# Microbial Challenge and Sanitization of the NGC Discover 10 Pro System Using Sodium Hydroxide

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Protein Purification

Bulletin 7260

## Abstract

When working to develop biologics or biotherapeutics, much effort goes toward ensuring no contaminating or hazardous residual components from the organisms responsible for protein expression remain after purification. However, there can be additional requirements for the instrumentation used for these purification processes. Herein, a microbial challenge test was performed on the NGC Chromatography System using a bacterial strain as well as two yeast strains commonly used in purification environments. These organisms were allowed to contaminate the entire NGC System for 24 hours, followed by sanitization. The sanitization of the system was completed via a cleaning-in-place (CIP) method with 1 M NaOH and resulted in a greater than 6 log reduction in the microorganisms, using colony forming units (CFU) as the testing indicator. Increased incubation with 1 M NaOH also resulted in reduction of endotoxins to acceptable levels. We conclude that the NGC System is capable of being sanitized and is ideal for the small-scale production of biotherapeutics.

## Introduction

The Bio-Rad NGC Chromatography System is used for purification in discovery phases to identify lead candidates, development phases in which optimization and processes are established, and again when those processes are transferred to manufacturing. In a cGMP environment, effective sanitization procedures for small-batch production of biopharmaceuticals are required for regulated laboratories. Validating equipment cleanliness is critical in the pharmaceutical industry and of utmost importance to avoid contaminating drug products with microorganisms, viruses, endotoxins, cleaning solution residues, and the like. Contaminating an entire chromatography system with living organisms, followed by full-system decontamination, can verify how well the system can be cleaned and can help to identify problem areas.

Sodium hydroxide is an effective antimicrobial chemical that can reduce microorganisms to safe levels. It has also been shown to be powerful in denaturing nucleic acids and proteins. Sodium hydroxide concentrations can range from 0.1 to 1 M with a contact time of 1 to 2 hours, depending on the types of contaminants that need to be removed (for example, endotoxin inactivation may require a contact time of more than 12 hours).

In this study, all modules of a fully equipped NGC System were contaminated with three different organisms (*Escherichia coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae*) typically used for the production of recombinant proteins. The focus of our sanitization experiments was cleaning-in-place (CIP) with no dismantling of the NGC Chromatography System before or after infection. After 24 hours, samples of the infection solutions were taken in order to count remaining organisms. This was followed by decontamination of the NGC System with 1 M NaOH. Twenty percent ethanol was used to flush out the NaOH. Then the NGC System was rinsed with a sterile 0.9% NaCl solution in order to sample for surviving microorganisms, which were tested with different agar plates. Acceptance criteria for a successful cleaning process was both a minimum 6 log reduction for colony forming units (CFU) and 1 CFU in 10 ml effluent in absolute numbers. Endotoxin levels were specifically measured after *E. coli* contamination and cleaning with NaOH. Herein, we show an effective sanitization based on the above criteria.

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## Materials and Methods

### Test Strains, Cell Culture Media, and Their Preparation

To challenge the NGC System, three organisms typically used in protein production were selected (Table 1). The *E. coli* strain BL21 (DE3) carries the vector EX-EGFP-B13 with ampicillin resistance for the expression of green fluorescent protein (GFP) (herein referred to as *E. coli*-B13). Both yeast strains (*P. pastoris*-GS 115 and *S. cerevisiae*-INVSc1) are auxotrophic organisms that cannot reproduce in the absence of specific amino acids (herein referred to as *P. pastoris*-GS 115 and *S. cerevisiae*-INVSc1, respectively). *P. pastoris*-GS 115 is auxotrophic for histidine; *S. cerevisiae*-INVSc1 for histidine, leucine, tryptophan, and uracil. Testing with these three organisms enabled better discrimination between their specific growth after decontamination, possible general background contamination of the NGC System before infection, and handling errors during sample recovery and analysis.

