# Endotoxin control in the process development lab: from upstream bacterial fermentation to downstream purification processes

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## Introduction

Although *E. Coli* has proven itself an important, if not vital, player in the production of biopharmaceuticals, its use in upstream fermentation does not come without challenges and costs for downstream purification. At the start of purification, clarified cell lysate may have an endotoxin level greater than 1 billion EU/mL. By the time the material becomes a final formulated drug product, its endotoxin level must be reduced many orders of magnitude to meet the FDA standard of 5 EU/kg. However, due to the small size of individual endotoxin molecules, the ability to micellize into aggregates with molecular weights as high as 1 MDa and interact with other molecules at higher concentrations, and its pH and heat resistance, the challenges of endotoxin removal are well known. Here, we test the endotoxin reduction capabilities of several processes, namely anion exchange membranes, surfactant-based protocols, and spin columns. We also evaluate cleaning procedures for their effectiveness at depyrogenation and quantify the endotoxin level of a process development lab environment where bacterial fermentations are regularly performed to mitigate endotoxin cross contamination in the process development lab.

## Materials

The material tested was an enzymatic protein produced by *E. Coli*. The endotoxin removal strategies were obtained from the following sources: Mustang Q (MQ) membrane capsules from Cytiva, Sartobind STIC PA (primary amine) anion exchange membrane from Sartorius, Triton X-100 from Sigma Aldrich, and Proteus endotoxin removal spin columns from Bio-Rad. Endotoxin testing was performed using the Endosafe nexgen-PTS and Endosafe LAL cartridges purchased from Charles River.

## Methods and Results

Environmental endotoxin:

The endotoxin level of the lab environment was quantified by placing 20 mL of endotoxin-free water in sterile petri dishes in locations around the upstream and downstream labs. Samples were left for 1 day before being tested for endotoxin. Low levels of endotoxin were measured in all locations except for an enclosed room in the downstream lab, showing that the presence of bacterial fermentation contributes to the endotoxin level of the lab. However, endotoxin levels may be able to be minimized through simple physical barriers. The environmental endotoxin results are shown in Table 1.

*Table 1: Environmental endotoxin levels*

| Sample | Final volume (mL) | Endotoxin (EU/mL) | Endotoxin (EU) |
|---|---|---|---|
| Downstream main lab | 4.2 | 0.301 | 1.26 |
| Downstream side lab (door closed) | 12.2 | < 0.05 | < 0.61 |
| Upstream main lab | 11.5 | 0.155 | 1.78 |
| Upstream fermentation | (20) | 0.206 | 4.12 |
| Biosafety cabinet | (20) | < 0.05 | < 1 |
| Waste room | 13.0 | 0.125 | 1.623 |

(volume in parenthesis indicates sample fully evaporated and had water added to test the endotoxin level)

Cleaning protocols:

Several cleaning protocols using 0.5 M NaOH (at room temperature) were evaluated for their ability to depyrogenate TFF and AKTA systems. The TFF system was first tested with new tubing, then post-use following a capture TFF step before and after a 30-minute cleaning cycle at a low flow rate. The AKTA Explorer systems were tested after being used for various capture and polishing columns. Three protocols were tested – 1) 30 minutes of contact time, 2) 30 minutes of contact time with a low flow rate, and 3) 300 minutes (5 hours) of contact time. In all cases, the endotoxin level post-cleaning was lower than pre-cleaning. The endotoxin reduction varied depending on the instrument and starting endotoxin level. Results indicate that longer contact times are effective at increasing depyrogenation and all results are shown in Table 2.

*Table 2: Endotoxin reduction achieved by cleaning procedures*

| Sample | Initial endotoxin (EU/mL) | Final endotoxin (EU/mL) | Endotoxin reduction |
|---|---|---|---|
| MasterFlex PharmMed BPT tubing (new) | N/A | < 0.05 | N/A |
| MasterFlex PharmMed BPT tubing (used) (30 min cleaning) | 0.136 | 0.089 | 35% |
| AKTA (30 min cleaning) | 0.486 | 0.322 | 34% |
| AKTA (30 min cleaning w/flow) | 2.88 | 2.37 | 18% |
| AKTA (300 min cleaning) | 2.37 | 0.630 | 73% |

Endotoxin reduction:

The Mustang Q and STIC experiments were performed using an AKTA Pilot or Explorer as appropriate for the process scale. The membranes underwent a sanitization and equilibration procedure before the product was flowed through the capsules and followed by a flush with equilibration buffer. Flowthrough was collected when the absorbance at 280 nm (A280) began to rise until the absorbance decreased back to a baseline.

