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Review Article

Upstream and Downstream Processes of rFVIII Recombinant Protein Manufacturing in Respect to Fluid Flow, Mixing, Heat and Mass Transfer

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ABSTRACT

The intrinsic blood coagulation pathways depend heavily on factor VIII, a glycoprotein cofactor. Hemophilia A, an X-linked dominant disease, is treated with FVIII, a very complex therapeutic protein commercially available. It is currently one of the most extensive and significant coagulation factors. Two isolated plasma and recombinant lyophilized FVIII concentrates are used to treat hemorrhagic illness in hemophilia A sufferers. Plasma-extracted products separated from humanoid blood can be substituted with recombinant FVIII (rFVIII) products, which are free of both humanoid and carnal proteins and transcribed in eukaryotic cells. In regard to fluid flow, mixing, heat, and mass transfer phenomena, the upstream and downstream manufacturing processes of rFVIII will be briefly reviewed in this publication.

***Keywords**: Recombinant protein rFVIII manufacturing, Upstream and downstream processes, Cell growth in bioreactors, Fluid flow, Heat and mass transfer*

rFVIII Rekombinant Protein Üretiminde Sıvı Akışı, Karıştırma, Isı ve Kütle Transferi: Yukarı Akış ve Aşağı Akış Süreçleri

ÖZ

Ana kan pıhtılaşma yolları, büyük ölçüde bir glikoprotein kofaktörü olan faktör VIII'e bağlıdır. X'e bağlı baskın bir hastalık olan Hemofili A, ticari olarak temin edilebilen çok karmaşık bir terapötik protein olan FVIII ile tedavi edilir. Şu anda en kapsamlı ve önemli pıhtılaşma faktörlerinden biridir. Hemofili A hastalarında hemorajik hastalığı tedavi etmek için iki izole edilmiş plazma ve rekombinant liyofilize FVIII konsantresi kullanılır. İnsan kanından ayrıştırılan plazmadan elde edilmiş ürünler hem insansı hem de hayvansal protein içermeyen ve ökaryotik hücrelerde kopyalanan rekombinant FVIII (rFVIII) ürünler ile ikame edilebilir. Sıvı akışı, karıştırma, ısı ve kütle transferi olayları ile ilgili olarak, rFVIII'in yukarı akış ve aşağı akış üretim işlemleri bu makalede kısaca gözden geçirilecektir.

***Anahtar Kelimeler:** Rekombinant protein rFVIII üretimi, Yukarı akış ve aşağı akış süreçleri, Biyoreaktörlerde hücre çoğalması, Sıvı akışı, Isı ve kütle transferi*

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I. INTRODUCTION

Hemophilia, a bleeding disorder, is treated with recombinant activated factor VIII (rFVIII), a protein. A kilogram of rFVIII protein costs roughly $9.6 billion and is produced using the Chinese Hamster Ovary (CHO) cell line [10]. Hemophilia is a collection of diseases brought on by a lack of the blood-clotting factor VIII (FVIII), an X-linked genetic coagulopathy [2], [5]. They can be identified by the chronic bleeding that occurs after an injury, trauma, menstruation, or surgery. Sometimes the bleeding occurs spontaneously and without apparent cause. The blood coagulation system cannot function without blood clotting factor VIII (FVIII). Recombinant FVIII is used to treat hemophilia A, which is caused by an inability to produce functional FVIII [8]. In general, biologic drug use in patients requires large-scale expansion systems while maintaining the quality of the cells. Single-use bioreactors have been suggested by regulatory agencies (European Medicines Agency (EMA) and US Food and Drug Administration (FDA) due to lowering the risk of product contamination [28].

