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# Broad application and optimization of a single wash-step for integrated endotoxin depletion during protein purification

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ABSTRACT

Endotoxins contaminate proteins that are produced in *E. coli*. High levels of endotoxins can influence cellular assays and cause severe adverse effects when administered to humans. Thus, endotoxin removal is important in protein purification for academic research and in GMP manufacturing of biopharmaceuticals. Several methods exist to remove endotoxin, but often require additional downstream-processing steps, decrease protein yield and are costly. These disadvantages can be avoided by using an integrated endotoxin depletion (iED) wash-step that utilizes Triton X-114 (TX114). In this paper, we show that the iED wash-step is broadly applicable in most commonly used chromatographies: it reduces endotoxin by a factor of $10^3$ to $10^6$ during NiNTA-, MBP-, SAC-, GST-, Protein A and CEX-chromatography but not during AEX or HIC-chromatography. We characterized the iED wash-step using Design of Experiments (DoE) and identified optimal experimental conditions for application scenarios that are relevant to academic research or industrial GMP manufacturing. A single iED wash-step with 0.75% (v/v) TX114 added to the feed and wash buffer can reduce endotoxin levels to below 2 EU/ml or deplete most endotoxin while keeping the manufacturing costs as low as possible. The comprehensive characterization enables academia and industry to widely adopt the iED wash-step for a routine, efficient and cost-effective depletion of endotoxin during protein purification at any scale.

## 1. Introduction

*E. coli* is the organism of choice for recombinant protein expression whenever mammalian cells can be avoided: it features simple and cost-effective cultivation, high yields and easy scale-up from laboratory to industrial scale.

However, up to three quarters of *E. coli*'s outer membrane consists of endotoxins which are released during cell disruption and may bind tightly to the produced protein [1]. Excessive endotoxin in the human bloodstream may cause fever, organ damage and even death [2]. Thus, endotoxin depletion is crucial for biopharmaceuticals, especially when the patient receives high doses of recombinantly-produced proteins. Therefore, current guidelines limit the amount of endotoxin that can be administered. For example, the US Pharmacopoeia specifies 5 EU per kilogram body weight as the maximum allowed amount of endotoxin during intravenous application [3]. Similar doses also apply for academic or pre-clinical research to avoid experimental artefacts, especially when working with *in vitro* and *in vivo* systems that react upon endotoxin stimulation [4]. Thus, low endotoxin levels must be achieved while maintaining reasonable production costs and yields.

Different methods can be used to selectively deplete endotoxins during protein purification. In large-scale downstream processes, endotoxins are effectively removed by anion-exchange chromatography in flow through mode or by membrane adsorbers [5–7]. At laboratory scale, endotoxins are removed by Triton X-114 (TX114) phase separation [8] or specifically developed affinity resins like the EndoTrap® (Hyglos). Nevertheless, these methods have significant disadvantages, as they can either stress the protein, lead to protein loss, require an additional downstream processing step, are hard to scale, are not GMP manufacturing compatible, restrict the choice of buffers or are costly. Thus, a method to deplete endotoxins that avoids these disadvantages and is broadly applicable, reliable, fast and cost-effective would be of great value for high quality protein purification.

In 2006, Reichelt et al. developed a method that potentially fulfils these criteria: they integrated a simple TX114 wash-step into their chromatography that removed > 99% of endotoxins [9]. This wash-step did not influence the biological activity of purified antibody fragments [10]. The wash-step was successfully integrated in NiNTA-,

*Abbreviations:* AEX, anion exchange chromatography; CEX, cation exchange chromatography; CIP, cleaning in place; DoE, Design of Experiments; EU, endotoxin units; GMP, good manufacturing practice; GST, glutathione S-transferase affinity chromatography; HIC, hydrophobic interaction chromatography; iED, integrated endotoxin depletion; MBP, maltose binding protein affinity chromatography; NiNTA, nickel nitrilotriacetic acid chromatography; SAC, streptavidin affinity chromatography; TX110, Triton X-110; TX114, Triton X-114

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GST-, and CEX-chromatography and even used under denaturing conditions [9–11]. The purification yields were consistently above 90%. Nevertheless, the protocol employed very low flow-rates and high wash-volumes. Furthermore, not all relevant chromatographies and conditions have been investigated yet.

In theory, this wash-step bears the potential to be broadly applicable in many chromatographies and under different conditions and scales, but a more comprehensive investigation and optimization would be necessary to enable its widespread adoption.

Therefore, this paper expands the integrated endotoxin depletion wash-step (iED) in three ways:

i. Test its broad applicability in all commonly-used chromatography media and conditions.
ii. Identify all factors influencing its efficiency and quantify their effect
iii. Pinpoint optimal experimental conditions for three different application scenarios relevant to academia and industry

## 2. Materials and methods

### 2.1. Chromatography setup

For chromatography, an automated FPLC system was used at 2 to 8 °C unless stated otherwise (ÄKTA™ avant 25, GE Healthcare). A standardized inlet system was developed, using inlet A5, A6 and A7 for 20% ethanol, 1 M NaOH and H2O, respectively, and inlet B5, B6 and B7 for 20% ethanol with 0.2 M acetate, 2 M NaCl and $H_2O$, respectively. Outlet 1 was connected to a nickel waste container. This setup facilitates that almost any column can be CIPed right after use without switching buffers using broadly applicable CIP programs and that NiNTA chromatography can be automated for sequential runs of different proteins.

