



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 H₂O, 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 MgSO₄, 100 mg/l Ampicillin sodium salt or 100 mg/l Kanamycin sulfate, 0.2 g/l PPG2000 and inoculated to an OD₆₀₀ of 0.1. The production-culture was grown at 37 °C with 225 rpm. After reaching an OD₆₀₀ 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 7000g 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 17000g 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 * 10³, 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 NiNTA 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 25000g 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 NiNTA 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 NiNTA 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

conditioned for at least 4 h before phase separation was visually assessed.

2.8. SDS-PAGE

SDS-PAGES were performed using the Bolt®-system (Thermo Fisher Scientific) with 4–12% Bis-Tris Plus precast gels. Gels were stained with Quick Coomassie™ Stain (Serva Electrophoresis), having a detection limit of 5 ng per band.

2.9. Quantification of endotoxin levels

The endotoxin level was determined using the EndoLISA® ELISA-based Endotoxin Detection Assay (Hyglos) according to manufacturer's instructions. Fluorescence was measured using an Infinite® F200 pro fluorescence reader (Tecan) equipped with a 380/20 nm and a 465/35 nm excitation and emission band-pass filter, respectively. To ensure measurements are within the standard curve, proteins were diluted 10 to 1,000,000-fold with endotoxin free water. For data analysis, a linear fit was used to fit the standard curve according to the manufacturer's instructions, where fluorescence values ranging from 0.05 to 50 EU/ml were within the dynamic range.

2.10. Determination of efficiency factors and relative costs

The endotoxin reduction factor (ERF) is calculated by Eq. (A1):

$$\text{ERF} = \frac{\text{Endotoxin}_{\text{feed}}}{\text{Endotoxin}_{\text{eluate}}} \quad (\text{A1})$$

The endotoxin reduction efficiency (ERE) is calculated by Eq. (A2):

$$\text{ERE} = \frac{\text{ERF}}{\text{runtime}} \quad (\text{A2})$$

The iED wash-step improvement factor (iEDws-IF) is calculated by Eq. (A3):

$$\text{iEDws-IF} = \frac{\text{ERF}_{\text{w/iED}}}{\text{ERF}_{\text{w/o iED}}} \quad (\text{A3})$$

To calculate the cost increase of the iED wash-step chromatography, we assumed that personal- and facility-costs are much higher than TX114 wash buffer costs. Thus, costs for chemicals and buffers were neglected and only the runtime of the chromatographies wash-steps factored into the equation. The cost increase was normalized to the longest iED wash-step that was tested. The costs were calculated by Eq. (A4):

$$\text{cost increase}_{\text{iED wash step}} = \frac{\text{runtime}_{\text{iED wash step}} + \text{runtime}_{\text{wash}}}{\text{runtime}_{\text{longest iED wash step}} + \text{runtime}_{\text{wash}}} \quad (\text{A4})$$

2.11. Design of Experiments and sweet-spot analysis

For experimental planning, analysis and subsequent visualization of the DoE-guided experiments, the software MODDE version 11 (Umetrics) was used. As objective, the response surface optimization (RSM) was selected and the suggested experimental design central composite face centered (CCF) with a star distance of 1 chosen. The experiment included 27 conditions, including 3 center points, and was executed in the randomized order as predetermined by the software. The software's model equation used for fitting is:

$$\begin{aligned} y &= \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 + \dots + \beta_{12} \cdot x_1 \cdot x_2 + \dots + \beta_{11} \cdot x_1^2 + \beta_{22} \cdot x_2^2 \dots + \varepsilon \\ &= \beta_0 + \sum_{i=1}^n \beta_i \cdot x_i + \sum_{j<i} \sum \beta_{ji} \cdot x_j \cdot x_i + \sum_{i=1}^n \beta_{ii} \cdot x_i^2 + \varepsilon \end{aligned} \quad (\text{B1})$$

β_0 = Intercept β_1 = regression coefficient (linear effects) β_{ij} = regression coefficient (interaction effects) β_{ii} = regression coefficient (quadratic

effects)

For evaluation, insignificant terms were removed until the model was not further improved. The experiment was valid for interpretation when the fitting quality parameters were above the cutoffs defined by the software: R^2 , Q^2 , model validity and reproducibility were > 0.5 , 0.2 , 0.25 and 0.6 , respectively.