The Triton X-100 protocol involved an initial incubation step with a surfactant-spiked buffer, repeated concentration and dilution cycles with the spiked buffer to remove endotoxin, then a second set of concentrations and dilutions with unspiked buffer to remove the Triton X-100. Concentration was done in Amicon centrifugal filter units in 15-minute increments at 3000 rpm (1860 g) and 20°C.

The Proteus spin columns were used following the provided instructions. After equilibrating the column, the material to be purified was mixed with the resin for 2 hours at room temperature. The column was then centrifuged as instructed at 750 g for 10 min and the flowthrough was tested for protein concentration and endotoxin. For all experiments, yield was quantified by A280 measurements from a NanoDrop One and endotoxin was measured using the Endosafe nexgen-PTS.

*(Endotoxin reduction cont.)*

Tables 3 and 4 and Figures 1-3 show the endotoxin reduction and yield results. All methods demonstrated some efficacy at removing endotoxin from the starting material, ranging from 43-99.9% of endotoxin removed. In general, the percent of endotoxin removed was greater for material starting at higher endotoxin levels. Protein recovery yields were overall high, averaging 87%, though the yield was unable to be quantified for the Triton X-100 protocol due to absorbance at 280 nm from Triton that was not removed from the final product.

*Table 3: Endotoxin reduction capabilities of tested procedures*

| Sample | Initial volume (mL) | Initial endotoxin (EU/mL) | Initial endotoxin (EU) | Final volume (mL) | Final endotoxin (EU/mL) | Final endotoxin (EU) | Endotoxin reduction |
|---|---|---|---|---|---|---|---|
| MQ[1] (10 mL) | 77 | 738 | 56800 | 143 | 60.4 | 8640 | 85% |
| MQ (60 mL) | 553 | 6970 | 3850000 | 1130 | 50 | 56300 | 99% |
| MQ (2 x 60 mL) | 3850 | 9090 | 35000000 | 4820 | 6.78 | 32700 | 99.9% |
| MQ (260 mL) | 15800 | 287 | 4540000 | 17300 | 1.17 | 20200 | 99.6% |
| Spin column | 11.5 | 30.3 | 348 | 11.9 | 16.8 | 200 | 43% |
| STIC (1 x 1 mL) | 103 | 6.91 | 712 | 130 | 1.64 | 213 | 70% |
| STIC (2 x 1 mL) | 275 | < 500 | < 13800 | 300 | 62.9 | 18900 | < 86% |
| STIC (3 x 1 mL) | 307 | 26.8 | 8230 | 350 | 9.77 | 3420 | 58% |
| STIC (4 x 1 mL) | 1580 | 39.1 | 61700 | 1580 | 2.37 | 3740 | 94% |
| Triton X-100 | 36 | 342 | 12300 | 2.7 | 144 | 389 | 97% |

[1]MQ = Mustang Q

*Table 4: Protein recovery yield results for endotoxin reduction procedures*

| Sample | Initial volume (mL) | Initial conc. (mg/mL) | Initial protein (mg) | Final volume (mL) | Final conc. (mg/mL) | Final protein (mg) | Yield |
|---|---|---|---|---|---|---|---|
| MQ[1] (10 mL) | 77 | 4.0 | 306 | 143 | 2.0 | 281 | 92% |
| MQ (60 mL) | 553 | 3.8 | 2110 | 1130 | 1.4 | 1570 | 75% |
| MQ (2 x 60 mL) | 3850 | 0.82 | 3160 | 4820 | 0.63 | 3040 | 96% |
| MQ (260 mL) | 15800 | 0.85 | 13400 | 17300 | 0.75 | 12900 | 96% |
| Spin column | 11 | 4.4 | 48.5 | 11.9 | 3.8 | 45.5 | 94% |
| STIC (1 x 1 mL) | 103 | 2.6 | 265 | 130 | 1.6 | 212 | 80% |
| STIC (2 x 1 mL) | 275 | 1.3 | 358 | 300 | 0.84 | 253 | 71% |
| STIC (3 x 1 mL) | 307 | 4.2 | 1470 | 350 | 3.4 | 1200 | 82% |
| STIC (4 x 1 mL) | 1580 | 7.1 | 11300 | 1580 | 7.0 | 11000 | 98% |
| Triton X-100 | 36 | 0.78 | 28.1 | 2.7 | 62* | 168 | N/A |

[1]MQ = Mustang Q *A280 unreliable because of absorbance from unseparated Triton X-100

