sodium hydrogen phosphate dihydrate suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,USP	137036
Potassium chloride suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,USP,JP	137009
Potassium dihydrogen phosphate cryst. suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,ACS	137039
di-Potassium hydrogen phosphate anhydrous suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,USP	137010
Citric acid monohydrate cryst. suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,USP,ACS	137003
tri-Sodium citrate dihydrat. cryst. suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,USP,ACS	137042
Calcium chloride dihydrate suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,USP	137101
Magnesium chloride hexahydrate cryst. suitable for the biopharmaceutical production EMPROVE® bio Ph Eur,BP,USP,JP,ACS	137008
Antifoam (Poloxamer 188 suitable for biopharmaceutical production Ph.Eur.,NF)	137065
Phenol red suitable for the biopharmaceutical production Ph Eur	137038
Benzonase® suitable for biopharmaceutical production EMPROVE® bio	101697
Benzonase® ELISA kit II for the immunological detection of Benzonase®	101681
Tween® 80 (Polysorbate) suitable for use as excipient EMPROVE® exp Ph Eur,JP,NF	817061
Triton® X-100 suitable for the biopharmaceutical production EMPROVE® bio Ph Eur	108643
D(+)-Glucose anhydrous suitable for the biopharmaceutical production Ph Eur,BP,USP	137048
Sucrose (saccharose) for density gradient ultracentrifugation suitable for biopharmaceutical production EMPROVE® bio Ph.Eur.,BP,NF,JP	107654
Glycerol anhydrous (vegetable) suitable for biopharmaceutical production EMPROVE® bio Ph Eur,BP,JP,USP,ACS	137028
L-Aspartic acid extra pure Ph Eur,BP,USP	100129
L-Glutamine suitable for use as excipient EMPROVE® exp DAB,USP	100286
L-Lysine monohydrochloride suitable for use as excipient EMPROVE® exp Ph Eur,BP,USP	105701

Please contact Merck Millipore for further details.

**Table 4: Device/format recommendations to process a cell culture based influenza vaccine.**

Step	Recommendations
MDCK or Vero cell propagation Media filtration	Prefilter (Polysep™ II) > Final filter (SHC)
Virus propagation Media filtration	Sterile filter (SHC > Durapore® 0.1µm)
Clarification	Polysep™ II 1.0/0.5µm prefilter > Durapore® 0.45µm
<b>OR</b>	
Clarification	Millistak™ DOHC > Durapore® 0.45µm
<b>OR</b>	
Clarification	Pellicon® 2 0.45µ V screen > Durapore® 0.45µm
UF/DF (Benzonase® removal)	Pellicon® 2, Biomax® 300kDa, C screen
UF/DF (Post sucrose gradient)	Pellicon® 2, Biomax® 500kDa, C screen
UF/DF (splitting agent removal)	Pellicon® 2, Biomax® 300kDa, C screen
Chromatography	Fractogel® TMAE, Fractogel® DMAE
UF/DF (Formalin® removal)	Pellicon® 2, Biomax® 300kDa, C screen
Sterile filtration	Durapore® 0.22µm

## References

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