In case of very high R^2 , Q^2 and reproducibility, a negative value for the model validity was accepted as suggested by the software [13].

To identify the sweet-spot, we first selected the cut-off values of the experimental results according to the rationale of the respective application scenario. Subsequently, the software analyzed the inputs and identified the sweet-spot. To calculate the regions where all or none of the requirements are met, MODDE uses a Nelder Mead simplex methodology, a numerical method used to find the minimum or maximum of an objective function in a multidimensional space.

3. Results

3.1. Broad application of the integrated endotoxin wash-step

We set out to complement already published knowledge by testing the iED wash-step on all commonly used chromatographies and under different chromatography conditions. We spiked *E. coli* lysate with the quad-tag protein (qTP) – an artificial fusion protein that integrates four affinity tags (Suppl. Fig. 1A and B) – or selected model-proteins. We then performed chromatographies with an additional 15 CV wash-step that either included TX114 for endotoxin depletion or lacked TX114 as control (Suppl. Fig. 1C). Finally, we compared the eluates' endotoxin levels and purification yields (Fig. 1).

For NiNTA-, MBP-, SAC-, GST-, Protein A- and CEX- chromatographies the iED wash-step depletes endotoxin consistently to levels below 35 EU/ml (Fig. 1A and Suppl. Fig. 2A). Endotoxin was reduced 200.000-fold by NiNTA- and Protein A- chromatography, 70.000-fold by GST- chromatography, approximately 40.000-fold by MBP- and SAC- chromatography and 2.000-fold by CEX- chromatography. In contrast, HIC and AEX-chromatography did not allow for an efficient endotoxin depletion. HIC-chromatography could not be tested as TX114 does not dissolve in commonly used high-salt binding-buffers. AEX chromatography only reduced the endotoxin level approximately 7-fold to 50,000 EU/ml and was not significantly improved by the iED wash-step. During all chromatographies, the iED wash-step did not influence the chromatographies' yields that remained above 95% (Fig. 1B and Suppl. Fig. 1D).

Without the iED-wash step, NiNTA-, GST- and Protein A- chromatography still allowed a 950-fold, 370-fold and 3100-fold endotoxin depletion, respectively. Therefore, even if TX114 is omitted, these chromatographies allow for a first, basic endotoxin depletion. Compared to that, MBP-, SAC- and CEX- chromatography without TX114 only lowered endotoxin levels 25-fold, 31-fold and 10-fold, respectively. Thus, except for AEX and HIC, the iED wash-step improves endotoxin reduction 70- to 2700-fold for the tested chromatographies (Suppl. Fig. 2B).

Furthermore, the iED wash-step depletes endotoxins effectively under denaturing conditions, at room temperature and when integrated with the solvent-detergent treatment. Under these conditions, TX114 buffers may turn cloudy or phase-separate depending on the experimental conditions. When TX114 buffers are used at temperatures above 14 °C, phase-separation occurs depending on the buffer's ionic strength (Suppl. Fig. 3A). To avoid an influence on the chromatography, the buffer can be stirred constantly or, ideally, phase separation is avoided altogether by reducing the buffer's ionic strength. The chromatography is not influenced when the buffer turns cloudy but does not phase separate. However, when chaotropic agents like urea or guanidine are used at high molarity, the buffers stay transparent without phase-separation.