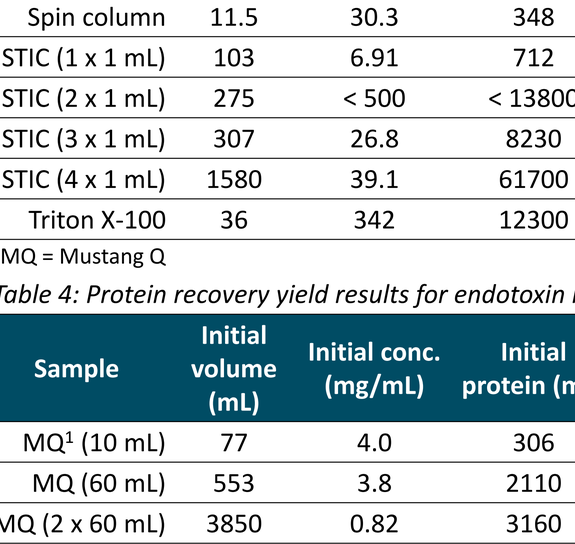


*Figure 1: Final endotoxin plotted against initial endotoxin*

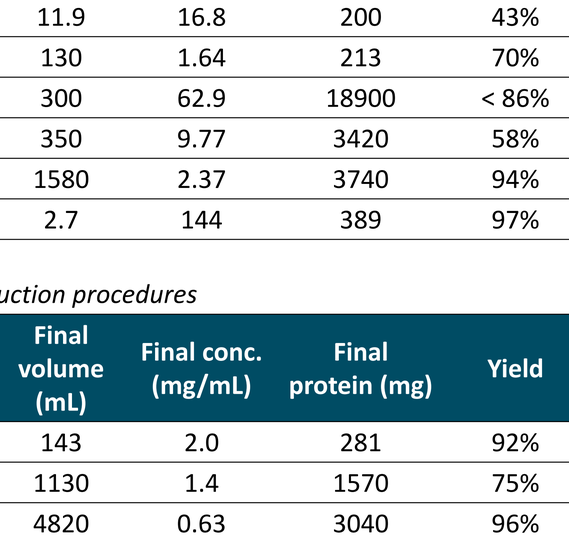


*Figure 2: Log endotoxin reduction*

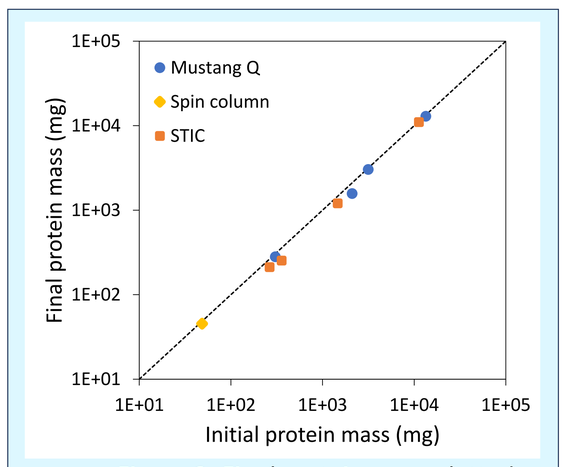


*Figure 3: Final protein mass plotted against initial protein mass*

## Conclusion

The use of bacterial fermentation to produce biopharmaceuticals can contribute to the overall endotoxin level of a process development lab, though our environmental endotoxin data suggest straightforward physical separation may be enough to maintain low endotoxin levels. Additionally, 0.5 M NaOH is shown to be able to remove endotoxin from equipment after use with bacterial-based materials. Contact time is an important factor in the extent of depyrogenation. For the protein of interest, all endotoxin reduction methods tested were effective at removing endotoxin while maintaining good yields except for the Triton X-100 surfactant-based protocol, which removed endotoxin but was not separated from the final product. However, determining which method is most suitable for endotoxin reduction of a protein solution is heavily dependent on the particular protein's characteristics, and other factors to consider include ease of use, time, cost, and scalability.

## References

*Proteus Endotoxin Removal Kit Handbook*. Retrieved July 25, 2024, from https://www.bio-rad-antibodies.com/static/uploads/ifu/pur030.pdf

Kondratova, L., Kondratov, O., Ragheb, R., & Zolotukhin, S. (2019) *Molecular Therapy - Methods & Clinical Development, 15*. Removal of Endotoxin from rAAV Samples Using a Simple Detergent-Based Protocol., 112–119. https://doi.org/10.1016/j.omtm.2019.08.013

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