



Sanitization of ÄKTA™ pilot 600 using sodium hydroxide

The ÄKTA pilot 600 chromatography system is designed to facilitate sanitization, with the high standards imposed by regulatory authorities for purity of the clinical products in mind. In this study, the predefined sanitization method developed for ÄKTA pilot 600 was assessed. In a microbial challenge test, parts were exposed to a bacterial strain recommended by the United States Pharmacopoeia (USP 38) and to yeast strains commonly used in production environments. Sanitization of the system was performed by running the predefined method using 1 M sodium hydroxide (NaOH) as sanitization agent. The results show excellent cleaning efficiency, with a log reduction factor of ≥ 6 for colony forming units (CFU) of the test microorganisms. Sanitization of the SNAP-connectors using 1 M NaOH was also successfully conducted.

Introduction

Bacteria and yeasts are found in many laboratory and production environments. Growing rapidly to large quantities under favorable conditions, these microorganisms can remain as contaminant of the bioproduct throughout manufacturing, with batch failure and related costs as a consequence. Hence, it is important to follow hygienic routines throughout the whole production process. Sanitization is commonly used for chromatography systems to maintain microbial presence at levels that minimize the risk of contaminating the bioproduct. A few important terms that are related to system and production hygiene are explained in Table 1.

ÄKTA pilot 600 is a bench-top chromatography system intended for process development, scale-up, and scale-down applications as well as in small- and intermediate-scale production. The system is designed to facilitate sanitization and is well-suited for use in both GMP and non-GMP environments (Fig 1). The system cabinet and the frontside with all valves and sensors are easy accessed to simplify external sanitization. The stainless steel surfaces of the cabinet and the optional column stand can be wiped off and have minimal areas where dust and liquid can get trapped. In addition, the novel SNAP-connectors are easy to remove and omit the need for O-rings, which might otherwise present a sanitary weakness. For external cleaning, the air trap, rinsing solution module, and



Fig 1. The ÄKTA pilot 600 chromatography system is designed to simplify the sanitization procedure, supporting biomanufacturers in meeting the high purity standards imposed by regulatory authorities for clinical products.

the optional filter module are easily removed, and the pegs left on the system are protruding to facilitate cleaning, thereby preventing collection of dust or liquid. The wet side is designed to minimize system volume and dead-legs in the flow path. Materials are tested for extractables and leachables when in contact with solutions commonly used in the biomanufacturing industry. The predefined sanitization method for the flow path and the pump rinsing system requires minimal manual handling. The sanitization method is based on NaOH to allow for good process economy and bioburden control.

In this study, microbial challenge testing was performed to evaluate the efficiency of the predefined sanitizing method (1). The principle of microbial challenge testing is to introduce a high concentration of a predetermined microbial organism into the equipment, after which the challenged equipment is treated with a sanitization agent. After a specified time period, the number of surviving organisms are counted. Here, challenge testing of ÄKTA pilot 600 was performed in duplicate using strains of both *E. coli* and yeast. After sanitization using NaOH, purified water was pumped through the system and the eluate was collected for evaluation of the system sanitization. In addition, the system was dismantled and sampled for remaining microorganisms at predetermined sites. Microbial challenge testing of the SNAP-connectors was performed separately.

Table 1. Explanation of relevant and related terms

Antimicrobial agents	Agents that minimize or destroy microorganisms <i>in vitro</i> . The term antimicrobial is general and all inclusive. Antimicrobial agents include sanitizers, sterilizers, and disinfectants. However, sanitizers, sterilizers, and disinfectants are not necessarily the same and sanitization, sterilization, and disinfection are not interchangeable terms.
CIP	Cleaning in place, where cleaning performed without disassembly of the equipment.
Disinfection	The destruction of potential pathogens (while cleaning is the removal of all kinds of contaminants, proteins, lipids, other particles, and microorganisms).
Sanitization	The use of chemical agents or steam to reduce a microbial population to acceptable, predetermined levels.
Sterile	Free from all living microorganisms. Antiseptics prevent microbial growth. Aseptic prevent bacterial entrance.
Sterilization	The act or process, physical or chemical, which destroys or eliminates all forms of life, especially microorganisms.

Materials and methods

Preparation of the test strains

Organisms selected for microbial challenge testing are listed in Table 2. The organisms were stored on nutrient agar at 4°C. One day prior to each test, the organisms were inoculated in 200 mL of culture medium and grown at 32°C to 37°C for 18 to 24 h. As culture medium, tryptic soy broth (TSB) was used for the *E. coli* strain, whereas yeast extract peptone dextrose (YPD) medium was used for *P. pastoris* and malt extract agar for *S. cerevisiae*. For use in the microbial challenge tests, the cultures were suspended in sterile 50 mM NaCl solution and diluted to an approximate concentration of 10⁶–10⁷ viable organisms/mL.