II. BASICS OF THE rFVIII RECOMBINANT PROTEIN MANUFACTURING in RESPECT TO FLUID FLOW, MIXING, HEAT AND MASS TRANSFER PHENOMENA

The basic manufacturing procedure for a recombinant protein, rFVIII, begins with the gene of interest (GOI) and ends with the protein of interest (POI), as shown in the simplified method in Figure 1 [10]. To create rFVIII POIs, the appropriate nucleic acid sequence must first be extracted from hamster GOIs. By using the hamster genome sequence that is readily available, direct DNA synthesis is used to clone DNA sequences. A suitable host system is then used to create GOIs' expression plasmids, creating a recombinant cell that may be frozen for later use. To start a production run, cultures are produced from vials of frozen cell banks. Harvests are often carried out when cultures in batch cultures reach a high cell density. Following harvest, the production medium and cells containing the rFVIII POIs undergo additional processing for purification, filling, finishing, and packaging before being distributed [3], [10]. The following section will go into detail on the manufacturing procedures. However, for the sake of simplicity, the filling, finishing, and packing procedures will not be described here.

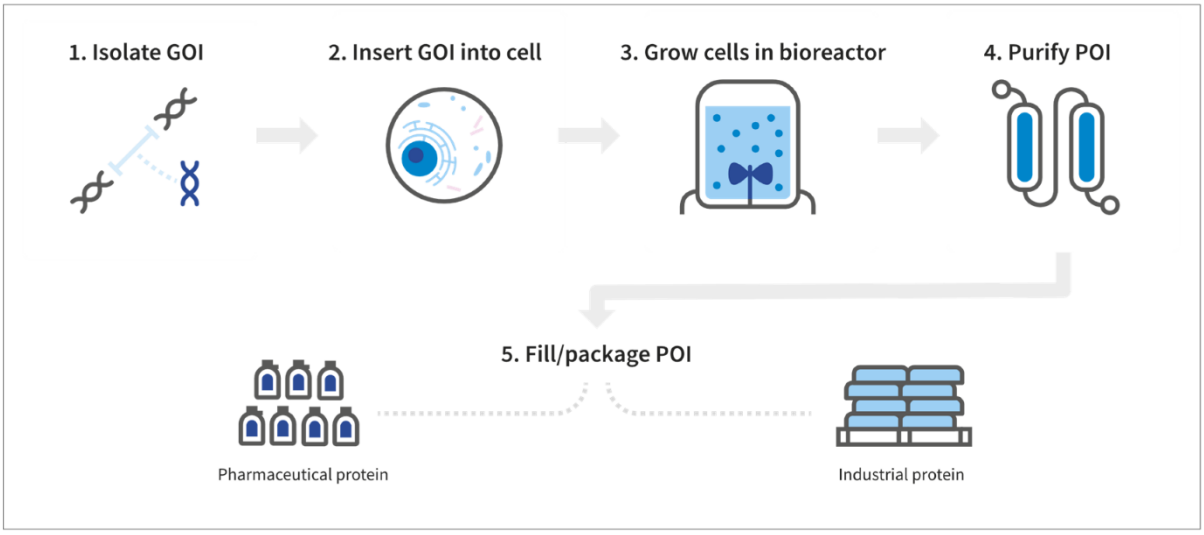

Figure 1. *Simplified process scheme of rFVIII from GOIs (FVIII) to POIs (rFVIII) [10]*

A. UPSTREAM PROCESS FOR GOIs (ANTI-HEMOPHILIC FACTOR PROTEIN) ISOLATION

GOI is an anti-hemophilic factor protein, and it is tested using a fluorescence-activated cell sorting method to detect the anti-hemophilic factor protein in peripheral blood mononuclear cells (PBMCs). The FVIII protein's schematic diagram is displayed in Figure 2 [9]. Factor VIII (FVIII) is an essential blood-clotting protein, also known as anti-hemophilic factor (AHF). In humans, factor VIII is encoded by the *F8* gene. Defects in this gene result in hemophilia A, an X-linked coagulation disorder. Factor VIII are produced in liver sinusoidal cells and endothelial cells outside the liver throughout the body [27]. ELISA (Enzyme-Linked Immunosorbent Assay) and Western blotting are frequently employed and are more appropriate methods to identify protein expression *in-vivo*. The simultaneous analysis of the levels of FVIII protein production by various kinds of cells is not possible with these approaches, though. For this reason, the GOIs' isolation procedure can make use of the flow cytometry method. Using the fluorescence-activated cell sorting method, the protoplasmic content of FVIII in patient cell and tissue samples as well as heterological gene processing systems may be determined and compared [6]. An ICS (indirect intracellular staining) technique can be used to investigate the internal and temporary processing levels of the cytoplasmic FVIII protein in the CHO eukaryotic cell population. This ICS method can also be utilized to find the internal processing level of the cytoplasmic FVIII protein in humanoid PBMCs.

