Sanitization of ÄKTApilot with sodium hydroxide

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Sanitization of ÄKTApilot™ with sodium hydroxide

Abstract

Sanitization of ÄKTApilot was assessed by subjecting the regular sanitization procedure to microbial challenge tests. The system was infected with solutions containing three strains of bacteria that are recommended by the United States Pharmacopoeia (USP XXV) and a strain of yeast commonly used in production environments.

Sanitization was performed by rinsing the system with sterile water followed by pumping 1 M NaOH through the system for approximately one and a half hours. The method showed excellent efficiency with at least 10^6 reduction in colony forming units (CFU) for the four test organisms.

Introduction

ÄKTApilot can be used in process development, scale-up and scale-down, as well as small-scale production. It is important that ÄKTApilot can be sanitized since regulatory authorities impose very high standards on levels of purity and microbial presence in products used in clinical applications. Therefore, microbial challenge tests are used to evaluate the efficiency of sanitizing procedures for regulatory purposes. Sodium hydroxide is the preferred anti-microbial agent since it is documented as being effective and is frequently used to sanitize both chromatography equipment and media to provide high levels of hygiene. Moreover, sodium hydroxide is inexpensive and readily available.

Microbial presence and survival

Bacteria and yeasts are found in many laboratory and production environments and grow rapidly after incubation. In large quantities, they can damage the function of chromatography columns and impair the performance of chromatography systems. Microorganisms adapt to a wide range of conditions. Some require nutrient-rich substrates or elevated temperatures. Many gram-negative bacteria produce endotoxins that can contaminate products. Certain groups, such as *Pseudomonas*, can proliferate in low nutrient, aqueous solutions and can be found in process chromatography buffers. Consequently, it is important to follow hygienic routines throughout the whole production process.

Fig 1. Microbial growth on agar plates.

Microbial challenge tests

The principle of microbial challenge tests is to introduce a high concentration of contaminants into the equipment or media and then treat with an anti-microbial agent. After a specified length of time, the numbers of surviving organisms are counted. In this study, an ÄKTApilot system was infected with four species of microorganisms. After infection, the system was sanitized by pumping 1 M NaOH through the system for approximately one and a half hours, after which the system was dismantled and microbial samples taken at predetermined sites.

Sanitization and other terms

Sanitization, defined as the use of chemical agents to reduce microbial populations, is commonly used in chromatography systems to maintain microbial presence at levels that minimize the risk of contaminating products. A few important terms that are related to system and production hygiene are explained in Table 1.

Table 1. Explanation of relevant and related terms

Sanitization is the use of any chemical agent to reduce a microbial population to acceptable, predetermined levels.

Sterilization is the act or process, physical or chemical, which destroys or eliminates all forms of life, especially microorganisms.

Disinfection is the destruction of potential pathogens.

Cleaning is the removal of all kinds of contaminants, proteins, lipids, other particles, and microorganisms.

An **antimicrobial agent** is an agent that minimizes or destroys microorganisms *in vitro*. The term antimicrobial is general and all inclusive: antimicrobials 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.

SIP Sterilization in Place. Note that SIP also can mean Sanitization in Place or Steaming in Place.

Sterile free from all living microorganisms. Antiseptics prevent microbial growth.

Aseptic prevent bacterial entrance.

Materials and Methods

The organisms chosen for the microbial challenge tests included three USP XXV recommended bacterial strains and a yeast (Table 2).

Table 2. Organisms chosen for the microbial challenge tests

Escherichia coli	ATCC 8739	Gram-negative bacteria
Pseudomonas aeruginosa	ATCC 9027	Gram-negative bacteria
Staphylococcus aureus	ATCC 6538	Gram-positive bacteria
*Pichia pastoris**	GS 115	Yeast

* The yeast, *Pichia pastoris*, was selected in favor of the USP XXV recommended yeast, *Candida albicans*, as it is more frequently used in process and production conditions.

Preparation of the test strains

The challenging organisms were stored on nutrient agar slants at 4°C. The day before each test, the organisms were inoculated in 20 ml Trypticase Soya Broth (TSB) and incubated at 30°C to 35 °C for 18 to 24 h. The yeast was cultivated in 50 ml Yeast Peptone D-glucose (YPD) medium. The bacterial culture was centrifuged for 10 min at 4500 rpm and the pellet was suspended in sterile peptone water and diluted to an approximate concentration of 10^6 viable organisms/ml.

