Virus purification using superheated buffer in place (SBIP) for packed bed chromatography

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Chromatography systems

Virus purification using superheated buffer in place (SBIP) for packed bed chromatography

Superheated buffer in place (SBIP) is a method for sterilizing packed chromatography beds used to process viruses and biomolecules that are too large for sterile filtration. Superheating occurs when pressurized liquids are heated above their normal boiling temperature; water-based buffer systems can be heated to well above 121°C, the temperature normally regarded as the standard to achieve sterilization. Sterilization by SBIP is achieved using a flow of superheated buffer over the chromatography medium (resin) in the packed bed and all the wetted parts of the system before introducing the sample. We have verified that a range of gel filtration (GF; also known as size exclusion) and ion exchange (IEX) chromatography media maintain performance and functionality after sterilization using SBIP. Process-scale testing with a custom-designed BioProcess™ system and a 56-liter BPSS 600 stainless steel column packed with Sepharose™ 4 Fast Flow demonstrates the utility of SBIP at process scale. SBIP allows repeated use of the medium in the packed bed while maintaining chromatographic performance, without the need to repack the column after cleaning in place (CIP).

Introduction

Vaccine production

Recent developments in the field of viral-based vaccine and gene therapy products have contributed significantly to a renewed focus on vaccines. Concurrently, regulatory authorities are imposing more stringent standards of safety and efficacy, similar to the requirements for protein-based drugs and other biopharmaceuticals. Regardless of origin from egg, bacteria, or cells, any purification process must deliver a sterile final product, defined by the absence of bacteria and fungi or their spores. The US Food and Drug Administration (FDA) Guidance for Industry recommends that vaccine manufacturers monitor sterility or bioburden and take all possible precautions to prevent contamination during purification. The European Medicines Agency (EMEA) recommends sterility testing after downstream processing of harvested viral vaccines (1). There is a need for validated sterilization processes appropriate for both investigational and licensed products (2). Due to the challenges of bringing safe biopharmaceuticals quickly and in a cost-efficient manner to market, vaccine manufacturers are also placing high demands on their production processes. As more vaccine candidate products move into late-stage clinical development during which scalable robust methods are critical for successful production, the need for better production and purification processes becomes clear.

Virus vaccine purification strategies

Virus purification processes usually include different combinations of microfiltration, ultrafiltration, and chromatographic separation. Common chromatography methods include IEX chromatography in nonbinding mode to remove contaminants and/or GF in group separation mode. Affinity chromatography may also be used. Any virus vaccine purification process will be limited by the fact that viruses are significantly larger than other common biomolecules such as proteins and peptides. Therefore, the purification methods that are regularly used in production of smaller biopharmaceuticals are not applicable to virus purification beyond their use in separating viruses from smaller

molecules present in the starting material. A final sterile filtration step is one the most frequent methods to achieve a sterile end product. However, the use of 0.2 µm filters for sterile filtration effectively excludes many viruses because they are too large to pass through the membrane; poor recoveries result. Furthermore, many viruses are sensitive to the high shear stress that can occur during sterile filtration and may be damaged or destroyed. Because sterile filtration is not an attractive option for virus sterilization, other methods should be considered.

Sterilization of a packed chromatography bed by superheated buffer in place

An alternative option to 0.2 µm filtration is the use of sterilization by superheated buffer in place (SBIP) in a fully closed chromatography system. Superheated water or buffer refers to an aqueous solution that is maintained in the liquid state above its normal boiling point by use of high pressure (Fig 1A). The absence of steam is a critical factor during superheating because steam formation or boiling would damage the packed bed. The use of SBIP allows an entire chromatography system, including the packed bed of the medium and all the surfaces of the internal flow paths, to be sterilized by pumping a superheated buffer solution at a temperature above 121°C throughout all wetted parts of the system before sample introduction. Furthermore, this process can allow repeated use of the medium in the packed bed without the need to repack the column after cleaning in place (CIP; 3) and SBIP.

The sterilization procedure for a closed chromatography system using SBIP consists of a sequence of three steps (Fig 1B): a heating phase: a continuous steady-state phase at a temperature above 121°C for a sufficient time to ensure sterilization; and a cooling phase. The whole cycle is performed with a constant flow of buffer over the packed bed and all wetted parts that will be in contact with the material to be processed.