### 2.2. Production of E. coli biomass for spike-in experiments

To generate *E. coli* biomass for spike-in experiments, an unmodified pASK-IBA3plus plasmid was transformed into chemically competent *E. coli* BL21(DE3). Then, a single colony was picked to inoculate a pre-culture in LB-antibiotic medium. The pre-culture was incubated overnight at 37 °C under vigorous shaking. For the main production-culture, Terrific Broth medium (Thermo Fisher Scientific) was supplemented with 2 mM $MgSO_4$, 100 mg/l Ampicillin sodium salt or 100 mg/l Kanamycin sulfate, 0.2 g/l PPG2000 and inoculated to an $OD_{600}$ of 0.1. The production-culture was grown at 37 °C with 225 rpm. After reaching an $OD_{600}$ of approximately 1, 200 µg AHT per liter of culture was added. After another six hours of incubation, the culture was split into 50 ml aliquots and the bacteria were harvested by centrifugation at 7000*g* for 15 min at 4 °C and stored at −20 °C until further processing.

### 2.3. Chromatography for integrated endotoxin depletion from supernatants

The *E. coli* biomass for purification was prepared as described above. Biomass aliquots of 50 ml culture were used per chromatography run which corresponded to approximately 0.5 g biological wet weight. The resuspension-buffers were adapted depending on the chromatography (Suppl. Table 2). Cell disruption was performed by sonication for 5 min on ice using a HD 2070 sonicator (Bandelin) with a MS 73 microtip at 100% amplitude and 50% pulsation. The cell lysate was clarified by centrifugation at 17000*g* for 30 min at 4 °C. After the supernatant was clarified and filtered, the target protein was added. For the affinity-tag based chromatographies NiNTA, SAC, MBP and GST, an artificial quad-tag protein (qTP) (Suppl. Fig. 1A and B) was added. For the Protein A, CEX and AEX chromatography, a polyclonal antibody mixture, lysozyme and human serum albumin were added, respectively. The final concentration of the respective protein was 0.1 mg/ml.

The chromatographies were run according to the manufacturer's instructions using the respective buffers and columns (Suppl. Table 2). In brief, the column was equilibrated with buffer A and the feed was loaded. Then, the column was washed with 15 CV iED-buffer A or buffer A without TX114 as control. TX114 was added on a volume per volume (v/v)-basis unless stated otherwise. Then, the column was washed with 15 CV buffer A. Elution was performed with 5 CV of 100% buffer B for NiNTA-, MBP-, SAC-, GST- and Protein A-chromatography and a linear gradient from 0% to 100% buffer B for AEX- and CEX-chromatography. To determine the chromatography's yield, the elution peaks were integrated and the amount of protein calculated using the lambert-beer-formula; the UV-cell had a path-length of 0.2 cm. The extinction coefficients for the qTP, polyclonal antibody mixture, lysozyme and HSA are 116.78, 210.0, 37.97 and 34.45 $M^{-1} * cm^{-1} * 10^3$, respectively. The chromatographies were run in triplicates, alternating between iED- and control-purifications after each run. To avoid endotoxin carryover, the columns were stripped, CIPed and recharged after each run.

For the DoE-guided characterization, the chromatographies were run according to the DoE's predetermined run-order (Suppl. Table 1). This run-order was implemented in the method's scouting-module that varies the different feed sample lines, buffer inlets and flow-rates.

### 2.4. Chromatography for integrated endotoxin depletion from insoluble fractions

The *E. coli* insoluble fraction for purification was essentially prepared as described above with minor modifications. The biomass was resuspended in NiNTA buffer A, sonicated and clarified. Then, the resulting supernatant was discarded, the pellet resuspended in NiNTAdenat buffer A using an Ultra-Turrax T25 digital (IKA) with the dispersing element S25N-8G at 10 000 to 14,000 rpm for 30 to 60 s and the qTP added. Subsequent steps were carried out as described above.

### 2.5. Production, isolation and washing of inclusion bodies

To test the iED wash-step on proteins produced in inclusion bodies, the protein CTA1-DD was used. The sequence of CTA1-DD was described elsewhere [12]. Production was carried out as described for the qTP but with the plasmid pASK-IBA3plus_CTA1DD-6× his that carries the CTA1-DD gene fused to a C-terminal 6× His-tag. The *E. coli* biomass derived from the CTA1-DD expression was resuspended in lysis buffer as described above. Cell disruption was performed by high-pressure homogenization with a PANDA2000 (GEA Niro Soavi) at 800–1200 bar by 3 passages. Then, the cell lysate was clarified by centrifugation at 25000*g* for 30 min at 4 °C and the supernatant discarded. The insoluble fraction contained the CTA1-DD inclusion bodies that were washed three times either using the control buffer (1× PBS, 1% TX110, 1 mM EDTA at pH 7.4) or the endotoxin depletion buffer (1× PBS, 1% TX110, 1% TX114, 1 mM EDTA at pH 7.4). The endotoxin was measured after each step from resuspended pellets using NiNTAdenat buffer A.

### 2.6. Spin-column purification for integrated endotoxin depletion from supernatants

The *E. coli* insoluble fraction for purification was essentially prepared as described above with minor modifications. The biomass was resuspended in NiNTA buffer A, sonicated and clarified. Then, the resulting supernatant was discarded, the pellet resuspended in NiNTAdenat buffer A using an Ultra-Turrax T25 digital (IKA) with the dispersing element S25N-8G at 10000 to 14,000 rpm for 30 to 60 s and the qTP added. Subsequent steps were carried out as described above.

### 2.7. Determination of Triton X-114 phase-separation

10 ml of the TX114 buffers were filled into 50 ml conical tubes and incubated under temperature controlled conditions. Starting at the lowest temperature, the buffers were thoroughly mixed and