Preparation and infection of the system

All detachable parts of the system were soaked in 1 M NaOH for 1 h before rinsing with 70% ethanol and reassembling. The whole system was then filled with 1 M NaOH for 1 h and flushed with sterile water until pH was neutral.

The system was infected with 250 ml bacterial or yeast suspension via the sample pump and pumps A and B at a flow rate of 10 ml/min. The air trap was filled manually with suspension. To simulate chromatographic conditions, the flow direction and valves were switched several times. Thereafter, the system was left for 16 to 20 h at room temperature.

Sanitization for first time use

Before using ÄKTApilot for the first time, it is important to remove the background microflora. This achieved by circulating 4 liters of 1 M NaOH through rinsing loops at a flow rate of 400 ml/min for 12 h. Thereafter, flush the system with sterile water to eliminate NaOH. The system is considered clear when the outlet pH drops below 7.5.

Regular sanitization procedure

Before starting the sanitization procedure, it is important to restrict the flow of effluent from the system with a manifold so that a back pressure of 0.3 MPa is attained. The function of the manifold is to ensure that the whole system floods with NaOH and that no infected air pockets remain.

The regular santization procedure is described in Table 3. The first and second stages consist of pumping 5 liters sterile water followed by 5 liters 1 M NaOH through rinsing loops at a flow rate of 400 ml/min. Thereafter, sanitization is conducted by circulating 4 liters of 1 M NaOH at a flow rate of 400 ml/min for 75 min. Finally, flush the system with sterile saline solution to eliminate NaOH. When the pH of the outlet solution was less than 7.5, the system was considered clear.

Table 3. The regular sanitization procedure

Function	Solution	Volume	Time
Rinsing	sterile water	5 l	1 loop = 12.5 min
Rinsing	1 M sodium hydroxide	5 l	1 loop = 12.5 min
Sanitization	1 M sodium hydroxide	4 l (circulating)	6 loops = 75 min
pH neutralization	sterile physiological saline solution	10 l	2 loops = 25 min

Microbial testing

Microbial samples were taken at various points within predetermined sites (Fig 2) with one of the following methods:

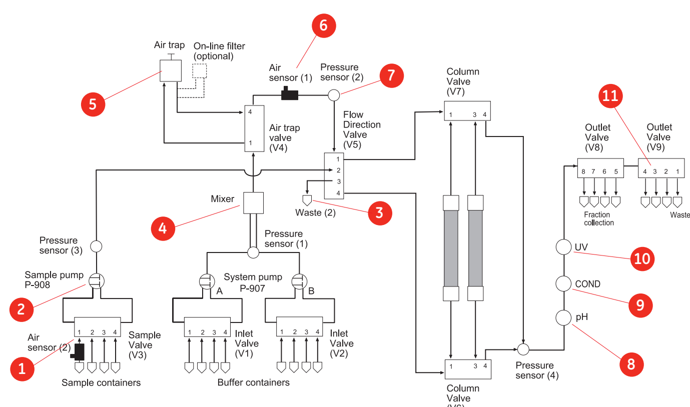

Fig 2. The microbial sampling sites in the system.

Test method 1, direct filtration

Sample solutions (ca. 25 ml for each sample) were collected in sterile beakers and then passed through a 0.45 mm cellulose membrane filter. The filter was incubated on TSA-plates (YPD plates used with *Pichia pastoris*) at 30°C to 35°C for 5 days. Subsequently, the number of CFU (Colony Forming Units) was counted and the number of viable microorganisms in the sample was estimated.

Test method 2, direct streak

Sample solutions (ca. 100 µl for each sample) were collected with sterile pipettes and directly spread on TSA-plates (YPD-plates used with *Pichia pastoris*) which were incubated at 30°C to 35°C for 5 days. Subsequently, CFUs were counted to give an estimate of the number of viable microorganisms in the sample.