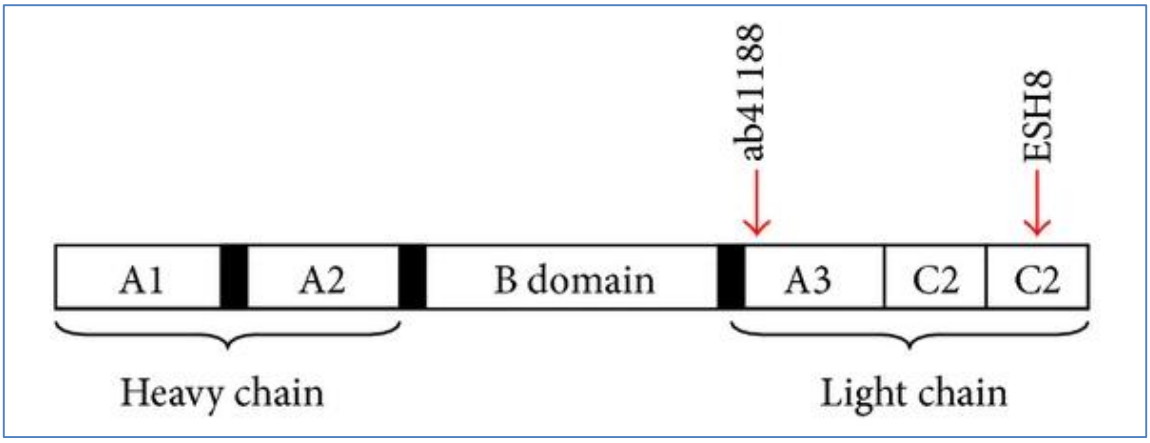

Figure 2. *FVIII protein flowchart illustrating the binding antigenic determinant of ESH8 and ab41188, two anti-FVIII laboratory-produced molecules. While ESH8 binds to the C2 region of the FVIII protein, the laboratory-produced molecule ab41188 binds to the N terminus of the A3 region [9]*

A.1. PMBC Preparation Process

The following steps are a part of the PMBC preparation process flow diagram shown in Figure 3 [9]. Heparinized venous blood is gathered by the National Institutes of Health Blood Bank from healthy, unrelated registered blood donors [19]. Centrifugal force is used to separate mononuclear cells on a lymph-cell isolation reagent. Following a 1 in 1 blood dilution with phosphate-buffered saline (PBS) at pH level of 7.4, 5 milliliters of lymph-cell isolation reagent can be added to the 50 milliliter BD Falcon tube. After centrifuging at 250 g for 30 minutes to separate the platelets, the white blood cells and platelets comprising mononuclear cells are collected in a 50-milliliter tube and rinsed twice with PBS and bovine serum albumin. Prior to flow cytometer measurement, cells are suspended in 200 liters of 1 percent paraformaldehyde in PBS. After centrifugation, intracellular staining is carried out using principal mice anti-humanoid FVIII unlabeled laboratory-produced molecules ab41188 by Abcam

Incorporated [20] and ESH8 by American Diagnostica Incorporated. Laboratory-produced molecule ab41188 connects the N terminus of the A3 region of the FVIII protein as ESH8 connects to a C2 region in the protein. After trypan blue staining, the viable cell count is calculated using a Cellometer cell counter.

A.2. Plasmid Vector Preparation

The following steps comprise the plasmid vector preparation [9]: To produce the FVIII protein, a plasmid vector with a framework derived from the pcDNA3 cellular inclusion containing an ampicillin-resistant gene was developed. The complementary DNA with polyA tail (cDNA-polyA) cassette is contained in the plasmid vector and is controlled by a potent CMV (cytomegalovirus) activator and a favorable neomycin (Neo) antibiotic resistance gene. One can use the HOW1-WT plasmid, which has cDNA, for the H3 haplotype of the human FVIII (Factor VIII) long isoform [12]. The plasmid vector is created by genOway in France [21] and purified using CsCl (caesium chloride) differential centrifuge by Loftstrand Labs Ltd in Maryland, USA [22].