Preparation and infection of the system

When using ÄKTA pilot 600 for the first time, it is important to remove the background microflora by circulating the system with 4 L of 1 M NaOH at a flow rate of 400 mL/min for 12 h, after which the system is flushed with sterile purified water (PW) to remove NaOH. The system is considered free from NaOH when the outlet pH drops below 7.5.

Table 2. Organisms used in the microbial challenge tests

<i>Escherichia coli</i>	Gram-negative bacterium (ATCC 8739)
<i>Pichia pastoris</i>	Yeast (GS 115)
<i>Saccharomyces cerevisiae</i>	Bakery yeast

For challenge testing, detachable parts of ÄKTA pilot 600 were soaked in either 1 M NaOH or 70% ethanol for more than 4 h before rinsing with sterile PW or autoclaved. After reassembly, the whole system was filled with 1 M NaOH and left for 18 to 24 h before flushing with 0.9% NaCl solution and PW until pH was neutral. The system was challenged with *E. coli* or *P. pastoris* suspension via the pumps A and B at a flow rate 360 mL/min. To simulate chromatographic conditions, the flow direction and valves were switched several times. The system was thereafter left for 18 to 24 h at room temperature. The pump rinsing system was challenged with the same organisms as for the wet side.

Sanitization of the system

The predefined sanitization method is outlined in Table 3, and a screenshot of the method in the UNICORN™ system control software is shown in Figure 2. To enable sanitization of the air trap, the sanitization procedure is to connect a tubing between the manual air trap valve and the IP port on the column valve, with the manual air trap valve fully opened allowing the method to automatically fill the air trap.

According to the procedure in the operating instruction (29261688), the pH sensor was removed and replaced with a dummy, and the pH monitor was washed manually with a syringe during the sanitization method. The pump rinsing system was sanitized simultaneously with the method in progress, with the same solution as for the wet side. The rinsing solution was replaced manually during progression of the method. Inlet tubing was switched manually between the solution containers. In contrary to recommended storage in 20% ethanol, sterile PW was used as storage solution in this study to enable detection of surviving challenging organisms.

Table 3. The predefined sanitization method

Function	Solution	Volume	Time
Rinsing	20% EtOH	6 L	10 min
Sanitization	1 M NaOH	6 L	10 min + 1 h
Flush	0.9% NaCl	6 L	10 min
Storage solution	20% EtOH*	6 L	10 min

* Recommended storage solution

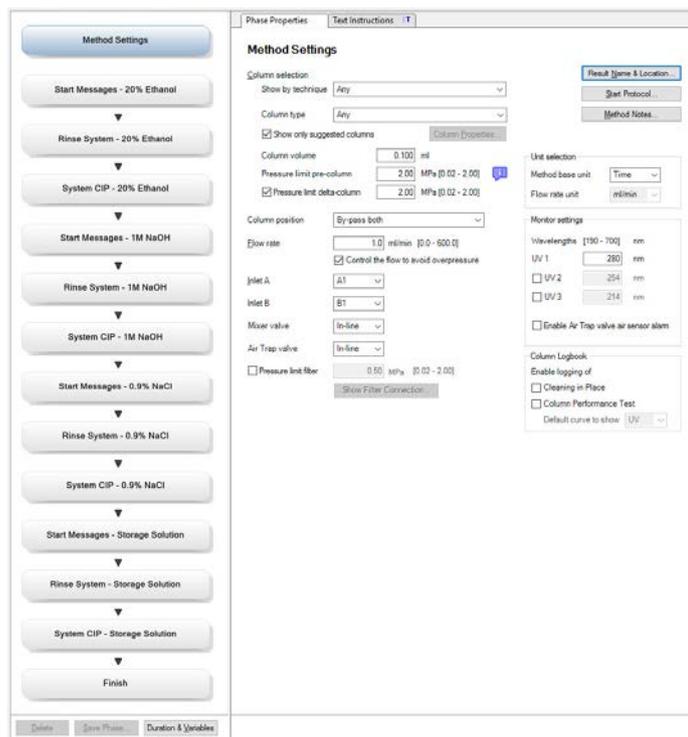


Fig 2. Screenshot of the sanitization method in the UNICORN method editor software.