Criteria for acceptance of SBIP technology

The SBIP technology requires that both the medium and the system hardware can withstand prolonged heating under high pressure at temperatures above 121°C. These criteria require extensive testing of the stability, function, and safety of the medium exposed to SBIP compared to unexposed medium. In addition, the critical system components must be maintained in good functioning order. SBIP must clearly satisfy the requirements of the regulatory authorities as well as the expected performance demands of preserving vaccine function and obtaining high virus recovery. It becomes clear that in the absence of sterile filtration, all steps in the purification process need to be rendered sterile while maintaining functionality and performance of the packed bed during the subsequent separation process, and that this sterilization must occur before the sample is loaded. General performance requirements for a medium in a packed bed are that the set specifications, height equivalent of a theoretical plate (HETP) of > 3000 theoretical plates/m and a peak asymmetry between 0.8 and 1.8 after repeated sterilization cycles, are met.

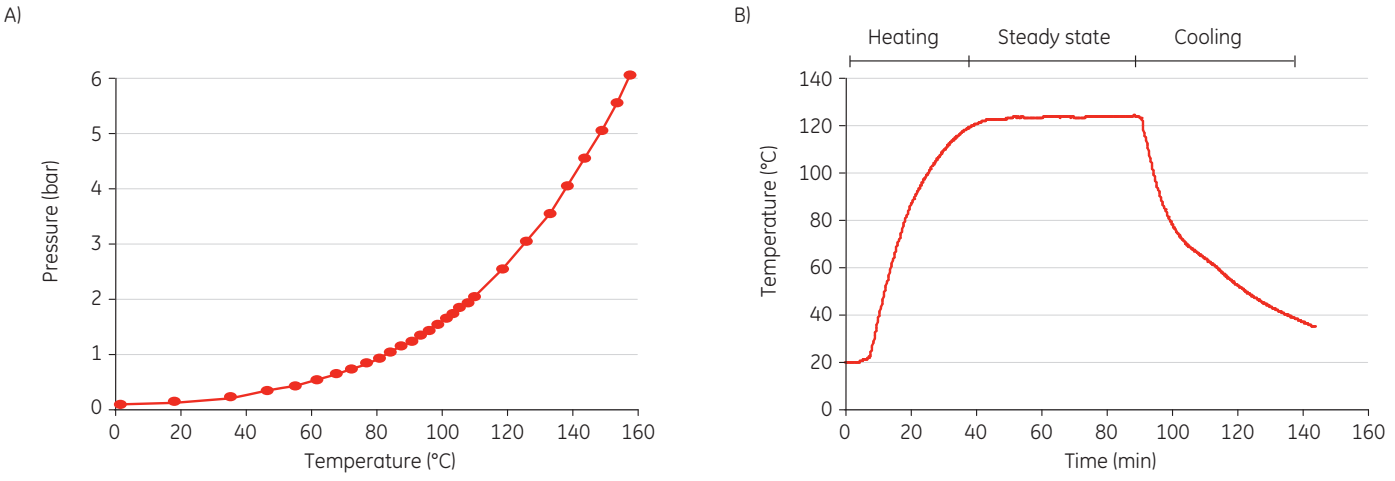

Fig 1. (A) Graph showing the required pressure to keep water liquid at a specific temperature. (B) The sequence of steps in a SBIP run: the heating phase; the steady-state phase; and the cooling phase.

Materials and methods

Initial testing and evaluation of media

The effect of column sterilization by repeated heating with superheated buffer was evaluated with the following IEX and GF media: Sepharose 4 Fast Flow, ANX Sepharose 4 Fast Flow (high sub), Capto™ Q; Capto S; SOURCE™ 30S; Sephacryl™ 400 HR; Sepharose Big Beads; and Sepharose SP XL. Testing was performed using an Accelerated Solvent Extractor (ASE™) from Dionex. The ASE is designed to extract samples at high temperature and pressure. Therefore, the temperature and pressure of the ASE runs is higher than required for SBIP. Use of an ASE enabled proof-of-principle for SBIP before proceeding to process scale. The extraction cells containing the media were sterilized with static fluid extraction for 12 min at 34.4 bar and 150°C. This procedure was repeated 25 times. Sterilization of ANX Sepharose 4 Fast Flow (high sub) and Sepharose SP XL requires the presence of a counter ion in the surrounding liquid to prevent hydrolysis of the medium; therefore, 0.1 M Tris-HCl, pH 7.5 and 0.2 M sodium acetate, respectively was used as the liquid during testing. Testing of all other media was performed with 0.1 M NaCl.