The iED wash-step can also be integrated into more complex

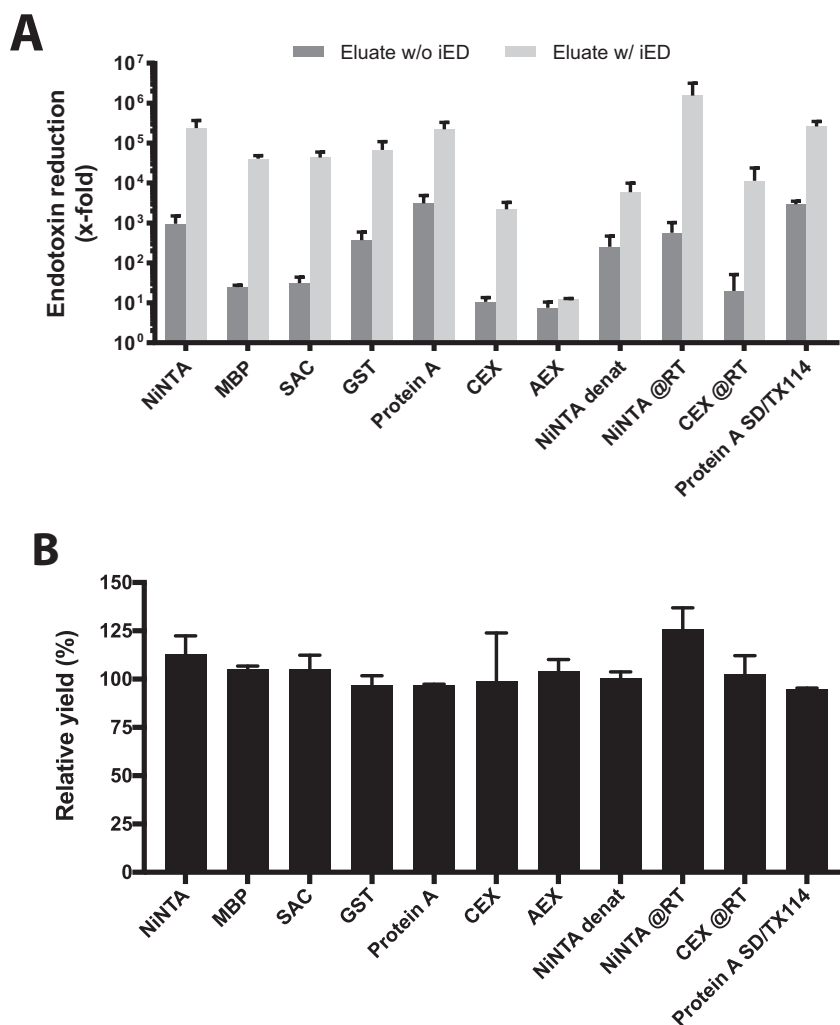


Fig. 1. The integrated endotoxin depletion wash-step is broadly applicable.

Proteins were purified using different chromatographies and conditions. A) Comparison of the endotoxin reduction levels after the chromatography without integrated endotoxin depletion (medium grey) and with integrated endotoxin depletion (light grey). B) From the same experiments, the relative yield was determined by comparing the amount of protein recovered by the iED wash-step to its control. Results of three independent experiments are shown as mean \pm S.D.

chromatographies with on-column refolding under reducing conditions (data not shown), upstream processes like inclusion body washing (Suppl. Fig. 3C) and spin-column purifications (Suppl. Fig. 3D). Under all these conditions, the iED wash-step did not impact the chromatographies' yields. We integrated the iED wash-step into the capture step of our routine protein purification protocols for many different proteins and reliably deplete endotoxins (Suppl. Fig. 3B).