The following cell culture media were used:

- Lysogeny broth (LB) for general bacterial growth and lysogeny broth with 0.1% ampicillin (LB Amp) for the cultivation of ampicillin-resistant *E. coli*-B13 (1% tryptone, 0.5% yeast extract, 1% NaCl)
- Yeast extract peptone dextrose (YPD) for the general cultivation of yeast (1% yeast extract, 2% peptone, 2% dextrose)
- Minimal dextrose (MD+) with specific amino acids for the specific cultivation of both auxotrophic yeast strains [1.34% Yeast Nitrogen Base (YNB) with ammonium sulfate, 1% dextrose, 4 x $10^{-5}$ % biotin, 0.0004% specific amino acids (histidine for *P. pastoris*-GS 115; histidine, leucine, tryptophan, uracil for *S. cerevisiae*-INVSc1)]. Neither yeast strain will grow on amino acid–deficient media

**Table 1. Organisms used for the contamination and sanitization experiments.**

| Organism | *Escherichia coli* | *Pichia pastoris* | *Saccharomyces cerevisiae* |
|---|---|---|---|
| Specification and selectivity | BL21 (DE3) with vector EX-EGFP-B13 — ampicillin resistance | GS 115 — auxotrophic for histidine | INVSc1 — auxotrophic for histidine, leucine, tryptophan, uracil |
| Growth medium for infection | LB Amp | YPD; Minimal dextrose (MD) with specific amino acid | YPD; Minimal dextrose (MD) with specific amino acids |
| Microbial agar testing conditions after decontamination | LB; LB Amp; YPD | YPD; MD with and without specific amino acids | YPD; MD with and without specific amino acids |

All three organisms were stored on their respective nutrient agars at 4°C. Due to the different reproduction times and growth conditions, *E. coli*-B13 was inoculated in culture medium and grown at 37°C for 24 hours, while both yeast strains were grown at 30°C for 48 hours. Prior to the contamination experiments, the cell cultures were suspended in sterile 50 mM NaCl solution and diluted to an average concentration of 5 x $10^6$ viable organisms/ml.

### NGC System Configuration and Preparation

The microbial challenge experiments were performed on an NGC Discover 10 Pro Chromatography System. A summary of the modules in the system, which was operated in an open lab environment, is shown in Table 2 and Figure 2. Flow rate was set to 10 ml/min for all experiments. The system contains two types of tubing: PEEK tubing (ID of 0.020" and 0.030") and transparent PTFE tubing (ID of 0.062").

Prior to infection and decontamination, the NGC System (including the pumps' backwash systems) was cleaned with 1 M NaOH and 20% ethanol. In order to detect any remaining background microflora, 20% ethanol was removed with sterile 0.9% NaCl solution and a few control samples were taken in a regular manner.

### Challenging with Individual Organisms

All infection and decontamination experiments were carried out in duplicate for each individual organism. Storage solution (20% ethanol) was removed from the system and replaced with a sterile 0.9% NaCl solution. Inlet tubing was connected to the buffer blending valve, buffer inlet valve, and the sample inlet valve. Open ends of tubing were immersed in a bottle of the cell suspension of the contaminating organism. The bottle was covered with aluminum foil to avoid any cross-contamination with airborne microorganisms. NGC System modules were challenged together via system and sample pumps. After incubation for 18 to 24 hours, in-line samples of the challenging organism were collected. The cell count of the corresponding samples ("survival test") was never below 3 x $10^6$ (data not shown). The NGC System was decontaminated at a flow rate of 10 ml/min with a preconfigured cleaning method (Figure 1).

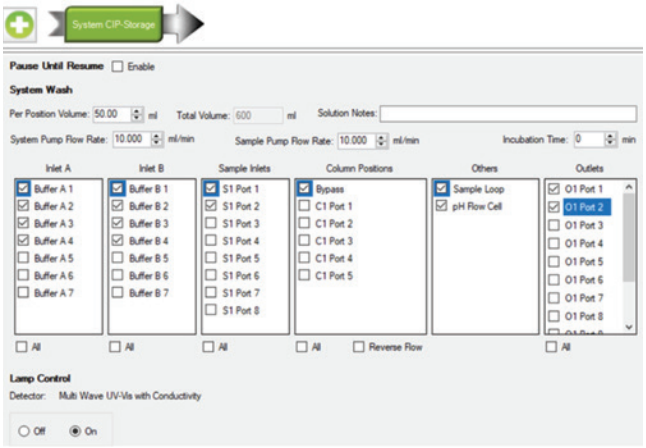


**Fig. 1. CIP method used for the sanitization experiments.**

The order of operations for each of the experiments was as follows:

1. Cell suspension was removed with 20% ethanol (10 ml per position).
2. The NGC System was flushed with 1 M NaOH (50 ml per position); 60 minutes incubation.
3. The NGC System was flushed with 20% ethanol (10 ml per position).
4. The NGC System was flushed with sterile 0.9% NaCl solution (10 ml per position). To confirm that NaOH was completely removed, pH was measured.
5. Sample elution was performed with sterile 0.9% NaCl solution (50 ml per position) and the samples were collected in either sterile tubes or Erlenmeyer flasks.