Microbial testing

Microbial sampling was conducted at various time points at predetermined sites using one of the following methods:

Test method 1

PW was pumped through the system and samples (minimum 50 mL) were collected in sterile tubes, and then filtered through a 0.45 µm cellulose nitrate membrane filters. The filter was incubated on agar plates at 30°C–35°C for 5 days, after which the plates were inspected for CFUs.

Test method 2

Surface samples were taken with swabs. The swab was inserted into the tube containing the isotonic swab rinse solution. After dissolution, the whole solution was put into molten agar and solidified in petri dishes. The plates were incubated at 30°C–35°C for 5 days, after which the plates were inspected for CFUs.

Test method 3

Detachable system parts were aseptically removed and transferred to a sterile tube subsequently filled with 50 mL of sterile peptone water, and then vigorously shaken for at least 20 min. The solutions were filtered through a 0.45 µm cellulose nitrate membrane filter. The filter was incubated on agar plates at 30°C–35°C for 5 days, after which the plates were inspected for CFUs.

Test method 4

Samples of challenging organism suspensions were diluted in series in 0.9% NaCl. Samples from the diluted suspensions were plated on agar plates and incubated at 30°C–35°C for 1–2 days, after which the plates were inspected for CFUs. The concentration of challenging organism was determined in the sampled suspensions.

Infection and sanitization of the SNAP connectors

For the SNAP-connectors, three studies were performed: one with *S. cerevisiae* and two with *E. coli*. The challenging organism was applied at 30% of the maximum flow rate (360 mL/min). The low flow rate was applied to prevent damage of the challenging organism. Sanitization using 1 M NaOH was performed at 75% of the maximum flow rate (900 mL/min). Samples were taken with swabs and microbial testing was conducted according to Test method 2 (see Section Microbial testing).

Results

Sanitization of the system

It is common practice to define sanitization as a 6 log reduction in CFUs achieved after a sanitization procedure. In this study, CFUs of the challenging organisms were counted at four phases during the sanitization study: in the inoculation solution, immediately after infection, prior to sanitization, and after sanitization. Results from determination of the concentrations of the challenging organisms in sample solutions are summarized in Table 4. The inoculum or starting concentration of the challenging organisms was in the range of $9\text{--}14 \times 10^6$ CFU/mL. After leaving the system for 16 to 20 h at room temperature, the concentrations were measured

again and was found to be between $8\text{--}17 \times 10^6$ CFU/mL. At this point, the sanitization method was conducted. Following sanitization, post-sanitization effluent analyses show no trace of viable challenging organisms.

System sampling modules are shown in Figure 3. The results presented in Table 5 show that the sanitization method eliminated both *E. coli* and *P. pastoris* from the wet side of the system and from the pump rinsing system with a more than 6 log reduction. In these studies, the pump rinsing system was challenged with the same amount of challenging organisms as was the wet side, and a few CFUs of *P. pastoris* were detected in the pump rinsing system in the second study. To prevent growth in the rinsing system, the rinse solution should be exchange as recommended in the operating instructions.

Table 4. Challenging organism viable count

	<i>E. coli</i> , viable count (CFU/mL)		<i>P. pastoris</i> , viable count (CFU/mL)	
	Study 1	Study 2	Study 1	Study 2
Inoculum, start concentration	13.4×10^6	11.4×10^6	11.4×10^6	9.6×10^6
Post-infection, effluent (outlet 6)	12.8×10^6	10.1×10^6	12.2×10^6	7.8×10^6
Pre-sanitization (after 16-20 h in RT), effluent (outlet 6)	16.6×10^6	13.6×10^6	8.2×10^6	8.7×10^6
Post-sanitization, effluent (outlet 1 to 6)	0	0	1*	0

* Growth of another organism than the challenging organism in 1 out of 6 outlets.

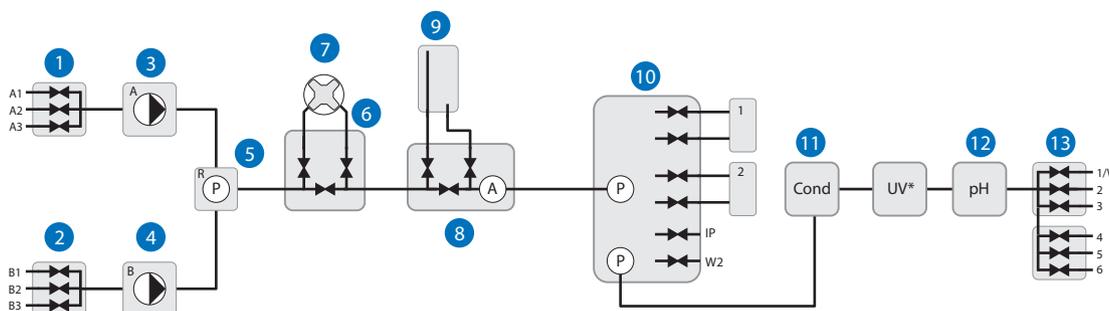


Fig 3. After sanitization using NaOH, purified water was pumped through the system and the eluate was collected for analysis. The system was also dismantled and sampled for remaining microorganisms at predetermined sites. All system modules are represented with one or more sampling points depending on the complexity of the module. *Due to its simple design, the component was tested as part of Test method 1.