3.2. Dependence of the iED wash-step efficiency on the feed's endotoxin level

To test the efficiency of the iED wash-step at different stages of a downstream-process, we investigated whether the feed's endotoxin level influences the endotoxin reduction efficiency. We serially diluted an *E. coli* lysate, spiked it with the qTP and measured the iED wash-step's endotoxin reduction factor (ERF, Fig. 2A). The higher the feed's endotoxin level, the higher the ERF. When the feed's endotoxin level is 10^6 EU/ml, the iED wash-step reduces it up to 10^5 -fold. With decreasing endotoxin levels in the feed, the ERF decreases linearly. Nevertheless, endotoxin levels were consistently reduced below 30 EU/ml (Fig. 2B).

3.3. Characterization of the iED wash-step by Design of Experiments

Next, we characterized the iED wash-step variables that are most relevant for its practical application using a NiNTA chromatography as model system. Expanding on the published protocols, we also analyzed whether an addition of TX114 to the chromatography's feed reduces the eluate's endotoxin level.

To investigate this multi-parameter system, we used a Design of Experiments approach (DoE) that is ideally suited for chromatography [14]. This allows for an in-depth analysis of the influence and interplay of individual, easily changeable process parameters affecting endotoxin depletion. The DoE design-space is comprised of four variables: the TX114 concentration in the feed, the TX114 concentration in the wash-buffer, the wash-step flow rate and the wash volume. As DoE-objective, we chose a Central Composite Face-Centered (CCF) optimization design that combines high- mid- and low variable settings resulting in 27 NiNTA chromatography runs (Table 1 and Suppl. Table 1). As readout, we measured the endotoxin level in the eluate and the relative yield referenced to the center-point (Suppl. Table 1).

The DoE model-fit had a high quality for both readouts. The R^2 - and Q^2 -values of the endotoxin content were 0.90 and 0.77, respectively, and the R^2 - and Q^2 -values of the relative yield were 0.82 and 0.49,

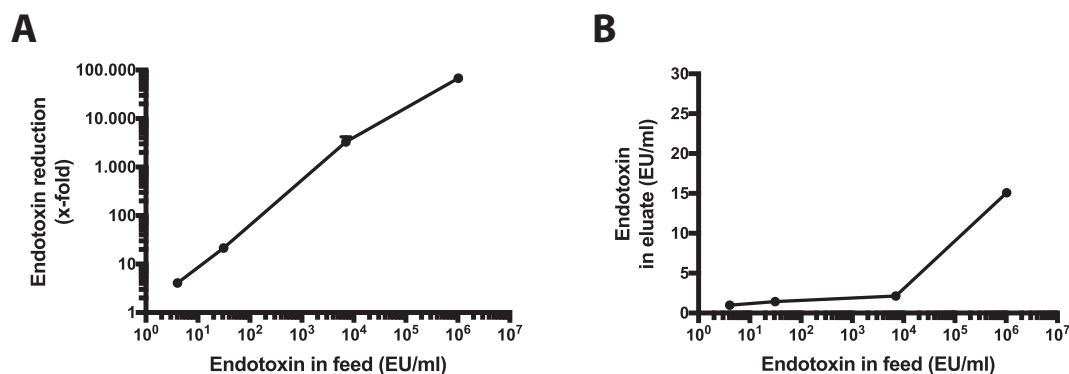


Fig. 2. The iED wash-step efficiency depends on the feed's endotoxin level.

A) The qTP was spiked into *E. coli* lysates with different endotoxin levels, purified by NiNTA and the resulting eluates' endotoxin levels measured. The endotoxin reduction factors in fold-change are plotted against the endotoxin levels of their feed solutions. B) The absolute endotoxin levels of the eluates from the same experiment are plotted against the endotoxin levels from their feed solutions. Results of three independent experiments are shown as mean \pm S.D.

Table 1

The design space of the DoE-guided iED wash-step characterization.

The DoE characterizes the impact of four chosen variables on selected readouts. The table lists the DoE's variables, their corresponding labels, units and range. The range defines the overall design space that the DoE characterizes. The measured readouts are the "endotoxin level in the eluate" and the "relative yield".