Following ASE treatment each medium was analyzed and compared to untreated medium with respect to particle size distribution, pore size distribution, and/or pressure flow properties using Coulter counter technology, pulsed gradient spin echo NMR, and by establishing pressure curves at different linear flow velocities, respectively. For ANX Sepharose 4 Fast Flow (high sub), the remaining ion exchange capacity was determined by titration with 0.0999 M $AgNO_3$ to the equivalence point after removing excess Cl^-. The performance of the medium was investigated by measuring the number of theoretical plates and the asymmetry after packing in 4.6/50 PEEK columns. HETP was determined using 10 µL of 2 M NaCl at 0.2 mL/min.

The functionality of untreated versus ASE-treated ANX Sepharose 4 Fast Flow (high sub) was evaluated by comparing the separation pattern for a protein mixture containing three proteins at a flow rate of 2 mL/min (2 bar) for 15 min. Briefly, the columns were calibrated in 20 mM piperazine, pH 6.2 at 0.1 mL/min, and a 100 µL sample containing GammaBind™ G type 2 (4 mg/mL), β-lactoglobulin A (11 mg/mL), and β-lactoglobulin B (11 mg/mL) in 20 mM piperazine, pH 6.2, was loaded on the column. Elution was performed using a linear gradient up to 0.3 M NaCl, 20 mM piperazine, pH 6.2, over twenty-one column volumes.

Evaluation of process-scale system functionality

The SBIP technology requires that both the medium and system hardware can withstand the conditions required to maintain a flow of superheated liquid above 121°C. A custom-built SBIP BioProcess system (Fig 2) was tested for the ability to maintain a flow of superheated water over all wetted parts of the system, including the packed bed, for the required amount of time to satisfy the requirements for sterility.

The system consists of a custom-made BioProcess system connected to a BPSS 600 column in which the buffer reservoir is heated by steam (Fig 3). To minimize heat losses over the column, additional heating of the column jacket column is performed using steam. A constant flow of buffer is applied throughout the system during the entire sterilization sequence. The sterilization efficiency and the performance of the packed bed were evaluated using Sepharose 4 Fast Flow as a model medium. Each SBIP sterilization cycle was performed with a constant flow of superheated 10 mM Tris (~pH 9.5–10.0) over the entire system, including the packed bed and all wetted parts. Each cycle consisted of: a heating phase for approximately

Fig 2. A schematic diagram of the SBIP BioProcess system, displaying the boiler reservoir (yellow), which is connected to the steel column (light blue-gray) outside the main frame of the system.

Fig 3. A schematic diagram of the flow system connecting the boiler reservoir (yellow) to the steel column (light blue-gray).

40 min until the set temperature (121°C) was reached; followed by a continuous steady state of at least 30 to 40 min above 121°C to ensure sufficient time for sterilization; and finally a cooling phase until room temperature was reached. The pressure was approximately 3 bar. The temperature was monitored at possible cold spots during each cycle to ensure that sufficient temperature was achieved throughout the system. A total of nine sequential SBIP cycles were performed. Cycle number 8 was an extended run with approximately 3 h at steady state (121°C) to verify the system temperature. The performance with regard to HETP and asymmetry of the packed bed was evaluated after 1, 4, and 8 SBIP cycles by injection of 1% acetone with water as buffer.

Results and discussion

Initial testing and evaluation of media

Table 1 shows the size distribution of Sepharose 4 Fast Flow and ANX Sepharose 4 Fast Flow (high sub) before and after ASE treatment. No significant influence on the size distribution was detected for either medium. In addition, the ion exchange capacity of ANX Sepharose 4 Fast Flow (high sub) before and after treatment was unchanged (Table 2).

Pressure flow curves for a range of IEX and GF media from GE Healthcare also showed no difference compared to the corresponding untreated medium (Fig 4).