Sample elution schemes and volumes for all individual organism experiments are mapped out in Figures 3–7. Samples were centrifuged in sterile conical tubes at 4,000 rpm for 15 minutes to concentrate surviving microorganisms. Supernatant was carefully removed and the bottom of each conical tube was wetted with 1 ml of a sterile 50 mM NaCl solution. On agar plates, in duplicate, 100 µl were aspirated and plated with different growth conditions using standard sterile techniques.

Samples obtained from experiments with *E. coli*-B13 were tested on both LB ± 0.1% ampicillin and YPD agar plates at 37°C and then evaluated for CFUs after 24–48 hours. Samples received from experiments with both yeast strains were tested on MD ± amino acid supplementation, as well as on YPD agar plates, at 30°C and then evaluated after 3 to 5 days.

### Challenging with Combination of Organisms

For contamination, 200 ml of each solution with known cell counts were mixed and applied. One day later, the cell counts in samples received from the NGC System were measured again. Decontamination was performed as previously described and the NGC System was eluted twice with 0.9% NaCl solution (immediately after and 120 hours post-decontamination). This was done to ensure decontamination was complete and regrowth of the contaminating organisms was not possible. The samples were tested for CFUs on the corresponding agar plates and the plates were inspected 120 hours after plating. For the two yeast strains, individual CFU numbers in the cell mixture described above could not be obtained. Therefore, they were counted on MD+ plates with a supplementation of all four amino acids.

Sample elution schemes and volumes for the combination experiment are shown in Figures 3–8.

### Endotoxin Testing

The LAL Chromogenic Endotoxin Quantitation Kit (Pierce, #88282) was used to measure the endotoxin content of the relevant samples. The kit detects endotoxin levels as low as 0.1 EU/ml. All solutions and general lab equipment were pyrogen free in the sampling process. The sampling solution was 0.9% NaCl in water. The first four sampling elution strategies can be found in Figures 3–6; sampling volumes were 5 ml, 5 ml, 10 ml, and 10 ml, respectively. The fifth sampling strategy is shown in Figure 9. Samples were taken at three different points throughout the experiment. Samples were analyzed, in duplicate, for their endotoxin content.

1. Sample A: the system was cleaned with 1 M NaOH. After incubating for 24 hours, the system was flushed with 0.9% NaCl solution until the pH was neutral. At that point, sample A was taken and served as a control.
2. Sample B: the system was challenged with *E. coli*-B13 in LB Amp (3 x $10^7$ CFU/ml) for 24 hours and decontaminated (incubated with 1 M NaOH for 1 hour). The system was flushed with 0.9% NaCl solution until the pH was neutral. At that point, sample B was taken.
3. Sample C: the system was incubated with 1 M NaOH solution for 24 hours. The system was flushed with 0.9% NaCl solution until the pH was neutral. At that point, sample C was taken.

### Sample Elution Flow Paths and Volumes

Over the course of contamination, decontamination, and validation, multiple flow paths were needed due to the

complexity of the NGC System. In order to test all potential sites of contamination, multiple flow paths were used for testing. The air sensor modules in the system were thoroughly tested, but are not shown in the fluidic schemes. The fluidic schemes are shown in Table 2 and Figures 2–9.

**Table 2. NGC module legend.**

| Module | Module name |
|---|---|
| 1 | Buffer blending valve |
| 2 | Buffer inlet valves A and B |
| 3 | F10 system pumps A and B with mixer |
| 4 | Sample inlet valve |
| 5 | F100 sample pump |
| 6 | Sample inject valve |
| 7 | Waste position of module 6 |
| 8 | Sample loop (1 ml) of module 6 |
| 9 | Column switching valve |
| 10 | Multi-wavelength UV/Vis detector |
| 11 | Conductivity monitor |
| 12 | pH valve |
| 13 | Outlet valve |
| 14 | Fraction collector |

Note: Five out of 12 outlet positions of the outlet valve were tested. The air sensor modules were tested, but are not shown.