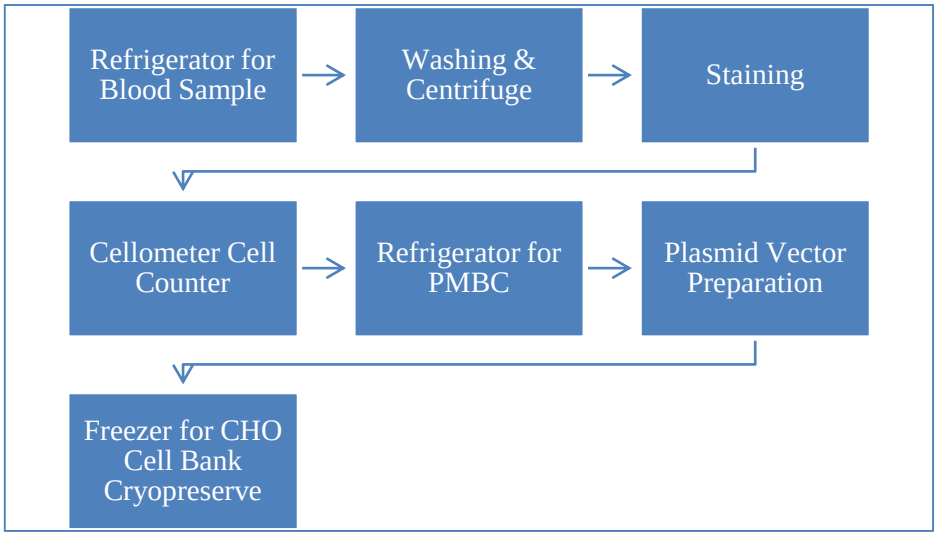

Figure 3. *GOIs isolation process steps*

B. UPSTREAM PROCESS FOR INSERTING GOIs INTO CELL

The steps involved in inserting GOIs into a cell include preparing the cell culture, transfecting the prepared culture, incubating the culture, and harvesting the culture. After harvesting, Flow Cytometry analysis is used to examine intracellular FVIII expression.

B.1. Cell Culture and Transfection

Factor VIII gene constructs are transferred with a GeneJet DNA *in-vitro* immunoblotting solution produced by SignaGen Laboratories in MD, USA [24] in a 3-in-1 compound proportion. Following six hours of contact time, full DMEM cultivation media with ten percent FBS is substituted for the immunoblotting solution including media. CHO cells are collected after 16 hours of transfection time and a fluorescence-activated cell sorting method is used to identify the cytoplasmic FVIII expression. CHO cell banks are cryopreserved (cryopreservation is the process of cooling and storing cells) in the freezer at -80°C until the production-scale process.

B.2. Flow Cytometer Analysis

A procedure is used to cultivate CHO cells in Dulbecco's Modified Eagle Medium (DMEM) with ten percent FMS (Fetal Bovine Serum) and five percent CO_2 at 37°C. Using 0.025 percent trypsin EDTA (Ethylenediaminetetraacetic acid), cells in the exponential development phase are separated from a tissue culture dish. A 25 cm^2 flask is inoculated with 10^6 of these cells and 5-milliliter DMEM with ten percent FBS manufactured by Invitrogen-Thermo Fisher Scientific [23]. Pandey *et al* (2013) indicate that the fresh medium cell growth is reloaded after 24 hours of incubation with 60-80% confluence for an additional 1 hour before transfection [9]. After CHO cells have been transfected, transfection effectiveness can be measured by discoloration with a laboratory-produced molecule to the FVIII protein. After being extracted, microorganism cells are twice cleaned in a washing buffer. Utilizing IntraPrep permeabilization reagents, microorganism cells can be fixed and made permeable before being incubated for an hour at room temperature with 1 in 100 dilutions of laboratory-produced molecules in 0.2 percent Bovine Serum Albumin in phosphate-buffered saline. Laboratory-produced goat anti-mice secondary molecule coupled with Alexa Flour 488 (a powerful green-fluorescent pigment with illumination that works well with a 488-nanometer laser wavelength) can be used to identify the expression of the FVIII protein. Following these steps, the flow cytometry data can be examined and analyzed using FlowJo, a program developed by Tree Star, OR, USA [25]. The intensity of the cells located inside a predetermined gate can be used to define the fluorescence signal average and middle values. To assess the appropriate gating for the living cells, cells that have not been dyed and rendered permeable are displayed on forward scatter cells versus side scatter cells dot plot. Two optical detectors can be used in flow cytometry to measure the light scattered by cells: the side scatter cell measures scatter at a 90° angle from the laser while the forward scatter cell detects scatter along the laser's path. While the side scatter cell refers to the complexity or granularity of the cell, the forward scatter cell refers to the size of the cell. Then, for all samples in the same experiment, the same gating parameters should be used.