Table 1. Particle size distribution before and after ASE* treatment

	Particle size distribution			
Medium	**D_{50} (µm)†**	**< 45 µm (%)**	**45–165 µm (%)**	**> 165 µm (%)**
Sepharose 4 Fast Flow pre-ASE	96.90	0.21	99.32	0.47
Sepharose 4 Fast Flow post-ASE	96.93	0.22	99.29	0.49
ANX Sepharose 4 Fast Flow (high sub) pre-ASE	100.35	0.38	99.10	0.52
ANX Sepharose 4 Fast Flow (high sub) post-ASE	108.60	0.24	98.26	1.50

* ASE = Accelerated Solvent Extractor

† D_{50} is the amount (in %) of particles with the average particle diameter

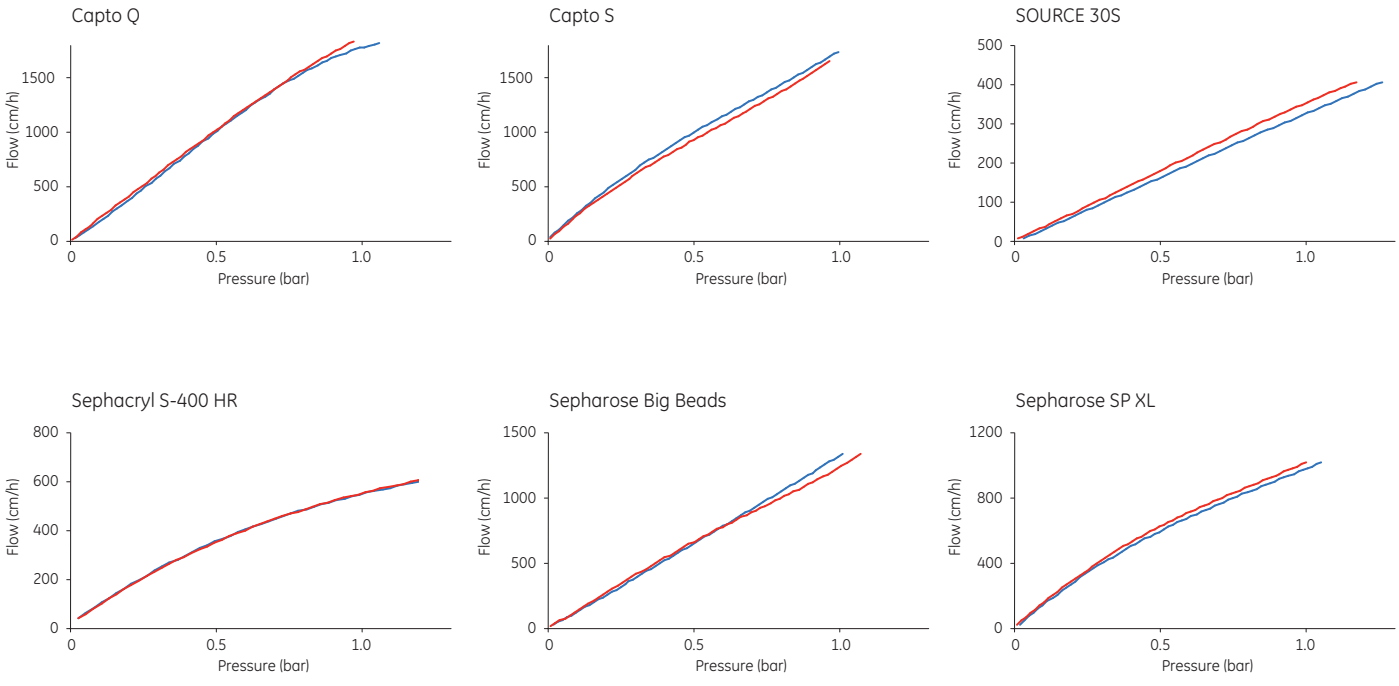

Fig 4. Pressure flow curves performed with untreated (blue) and ASE-treated media (red; 25 repeated cycles) for 12 min at 150°C and 34.4 bar) for Capto Q, Source 30S, Sephacryl 400 HR, Sepharose Big Beads, and Sepharose SP XL.

Table 2. Ion exchange capacity of ANX Sepharose 4 Fast Flow (high sub) before and after ASE treatment

Sample	Medium volume (mL)*	0.1 M $AgNO_3$†	Volume $AgNO_3$ (mL)	Capacity (mmol/mL medium)
Treated	3.735	0.0999	6.2157	0.1663
Untreated	3.735	0.0999	6.2005	0.1658

* Medium volume, dried by vacuum suction
† Concentration of $AgNO_3$ solution used for titration

Pore diffusivity was measured using pulsed gradient spin echo NMR for Capto Q, SOURCE 30S, Sephacryl S-400 HR, Sepharose Big Beads, Sepharose SP XL, Sepharose 4 Fast Flow, and ANX Sepharose 4 Fast Flow (high sub).