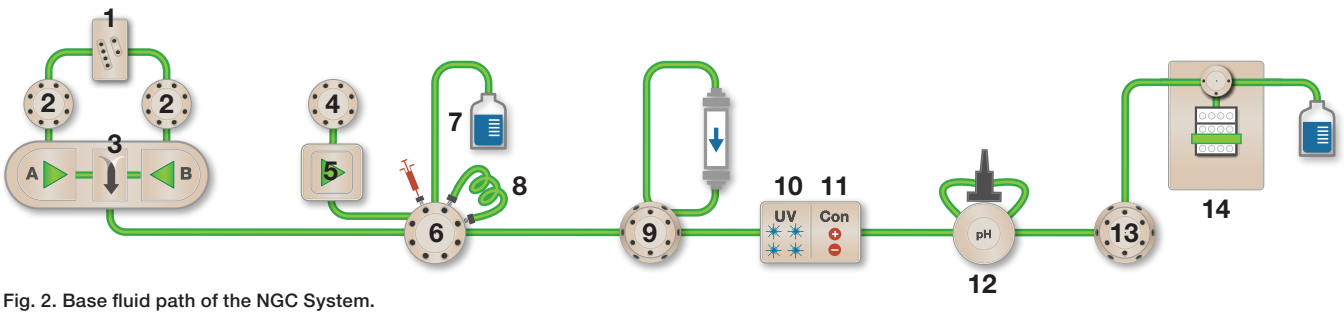


**Fig. 2. Base fluid path of the NGC System.**

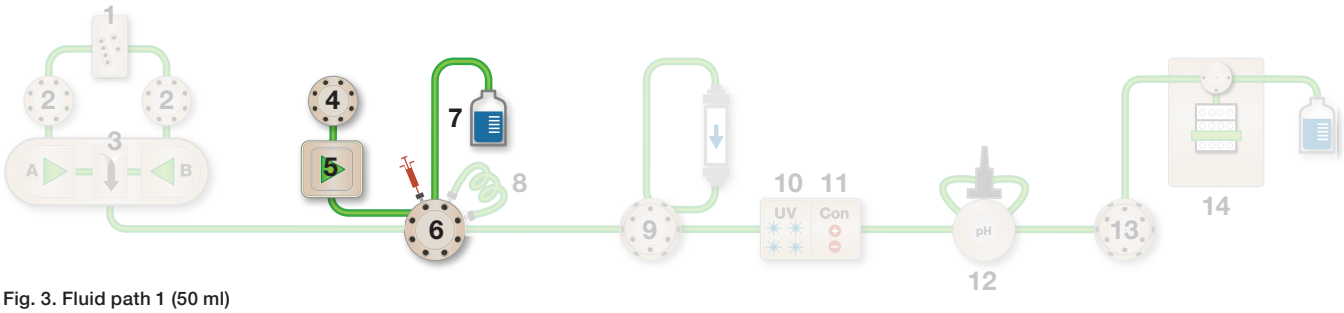


**Fig. 3. Fluid path 1 (50 ml)**

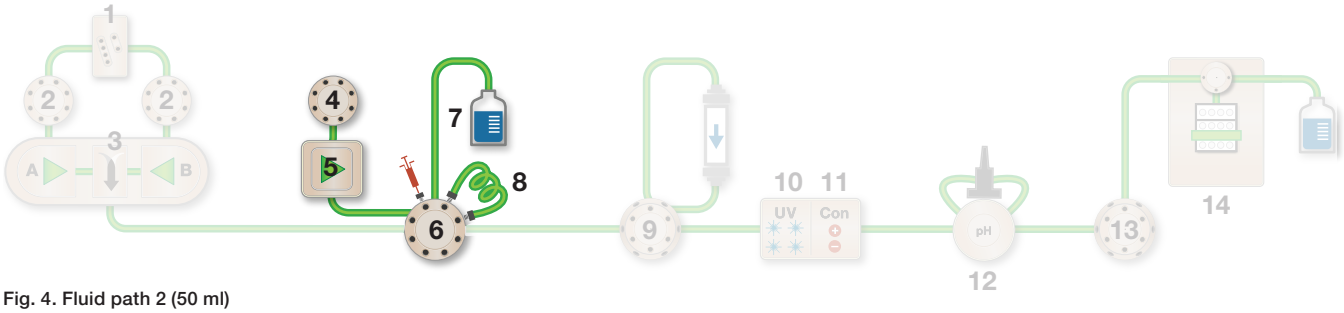