Test method 3, swab

Surface samples were taken with alginate swabs. Each swab was inserted into a tube containing isotonic swab rinse solution. Thereafter, it was mixed with molten agar and allowed to solidify on petri plates. The TSA-plates (YPD-plates used with *Pichia pastoris*) were incubated at 30°C to 35°C for 5 days. Subsequently, the number of CFUs was counted and the number of viable microorganisms in the sample was estimated.

Test method 4, peptone water filtration

Detachable parts were aseptically removed and placed in 50 ml sterile peptone water and then vigorously shaken for at least 20 min. The solutions were passed through a 0.45 mm cellulose membrane filter. The filter was incubated on TSA-plates (YPD-plates used with *Pichia pastoris*) at 30°C to 35°C for 5 days. Subsequently, the number of CFUs was counted and the number of viable microorganisms in the sample was estimated.

Test method 5, contact plates

TSA-contact plate (YPD-contact plate used with *Pichia pastoris*) was put directly on a surface for microbial sampling. The plate was incubated at 30°C to 35°C for 5 days. Subsequently, the number of CFUs was counted and the number of viable microorganisms in the sampled surface was estimated.

Results

The challenge organisms were counted at four phases during the sanitization study: in the inoculation solution, immediately after infection, prior to sanitization, and after sanitization. The results are shown in Table 4.

The inoculum or starting concentration of the challenge organisms was in the range of 0.8×10^6 to 4.1×10^6. All post-infection concentrations decreased slightly. After leaving the system for 16 to 20 h at room temperature, the concentrations increased to levels between 10 and 100 times the starting inoculum concentration. At this point, the sanitization procedure was conducted. Following sanitization, effluent analyses showed no trace of viable challenge organisms.

The results in Table 5 show that the regular sanitization procedure eliminated *Escherichia coli*, *Staphylococcus aureus*, and *Pichia pastoris* from the system. At two points, two individual surviving *Pseudomonas aeruginosa* were found after a sanitization.

Table 4. Challenge organism viable count at different phases

Viable Count (CFU/ml)	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Pichia pastoris*
Inoculum sample, start concentration	1.5×10^6	2.1×10^6	4.1×10^6	0.8×10^6
Post-infection, flow through sample (outlet F2)	8.8×10^5	1.8×10^6	1.6×10^6	0.2×10^6
Presanitization, flow through sample (outlet F2)	1.2×10^8	2.0×10^8	1.2×10^7	6.7×10^6
Post-sanitization, effluent 3. Waste (2)	0	0	0	0
Post-sanitization, effluent (outlet F2)	0	0	0	0

Conclusion

It is common practice to define sanitization as a 10^6 reduction in CFU achieved after a sanitization procedure. In this study, the bacteria concentrations were in the order of 10^7 to 10^8 after incubation and the yeast had a presanitization concentration of 6.7×10^6 (Table 4). After sanitization virtually no surviving organisms were found (Table 5).

This demonstrates that ÄKTApilot can be effectively sanitized with a simple treatment of 1 M NaOH for 87.5 min. However, it is important to remember that a sanitization procedure cannot alone guarantee good hygienic status of a chromatographic process. The process should always be used in conjunction with other well-designed and carefully controlled hygienic routines, as well as rigorous control of buffers, water, and other input material.

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

Sampling site (number of sampling points)	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Pichia pastoris*
	Viable Count (CFU/ml)			
1. Valve, inlet S1 (5)	0	2	0	0
2. Sample pump (8)	0	0	0	0
4. Mixer	0	0	0	0
5. Air trap (3)	0	2	0	0
6. Air sensor	0	0	0	0
7. Pressure sensor (3)	0	0	0	0
8. pH-cell (4)	0	0	0	0
9. Conductivity cell	0	0	0	0
10. UV flow cell	0	0	0	0
11. Outlet valve, F2 (4)	0	0	0	0

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

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GE Healthcare UK Limited, Amersham Place,
Little Chalfont, Buckinghamshire, HP7 9NA
UK

GE Healthcare Europe, GmbH
Munzinger Strasse 5, D-79111 Freiburg
Germany

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327
USA

GE Healthcare Japan Corp.
Sanken Bldg., 3-25-1, Hyakunincho, Shinjuku-ku, Tokyo 169-0073
Japan

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