Table 5. Number of CFUs remaining after sanitization with 1 M NaOH

Sampling site	No. of sampling points	<i>E. coli</i> , viable count (CFU/mL)		<i>P. pastoris</i> , viable count (CFU/mL)	
		Study 1	Study 2	Study 1	Study 2
1. Inlet A	1	0	0	0	0
2. Inlet B	1	0	0	0	0
3. Pump A	8	0	0	0	0
4. Pump rinsing system	8	0	1*	0	4 + 1†
5. Restrictor pressure sensor	18	0	0	0	0
6. Mixer valve	2	0	0	0	0
7. Mixer	2	0	0	0	0
8. Air-trap valve	10	0	0	0	0
9. Air-trap	10	0	0	0	0
10. Column valve	6	0	0	0	0
11. Conductivity monitor	1	0	0	0	0
12. pH module	4	0	0	0	0
13. Outlet	1	0	0	0	0

* Growth of another organism than the challenging organism in 1 out of 8 sampling points.

† Growth of challenging organism in 2 out of 8 sampling points.

Sanitization of SNAP-connectors

Sampling sites are shown in Figure 4, and the results are presented in Table 6. No challenging organisms were found on any of the sampling points after sanitization in any of the studies performed. The results indicate that the SNAP-connectors were effectively sanitized.

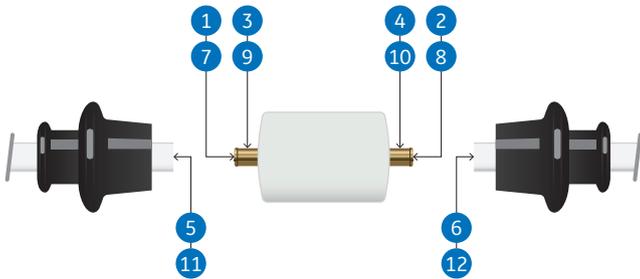


Fig 4. Sampling sites on the SNAP-connectors. For this study, two titanic pegs were inserted in a piece of peek-material.

Table 6. Number of CFUs remaining after sanitization with 1 M NaOH

Sampling site	Viable count (CFU/mL)	
	<i>E. coli</i>	<i>S. cerevisiae</i>
Small connector		
1. Inlet, inside	0	0
2. Outlet, inside	0	0
3. Inlet, outside	0	0
4. Outlet, outside	0	0
5. Hose inlet, inside	0	0
6. Hose outlet, inside	0	0
Large connector		
7. Inlet, inside	0	0
8. Outlet, inside	0	0
9. Inlet, outside	0	0
10. Outlet, outside	0	0
11. Hose inlet, inside	0	0
12. Hose outlet, inside	0	0

Conclusion

The predefined system sanitization method was evaluated using two challenging organisms, a bacterial *E. coli* strain and the yeast *P. pastoris*. Analyses of remaining microorganisms in sample solutions as well as on sampled system parts show efficient sanitization of the system using the predefined protocol and 1 M NaOH as sanitization solution. Although a few CFUs of the challenging organism were detected in the pump rinsing system in one out of four studies, a more than 6 log reduction of the challenging organisms was achieved on the sampled system parts. For the SNAP-connectors, a total of three sanitization studies were performed, using a bacterial *E. coli* strain and the yeast *S. cerevisiae* as challenging organisms. As shown for the wet side of the system, the results for the SNAP-connectors show no challenging organisms at any of the sampling points after sanitization.

Disclaimer

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Reference

1. Current good manufacturing practices for finished pharmaceuticals, 21 CFR 211. [Online] www.fda.gov/ohrms/dockets/98fr/98110s1.pdf. Accessed 8 January 2018.

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

gelifesciences.com/bioprocess

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GE Healthcare Japan Corp., Sanken Bldg., 3-25-1, Hyakunincho Shinjuku-ku, Tokyo 169-0073, Japan
For local office contact information, visit gelifesciences.com/contact.

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