**Fig. 4. Fluid path 2 (50 ml)**

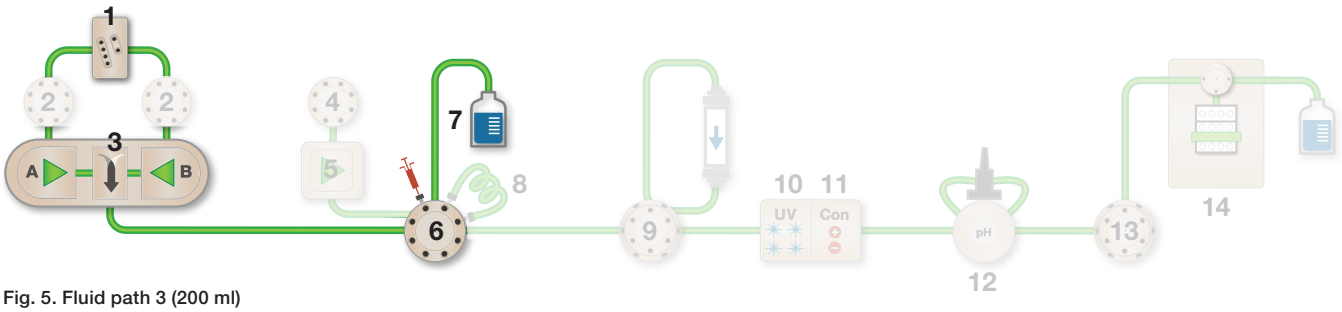


Fig. 5. Fluid path 3 (200 ml)

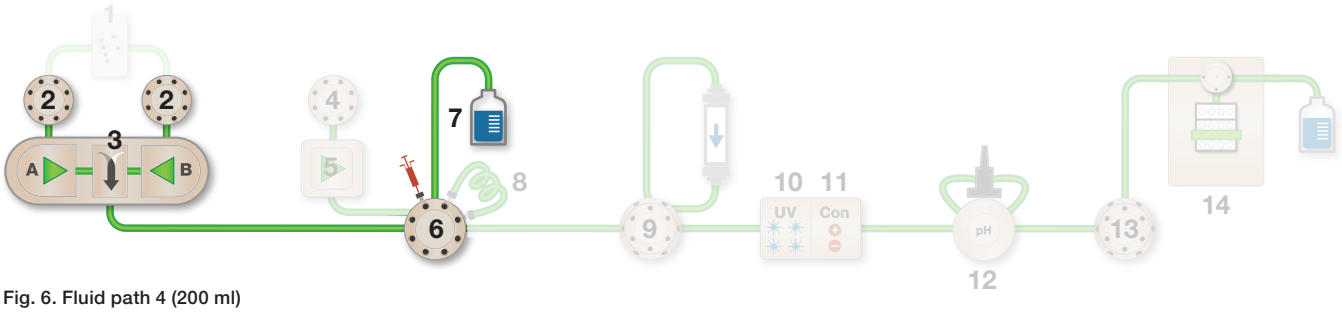


Fig. 6. Fluid path 4 (200 ml)

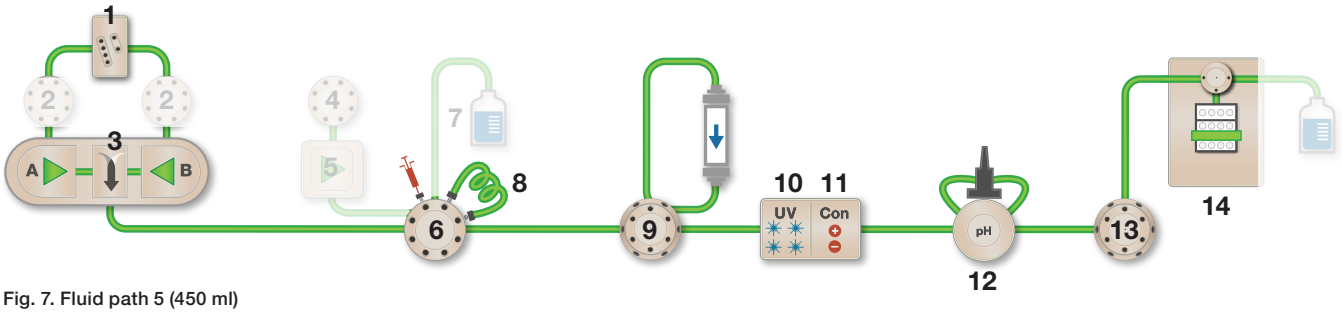


Fig. 7. Fluid path 5 (450 ml)