No significant difference in diffusivity was observed between the untreated medium and the medium sterilized using ASE (Fig 5).

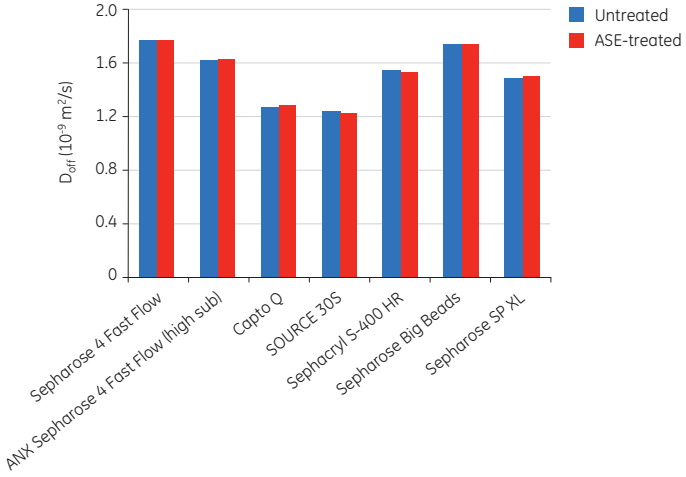

Fig 5. Results from measurement of porosity differences between ASE-treated media and untreated media with pulsed gradient spin echo NMR.

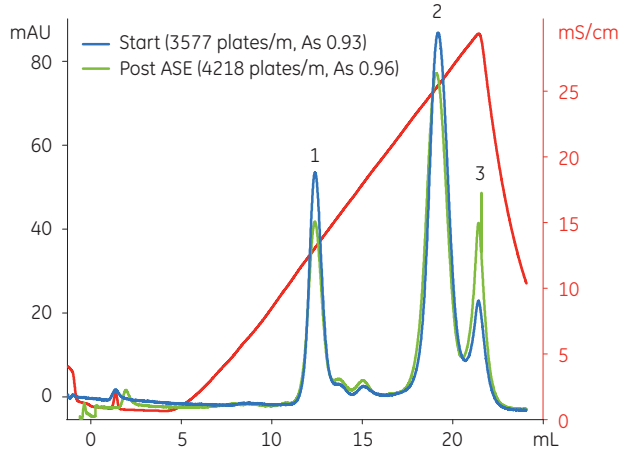

Fig 6. Elution profile of ASE-treated (green) and untreated (blue) ANX Sepharose 4 Fast Flow (high sub) loaded with a protein mixture containing β-lactoglobulin A (peak 1), Gammabind G type 2 (peak 2), and β-lactoglobulin B (peak 3). The red line measures conductivity. As = asymmetry.

Functional testing of ASE-treated ANX Sepharose 4 Fast Flow (high sub) (packed in the column after ASE treatment) was performed by determining the chromatographic elution profile for a mixture of three proteins (Fig 6). Untreated ANX Sepharose 4 Fast Flow (high sub) was used as a reference. Retention times of the three different proteins were identical between the treated and untreated media, indicating identical functional behavior. In addition, HETP and asymmetry were measured before and after ASE treatment of ANX Sepharose 4 Fast Flow (high sub). HETP was 3577 and 4218 plates/m before and after ASE treatment, respectively; asymmetry was 0.93 and 0.96, respectively.

The results of testing a range of IEX and GF media before and after ASE treatment suggest that sterilization by SBIP does not affect performance of the tested media with regard to particle size, pore size, pressure flow curves, ion exchange capacity, and column function compared to the corresponding untreated media. However, ANX Sepharose 4 Fast Flow (high sub) and Sepharose SP XL both require the presence of a counter ion in the surrounding liquid to prevent autohydrolysis.

Evaluation of process-scale system functionality

The custom-designed BioProcess system was initially tested to ensure that each SBIP cycle was able to deliver the required temperature of above 121°C by circulating hot buffer through the column and throughout the liquid flow path of the chromatographic skid. Figure 7 shows a graph of the temperatures measured by internal temperature sensors throughout a SBIP cycle. Sensors were placed at various points along the system. These results demonstrate that the custom-designed BioProcess system can achieve temperatures in excess of 121°C throughout the system.