Variable	Label	Unit	Min	Center	Max
TX114 concentration in feed	cTXf	%	0.0	1.0	2.0
TX114 concentration in wash-buffer	cTXw	%	0.0	1.0	2.0
Wash length	Wcv	CV	15	52.5	90
Wash flow rate	Wfr	ml/min	0.5	1.75	3.0

respectively. The center point showed very high reproducibility (Suppl. Fig. 4A). Thus, the DoE-model fit allowed data-interpretation.

The resulting model-coefficients identify and quantify the impact of the iED wash-step variables on the endotoxin level and the yield. The endotoxin level in the eluate is strongly reduced by adding TX114 to the feed and to the wash buffer (Fig. 3A, Suppl. Fig. 4B). Notably, increasing the TX114 concentrations deplete more endotoxins but with decreasing marginal effect. In contrast, the wash-step's flow-rate and the wash-volume have a limited impact. Larger wash-volumes and slower flow-rates lead to slightly lower endotoxin levels.

The chromatography yields are between 80% and 115% and are affected by changes of the iED wash-step variables (Fig. 3B and Suppl. Fig. 4C): larger wash-volumes and lower flow-rates decrease the yield. This effect is likely to be more pronounced for chromatographies with unfavourable on-off rates, as it would be the case for the *Strep-tag*[®] II [15]. Surprisingly, the addition of TX114 to the feed solution can increase the yield by 15%.

3.4. Identifying optimal iED wash-step settings for different application scenarios

After quantifying the influence of the iED wash-step variables, we isolated ideal conditions using a sweet-spot analysis. While the DoE characterizes the design space of multiple readouts, the sweet-spot analysis combines them along with other external factors – e.g. the personnel costs during a manufacturing plant operation – to identify optimal conditions. We performed sweet-spot analysis for three distinct application scenarios that are relevant to academia and industry (Fig. 4):

- What are the iED wash-step conditions that lead to the lowest possible endotoxin levels? The optimal conditions are centered around adding 1.25% TX114 to the feed and washing with 90 CV of a wash buffer including 1.5% TX114 at the slowest tested flow rate

of 0.5 ml/min.

- What are the iED wash-step conditions that lead to the lowest endotoxin levels possible while keeping the yield at 100%? The optimal conditions are centered around adding 1.25% TX114 to the feed and washing with 52.5 CV of a wash buffer including 1.25% TX114 at the fastest possible flow rate of 3.0 ml/min.
- What are the iED wash-step conditions that lead to the lowest endotoxin levels possible while keeping the run-time as short as possible and thus, personnel and production plant operation costs as low as possible? The optimal conditions are centered around adding 1.5% TX114 to the feed and washing with 15 CV of a wash buffer including 0.4% TX114 at the fastest possible flow rate of 3.0 ml/min.

4. Discussion

The iED wash-step depletes endotoxin efficiently in many chromatography types and under various conditions. Using the iED wash-step during capture-purification, the endotoxin reduction factor increases 20- to 2700-fold compared to the standard chromatography without TX114. However, the reduction factors of the different chromatographies cannot be compared directly, as the feeds endotoxin levels varied. This is likely a result of varying lysis efficiencies, as each feed was prepared with a different lysis buffer that was needed for the respective chromatography. Nevertheless, the iED wash-step consistently lowers endotoxin levels to below 35 EU/ml. According to the US Pharmacopoeia, endotoxin levels lower than 35 EU/ml would allow for application of an exemplary, injectable, non-intrathecal drug product with a maximum whole-body dose of 1 g at an assumed body weight of 70 kg, as it may be the case with monoclonal antibodies.

The iED wash-step does not affect yields that remain above 95%. This shows that the uncharged TX114 detergent does not impair chromatographies that are based on non-hydrophobic interactions.

Furthermore, the iED wash-step can be used under native- or denaturing conditions, reducing- or non-reducing conditions and at temperatures ranging from 4 °C to room temperature. In addition, the iED wash-step can be further integrated into inclusion body washes or solvent-detergent-treatments. The iED wash-step is independent of the purification scale: it can be utilized in small-scale purifications with spin-columns or magnetic beads as well as in large-scale purifications on automated FPLC-systems using pre-packed research- and bioprocess-columns.