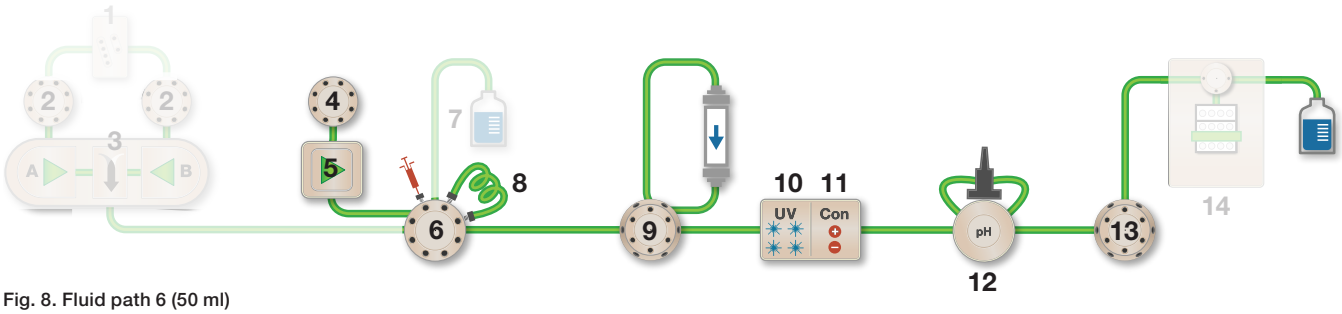


Fig. 8. Fluid path 6 (50 ml)

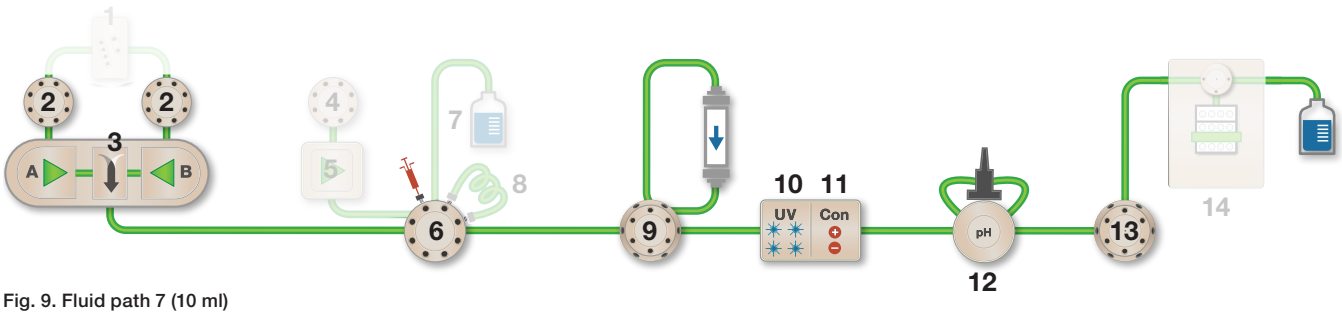


Fig. 9. Fluid path 7 (10 ml)

## Results and Discussion

### Challenging the NGC System with *E.coli*-B13

Different control samples were taken regularly to detect any remaining background microflora. No CFUs were detected on either YPD or LB agar plates after 24 hours incubation. The backwash system of the sample pump was also analyzed for background microflora, but no CFUs were seen (data not shown).

The *E. coli*-B13 experiments were multifaceted due to the complexity of the system. In the experiments, the NGC System components were infected together, in duplicate, using multiple flow paths (Figures 3–7). Complete system elution with sterile 0.9% NaCl solution was done in indicated flow paths and, in one case, repeated 24 hours after the first sampling.

Samples taken immediately after decontamination showed no signs of growth on LB Amp plates (Table 3). Resampling 24 hours after decontamination indicated no specific bacterial growth. Nonreproducible and unspecific colonies were seen on both YPD and LB agar plates, but greater than a 6 log reduction of bacterial growth was still achieved.

**Table 3. Experimental results from challenging with *E. coli*-B13 (in duplicate).** Data shown for the first sampling immediately following decontamination (four data points in microbial analysis) and for a second elution (two data points in microbial analysis) done 24 hours later. The elution done after the 24-hour incubation postdecontamination was performed only once.

| Microbial testing: 1st elution | Path 1 (50 ml) | Path 2 (50 ml) | Path 3 (200 ml) | Path 4 (200 ml) | Path 5 (450 ml) |
|---|---|---|---|---|---|
| # of colonies on YPD agar | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/1* | 0/0/0/0 |
| # of colonies on LB Amp agar | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 |
| # of colonies on LB agar | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 |
| **Microbial testing: 2nd elution** | | | | | |
| # of colonies on YPD agar | 0/0 | 0/0 | 0/0 | 0/0 | 0/2* |
| # of colonies on LB Amp agar | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| # of colonies on LB agar | 0/1* | 0/0 | 0/3* | 0/0 | 0/0 |

* Nonspecific events and not normalized against the elution volume.