C. UPSTREAM PROCESS FOR GROWING CELLS IN A BIOREACTOR WITH RESPECT TO FLUID FLOW, MIXING, HEAT, AND MASS TRANSFER PHENOMENA

Utilizing the pricey perfusion technique in stainless steel fermenters, recombinant FVIII for sale on the market can be created. CHO cells can also be used to make a complete recombinant human FVIII in recently developed batch-fermenting disposable single-use bioreactors [3], [4], [16], [26]. A single-use bioreactor (SUB) which has a 2000-liter volume, a 5:1 turndown ratio, adjustable stirred-tank mixing, and open-architecture control flexibility can be employed. It contains ports for monitoring sampling and liquid transfers as well as venting, sparging, and temperature sensor components. In contrast to conventional monitoring probes, it offers the advantages of 1) minimizing cleaning or sterilizing, 2) assisting in lowering the danger of cross-contamination, 3) allowing customization to the production volume, and 4) assisting in avoiding the costs associated with purchasing a new control system.

C.1. Preparation of Cell Culture Medium and Reagents for Cell Growth in a 2000 Liter SUB Bioreactor

The 2000-liter SUB can be utilized as a bioreactor [18]. The cell culture media preparation process in a 2000-liter SUB consists of the following: By hydrating 17.2 g/liter in a 1980-liter WFI (Water For Injection) water at about 90 percent of the ultimate volume (2200 liter), 34.056 kg of cell culture powder can be converted into enough CHO cell culture medium (ThermoFisher Catalog # 670006) to finish the

campaign (about 2200 liter). 4.456 kg of sodium bicarbonate is added at a concentration of 2.2 g/liter and stirred until dissolved after the cell culture medium has completely dissolved. Following the dissolution of 1.148 kg of l-glutamine at 0.58 g/liter, 1.98 kg of pluronic (a non-ionic surfactant) are added at 1.0 g/liter. After that, the solution is diluted to its full strength and blended for a further 30 minutes. Then, using 0.2-nanometer sterile filtration, the solution is transferred into gamma-irradiated, sterile Thermo Scientific BioProcess Containers (BPCs).

C.2. Cells, scale-up, and production scale process in a 2000 Liter SUB

During scale-up, CHO cells are defrosted from a cryopreserved cell bank and passaged on a schedule of three to four days, with seeding densities of roughly $2.5x10^5$ viable cells per milliliter. Only batch culture, with no feeding, is used for cell culture. The approximate culture volume (2000 liter) and vessel volume (2000 liter) make up the seed train volume (2x2000 liter). The dissolved oxygen (dO_2), pH, temperature, and agitation set points in bioreactors are monitored and maintained by bioreactor controllers. Temperature 37°C, pH 7.0, impeller speed 80 rpm, and a minimum dissolved oxygen level of 30% are the parameters required for CHO cultivation [11]. Cell population density, viability, and culture biochemistry are checked at least once each day. Although the non-fed batch cultures would generally be maintained for about 7-8 days [18], the 2000-liter bioreactors should be operated for a total of 21 days in order to evaluate sterility.