The only exceptions where the iED wash-step does not work are AEX- and HIC-chromatography. AEX is known to bind endotoxin tightly and is frequently used to deplete endotoxins in flow through mode [6]. It can be hypothesized that the charge-based endotoxin-AEX ligand interaction cannot be loosened by TX114 - a non-ionic detergent. Since

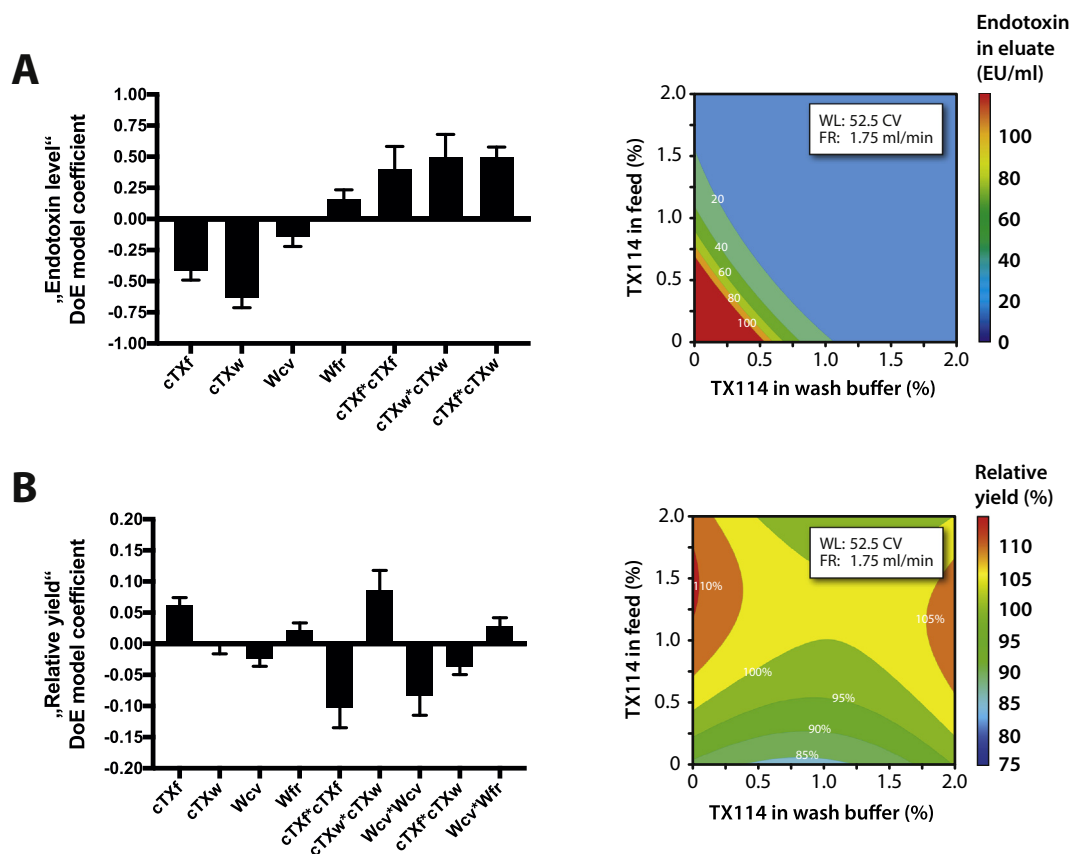


Fig. 3. The iED wash-step characterization identifies the most impactful parameters and quantifies their impact on endotoxin level reduction and relative yield. The DoE model coefficients are the primary output of the analysis and the resulting model-equation can be visualized in contour plots. The DoE quantifies the linear and quadratic impact of each variable and their pairwise interactions. Positive coefficient values lead to higher readouts, while negative coefficient-values lead to lower readouts. Coefficients that are not shown did not have an impact on the outcome and were removed during model-fitting. A) DoE-model coefficients plot for the eluate's endotoxin level (left panel) and a selected contour plot (right panel). B) DoE-model coefficients plot for the relative yield (left panel) and a selected contour plot (right panel). The selected contour plots visualize the model-equations for the most impactful variables TX114 concentration in the feed (cTXf) and TX114 concentration in the wash buffer (cTXw) with wash-length (WL) and flow-rate (FR) fixed at their center-values. The corresponding contour plots are shown in Supplemental Fig. 4.

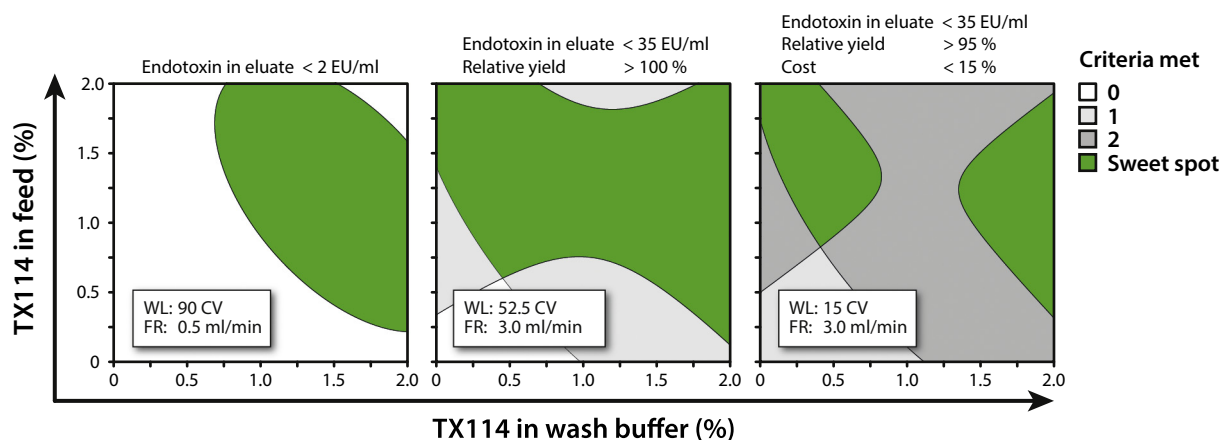