### Challenging the NGC System with *P. pastoris*-GS 115 and *S. cerevisiae*-INVSc1

The *P. pastoris*-GS 115 and *S. cerevisiae*-INVSc1 experiments were performed in duplicate. The elution scheme for *P. pastoris*-GS 115 is indicated in Figures 3–7. No viable *P. pastoris*-GS 115 cells were detected after decontamination (Table 4). No specific colonies were seen after a second elution with sterile 0.9% NaCl solution 24 hours after the first sampling round.

**Table 4. Results from challenging with *P. pastoris*-GS 115.** Data shown for the first sampling immediately following decontamination (four data points in microbial analysis) and for a second elution (two data points in microbial analysis) done 24 hours later. The elution done after the 24-hour incubation postdecontamination was performed only once.

| Microbial testing: 1st elution | Path 1 (50 ml) | Path 2 (50 ml) | Path 3 (200 ml) | Path 4 (200 ml) | Path 5 (450 ml) |
|---|---|---|---|---|---|
| # of colonies on YPD agar | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 |
| # of colonies on MD+ agar | 0/0/0/0 | 0/0/0/0 | 0/0/1*/0 | 0/0/0/0 | 0/0/0/0 |
| # of colonies on MD– agar | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 | 0/0/0/0 |
| **Microbial testing: 2nd elution** | | | | | |
| # of colonies on YPD agar | 0/0 | 0/0 | 0/0 | n/a | 0/0 |
| # of colonies on MD+ agar | 0/0 | 0/0 | 0/0 | n/a | 0/0 |
| # of colonies on MD– agar | 0/0 | 0/0 | 0/6* | n/a | 0/0 |

* Nonspecific events and not normalized against the elution volume.

Experiments were also done with *S. cerevisiae*-INVSc1 according to the same elution scheme as *P. pastoris*-GS 115. A single second elution of the system 24 hours after decontamination was performed. All samples tested negative for specific CFUs (Table 5).

**Table 5. Results from challenging with *S. cerevisiae*-INVSc1 (in duplicate).** Data shown for the first sampling immediately following decontamination (four data points in microbial analysis) and for a second elution (two data points in microbial analysis) done 24 hours later. The elution done after the 24-hour incubation postdecontamination was performed only once.

| Microbial testing: 1st elution | Path 1 (50 ml) | Path 2 (50 ml) | Path 3 (200 ml) | Path 4 (200 ml) | Path 5 (450 ml) |
|---|---|---|---|---|---|
| # of colonies on YPD agar | 0/0/0/0 | 0/1*/0/0 | 0/0/n/a** | 0/0/0/0 | 0/0/0/0 |
| # of colonies on MD+ agar | 0/0/0/0 | 0/0/0/0 | 0/0/n/a** | 0/0/0/0 | 0/0/0/0 |
| # of colonies on MD– agar | 0/0/0/0 | 0/0/0/0 | 0/0/n/a** | 0/0/0/0 | 0/0/0/0 |
| **Microbial testing: 2nd elution** | | | | | |
| # of colonies on YPD agar | 0/0 | 0/0 | 2*/0 | n/a | 0/0 |
| # of colonies on MD+ agar | 0/0 | 0/0 | 2*/0 | n/a | 0/0 |
| # of colonies on MD– agar | 0/0 | 0/0 | 0/0 | n/a | 0/0 |

* Nonspecific events and not normalized against the elution volume.
** Sampling error.

### Challenging the NGC System with a Combination of *E. coli*-B13, *P. pastoris*-GS 115, and *S. cerevisiae*-INVSc1

Equal volumes were mixed and the individual cell count was as follows: *E. coli*-B13, 2 x $10^7$ CFU/ml; *P. pastoris*-GS 115, 4 x $10^6$ CFU/ml; *S. cerevisiae*-INVSc1, 4 x $10^6$ CFU/ml. The system was infected and, 24 hours later, samples were taken so CFU numbers could be remeasured: *E. coli*, 13.3 x $10^7$ CFU/ml; yeast cumulative, 2.5 x $10^7$ CFU/ml. All three organisms grew extremely well under these conditions and the sheer number of organisms posed an elevated contest to the sanitization protocol, which was applied unchanged. Sampling with sterile 0.9% NaCl solution was done immediately after decontamination and was repeated 120 hours after the first sampling. During those 120 hours the NGC System was stored in 0.9% NaCl solution instead of the typical 20% ethanol. Module combinations and elution schemes are indicated in Figures 3–8. Test results are outlined in Table 6.