C.3. Downstream Processes for Purifying POIs (rFVIII)

To maximize the cleanliness and safety from pathogens of rFVIII, a purification procedure is required. This procedure entails 1) a cell elimination phase by centrifugal force separation and filtering, 2) 2 filtering operations, and 3) 5 chromatography purification column phases. The purification steps of the rFVIII are shown in Figure 4 [15].

- Step 1: Harvest (salt extraction and cell removal) by Bio20 STAX (Pall Corp.) disposable depth filter system
- Step 2: Capture (concentration and purification) by a multi-modal Cation Chromatography with Capto MMC resin
- Step 3: Purification by Cation Exchange Chromatography with SP Sepharose FF resin
- Step 4: DNA removal by charged filter with Sartobind Q Anion Exchange Membrane (Sartoris Stedim, Denmark)
- Step 5: Pathogen Safety # 1 (S/D-Chemicals removal)
- Step 6: Affinity Purification by VIIISelect Affinity Chromatography
- Step 7: Pathogen Safety # 2 by Planiva 20N Nanofilter with 20 nm pore size (N.V. Asahi Kasei Bioprocess, Belgium) [1, 7. 13, 14]
- Step 8: Concentration and Polishing by Anion Exchange Chromatography with Sepharose FF resin
- Step 9: Formulation and Polishing by Size Exclusion Chromatography with pre-packed Superdex 200 preparative-grade resin (GE Healthcare/Life Sciences)

C.3.1. Step # 1: Harvesting

FVIII can be detached from cells using a batch harvest method that involves adding sodium chloride to the cell solution until it reaches to 0.3 molar mass concentration level. Cells can be eliminated by centrifugation and filtering prior to the gathered sample being delivered straight to the extraction process without requiring dilution or additional modifications [15].

C.3.2. Step # 2: Capture Purification

In this step, 2000 kg of cell-free harvests can be fed to a 20-liter multifunctional cation exchange chromatography column for main substance purification and concentration after equilibrium with a 0.3 molar mass sodium chloride solution at a pH level of 7 with a rate of flow of 700 liters per hour. Total 50 times of the chromatography column volume are used in a stepwise process to wash the chromatography column one more time by decreasing the pH level to 6.5 while utilizing different sodium chloride concentrations. The final wash also contained a mixture of 10 percent ethylene glycol and 0.4 molar mass arginine. FVIII is extracted and gathered from the chromatography column by increasing the arginine concentration to 0.8 molar mass. The chromatography column is then regenerated with a nonionic detergent and high salt solution (0.5 molar mass sodium chloride and one percent octylphenol ethoxylate solution), low-level pH (1 molar mass acetic acid), high-level pH (1 molar mass sodium hydroxide), and eventually kept in a 0.05 molar mass sodium hydroxide solution prior to the start of the next production batch [15].

C.3.3. Step # 3: Cation Exchange Purification

This procedure can be carried out using a 20-liter chromatography column volume that has been pre-equilibrated with 0.1 molar mass sodium chloride, pH buffer solution at 6.5 level with a flow rate of 550 liters per hour. Before being added to the chromatography column, the effluent from Purification Step # 2 is reduced ten times to a conductivity equal to 0.1 molar mass sodium chloride at pH level of 6.5. By raising sodium chloride concentration to 0.15 molar mass, the column is further cleaned using a gradual increase with a total of 50 chromatography column volumes. FVIII is further extracted and gathered from the chromatography column by raising the sodium chloride concentration to 0.3 molar mass. The column is then cleaned using a non-ionic detergent and high-salt solution (0.5 molar mass sodium chloride and one percent octylphenol ethoxylate solution), low pH level (0.1 molar mass acetic acid), high pH level (1 molar mass sodium chloride), and finally stored in a weak sodium hydroxide solution at 0.05 molar mass prior to the start of the next production batch [15].

C.3.4. Step # 4: DNA Removal

FVIII is purified from a plasma fraction using anion exchange chromatography. At the highest pressure of 300 kPa, 30–40 liters of the portion are passed through a 2.5 m^2 anion exchange membrane that has been stabilized with the same solution employed to elute FVIII in Step #3. In the portion that flows through, FVIII is retrieved [15].