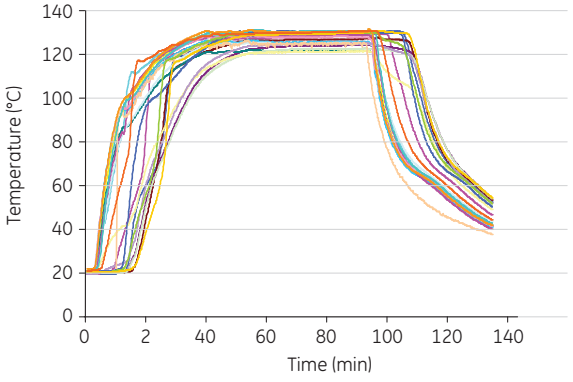

Fig 7. Temperature monitoring during a SBIP cycle showing temperature sensors (displayed with different colors) at critical points (potential cold spots) in the system.

Acetone was injected into the Sepharose 4 Fast Flow column (see *Materials and Methods*) to determine the influence of SBIP cycles on column performance. Chromatograms were obtained after each cycle, and HETP and asymmetry were determined (Fig 8). An overlay chromatogram (Fig 8A) from the evaluation of column performance after 1, 4, and 8 sequential SBIP cycles shows that the packed bed performance was not compromised after 8 SBIP cycles. In addition, HETP and asymmetry were relatively constant over 8 cycles (Fig 8B).

The ability of SBIP to sterilize the closed chromatography system was confirmed by the use of biological indicator (BI) spore pouches embedded in the packed bed of the column and throughout the system (Fig 9). After the SBIP cycle was completed, each BI spore pouch was incubated to monitor spore growth over several days. All 29 BI spore pouches in the column and system and the five negative controls were negative for growth, while all five positive controls were positive for growth.

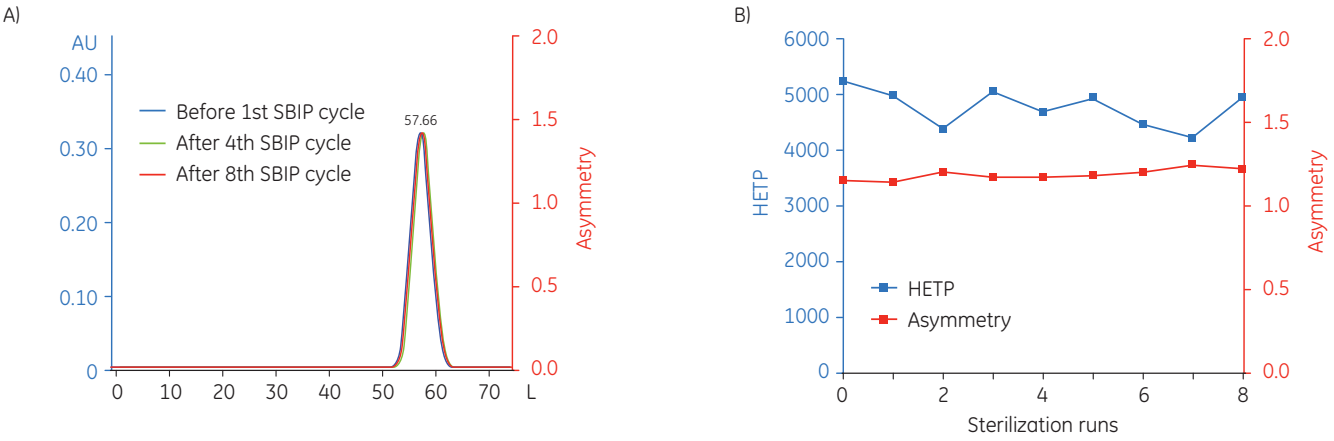

Fig 8. A) Overlay chromatogram from evaluation of column performance after the first (blue), fourth (green), and eighth (red) sequential SBIP cycle. AU = absorbance at 280 nm. B) Results of HETP (blue) and asymmetry (red) determinations after each cycle.