**Table 6. Challenging experiment with high cell density solution (mixture of *E. coli*-B13, *P. pastoris*-GS 115, and *S. cerevisiae*-INVSc1).** Data shown for the first sampling immediately following decontamination (two data points in microbial analysis) and a second elution (two data points in microbial analysis) done 120 hours later.

| Microbial testing: 1st elution | Path 1 (50 ml) | Path 2 (50 ml) | Path 3 (200 ml) | Path 4 (200 ml) | Path 5 (450 ml) | Path 6 (50 ml) |
|---|---|---|---|---|---|---|
| # of colonies on YPD agar | 0/0 | 0/0 | 1/1 | 0/0 | 0/0 | 0/0 |
| # of colonies on MD+ agar | 0/0 | 0/0 | 1/1 | 0/0 | 0/0 | 0/0 |
| # of colonies on LB agar | 0/0 | 2/0 | 0/0 | 0/0 | 2/2 | 0/0 |
| # of colonies on LB Amp agar | 0/0 | 0/0 | 0/0 | 0/0 | 2/2 | 0/0 |
| **Microbial testing: 2nd elution** | | | | | | |
| # of colonies on YPD agar | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| # of colonies on LB agar | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |

The inspection of the agar plates from the first sampling was done after 120 hours and two specific events were recorded. There were 10 CFUs (yeast) in 200 ml in the flow path from the buffer blending valve to the waste position of the inject valve. An additional 20 CFUs (*E. coli*-B13) in 450 ml were found in the flow path from the column switching valve to the outlet valve of the system. However, the CFU level was still below the GMP requirements (10 CFU/100 ml) for the manufacture of water for injection (WFI). Although the system was in 0.9% NaCl solution for that 120 hours, the agar plate inspection of the second sampling was negative and no further specific growth events were observed.

### Endotoxin Testing

During the course of this project, the NGC System was treated with *E. coli*-B13 solutions multiple times. Gram-negative bacteria such as *E. coli*-B13 release endotoxins into the surrounding environment upon cell death. Since bacterial endotoxins are potent inflammatory agents, it is important that they do not accumulate over time in a chromatography system. They can be removed with a proper sanitization agent like NaOH. Endotoxins are very stable lipopolysaccharides and may require cleaning agent incubation as long as 12 hours. Our sampling strategy for measuring endotoxin levels was done in three parts (see Materials and Methods). The results shown in Table 7 indicate that an extended incubation time in NaOH is necessary to obtain samples with endotoxin concentrations below 0.1 EU/ml (sample panels A and C). NGC System cleaning with the protocol previously described is based on a 1 hour incubation time for NaOH. This time is too short for complete endotoxin removal because one out of five samples of panel B showed an elevated endotoxin concentration of 0.5 EU/ml. Overall, the results demonstrate that endotoxin removal from the NGC System can be achieved by NaOH treatment with appropriate incubation times.

**Table 7. Endotoxin testing with three panels.** Sample panels included A (before contamination, B (after *E. coli*-B13 contamination of the NGC System and sanitization with NaOH for 1 hour), and C (sanitization with NaOH for 18 hours). Sampling was performed in sections (from left to right). Sampling solution was 0.9% NaCl.

| | Microbial Test | Path 1 (5 ml) | Path 2 (5 ml) | Path 3 (10 ml) | Path 4 (10 ml) | Path 7 (10 ml) |
|---|---|---|---|---|---|---|
| A | Endotoxin testing, EU/ml | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| B | Endotoxin testing, EU/ml | <0.1 | <0.1 | <0.1 | <0.1 | 0.5 |
| C | Endotoxin testing, EU/ml | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |

## Conclusion

The results of this study clearly demonstrate that massive microbial contamination of a comprehensively equipped NGC Chromatography System can be reduced by more than 6 log for colony forming units (absolute numbers were as low as 1 CFU/10 ml effluent). The cell types used for the challenge experiments represent an important class of organisms used for the expression and development of protein-derived therapeutic drugs. The results show excellent cleaning efficiency under extreme contamination. A few CFUs of the challenging organisms were detected after the infection with an *E. coli*-B13 and yeast cocktail, yet a minimum of 6 log reductions in CFUs was still achieved. In addition, endotoxin removal was investigated and a contact time for decontamination with NaOH was successful, but with an incubation time greater than 1 hour. These results for the NGC System instill confidence as pharmaceutical researchers set up and validate cleaning processes.

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