C.3.5. Step # 5: Pathogen Inactivation (Pathogen Safety # 1)

Two to five batches (100-250 kg) from Step #4 are combined before being mixed with 0.3 percent Tri(n-butyl) phosphate and 1 percent octylphenol ethoxylate solution at 22°C for 30 to 60 minutes [15].

C.3.6. Step # 6: Affinity Purification

At a rate of flow of 220 liters per hour, the antibody-stimulated mixture from Purification Step #5 is introduced to a 15-liter chromatography column that has been pre-equilibrated with a 0.3 molar mass sodium chloride, pH buffer level at 6.5. The sodium chloride concentration is gradually increased from 0.3 molar mass to 1 molar mass while washing the column. The total amount of wash buffer is 25 column volumes. FVIII is eluted from the column with 50% ethylene glycol. The column is then restored using

a low pH solution mixture (0.15 molar mass phosphoric acid and 0.17 molar mass acetic acid and 2.2 percent (vol/vol) benzyl alcohol) and a high-salt solution of 2 molar mass sodium chloride. Lastly, the column is kept in a 20 percent ethanol solution until the beginning of the subsequent production batch [15].

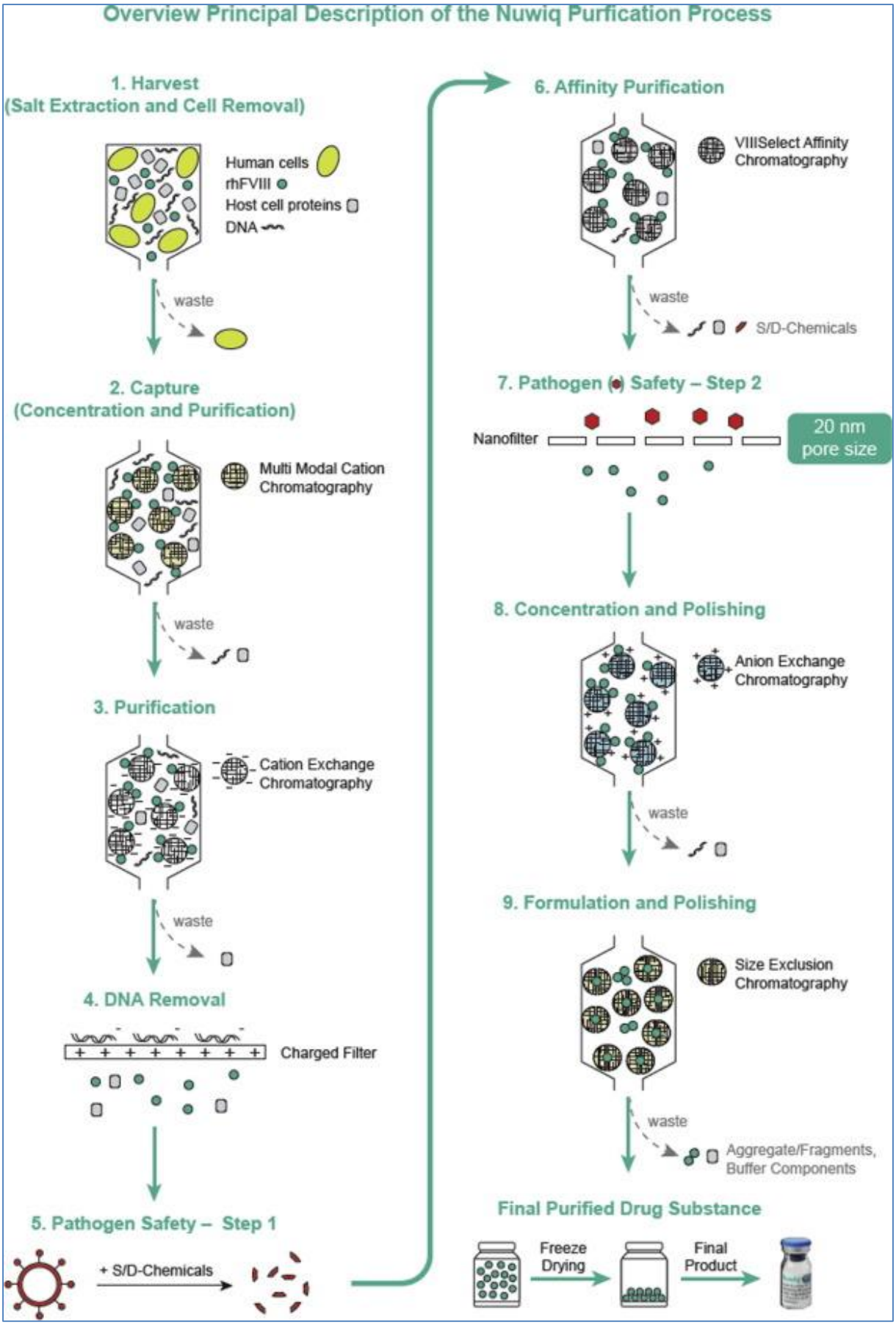

Figure 4. *Overview of rFVIII Purification Process Steps [15]*

C.3.7. Step # 7: Nanofiltration (Pathogen Safety # 2)

30-50 liter of FVIII encompassing portion from purification Step # 6 is watered-down 10-fold resulting in a sodium chloride concentration of 0.1 molar mass at pH level of 7.5. The mixture is nano-filtered through a 10 m^2 membrane filter surface area that has been balanced with a buffer solution containing 0.1 molar mass sodium chloride and a pH level of 7.5 at a pressure between 60 and 80 kPa [15]. Within the flow-through fraction, FVIII is retrieved [1], [7], [13], [14].

C.3.8. Step # 8: Anion Exchange Purification

The purification step # 7 filtrate is applied to a 5-liter column volume in this step, which was earlier balanced with 0.1 molar mass sodium chloride, pH buffer level of 7.5 at a 130 liter per hour flowrate. By raising sodium chloride content from 0.1 to 0.3 molar mass, altogether 15 chromatography column volumes are used to wash the column once more. FVIII is subsequently eluted and extracted from the chromatography column by increasing the sodium chloride concentration to 0.4 molar mass. After being renewed with a high-salt solution at 2 molar mass sodium chloride and high pH solution level at 1 molar mass sodium hydroxide, the chromatography column is then placed in a weak 0.01 molar mass sodium hydroxide solution before the start of the following manufacturing batch [15].