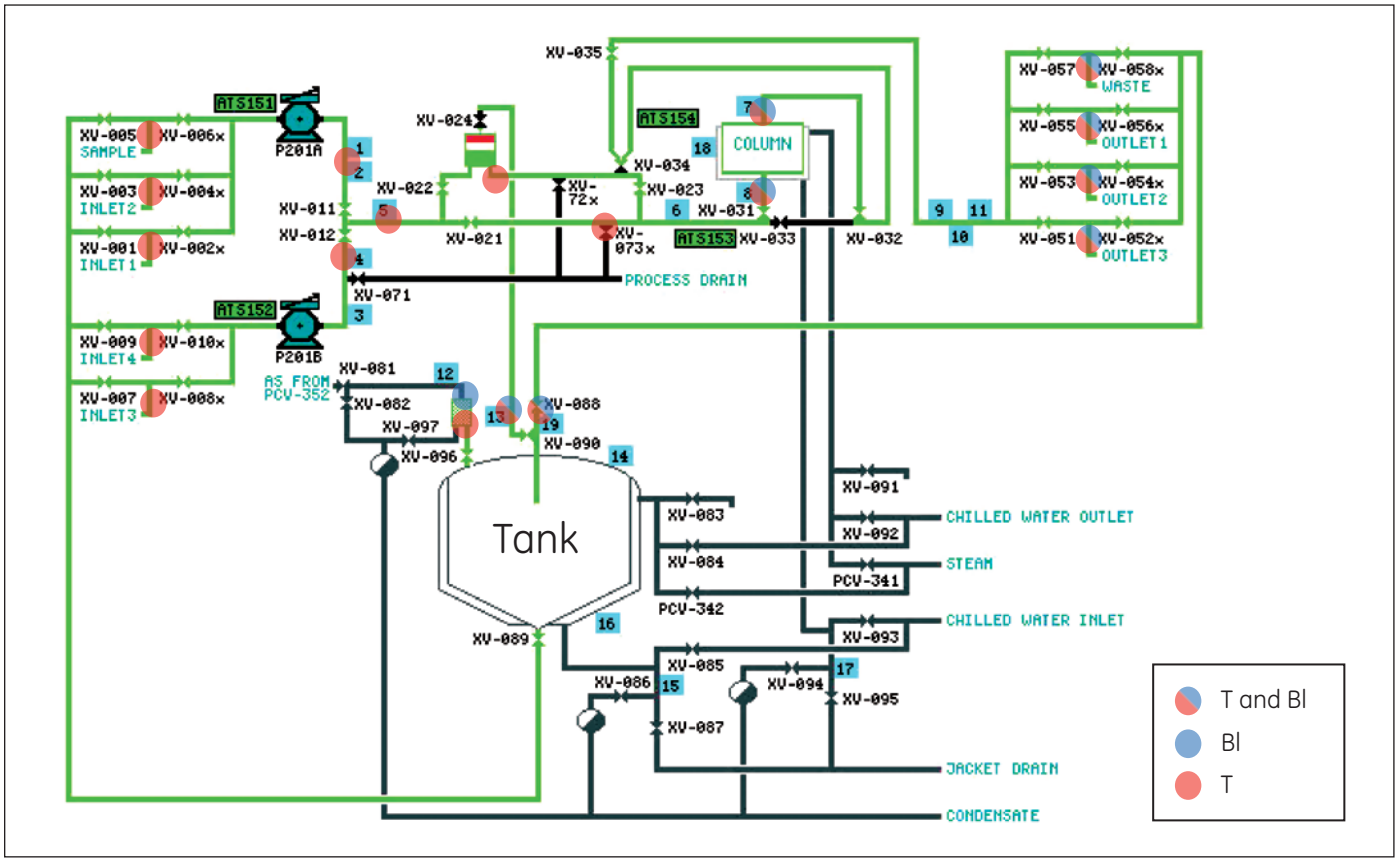

Fig 9. An overview of the system and its components with temperature sensors (T) and biological indicators (BI).

Conclusions

The data presented here show that sterilization with superheated buffer does not affect performance of a range of IEX and GF media and that the tested media are able to withstand 25 repeated ASE cycles at 150°C, 34.4 bar for 12 min. In addition, the results demonstrate that a process-scale SBIP system achieves sterility throughout the system. Also, performance of Sepharose 4 Fast Flow medium is maintained after 8 consecutive SBIP cycles. Therefore, the SBIP technology can be utilized for chromatography steps that require a sterilized final product, providing a viable alternative to final sterile filtration of the eluted product. This option is particularly attractive for the simultaneous purification and sterilization of viruses or other biomolecules that are too large or too labile for filter sterilization.

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