C.3.9. Step # 9: Size Exclusion Purification

The monomeric FVIII fraction is purified from the FVIII mixture coming from Step # 8 using size-exclusion chromatography. A total 50-liter chromatography column volume that had been pre-equilibrated with a solution mixture consisting of a modulated natural sugar, amino acid, and non-ionic cleansing agent at a pH level of 7 with a rate of flow at 650 milliliters per minute is used to process 2-4 liters of the solution. The monomer FVIII portion is extracted proportional to the holding time as assessed by absorption at 280-nanometer wavelengths. The column is then replenished with a high pH level mixture at 1 molar mass sodium hydroxide and ultimately kept in a weak sodium hydroxide mixture at 0.01 molar mass before the commencement of the subsequent production batch [15].

III. CONCLUSION

Through the use of single-use bioreactors, the efficacy of synthesized rFVIII proteins has been enhanced considerably over the course of the last three decades compared to the earlier synthesis of FVIII generated from plasma. A special mammalian cell line, cutting-edge purification techniques, and viral filtration steps are all used in the current scalable and transferable rFVIII manufacturing processes to manufacture reliably a unique, completely active, and extremely pure product that is free from infectious agents or impurities. The main advantages of SUBs in protein manufacturing are the elimination of cleaning, cleaning validation, and cross-contamination risk, as well as lower capital investment and speed to market. However, one of the disadvantages of employing SUBs is the buildup of accumulated plastic waste and its disposal as an environmental impact. Another disadvantage is the problem of the use of pressure devices which plastic components of SUBs may not be able to handle. High running costs and risk of leachables with possible cell growth impairment are other limitations/drawbacks and disadvantages. The full balance between the advantages and disadvantages of these more recently studied single-use bioreactors for the synthesis of FVIII may require further analysis for specific examples of its application.

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