Fig. 4. The sweet-spot analysis identifies optimal parameters for different application scenarios of the iED wash-step. The sweet-spots for three application scenarios were identified and the contour plots with the largest sweet-spot area shown. The sweet-spot is composed of overlaid response contour plots with three dimensions. The third dimension (z-axis) is colour coded and visualizes the regions where all (green) or none (white) of the requirements are met and the first (x-axis) and second (y-axis) dimension represents two factors. The other factors are held constant at levels shown in the white boxes in the lower left corner of the graph. The contour plot shows the optimal condition to achieve the lowest endotoxin level (left panel), the lowest endotoxin level while maintaining yields > 100% (center panel) and the lowest endotoxin levels with highest yields for minimal additional chromatography-step costs (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

endotoxins are positively charged, as is the purified protein during AEX chromatography, they bind and elute alongside the protein during chromatography. For HIC, the TX114 does not dissolve in high salt buffers and is presumed to interfere with the hydrophobic protein-ligand interaction.

The iED wash-step's efficiency depends on several factors. The endotoxin depletion is more efficient when the feed's endotoxin levels are higher. Thus, the iED wash-step is ideally integrated into the capture step during downstream-processing. The efficiency drops with lower feed endotoxin levels, making it less effective at later downstream-processing stages. Further, we noticed that endotoxin levels below 0.5 EU/ml were not reached and ionically-bound endotoxin on AEX-chromatography could not be removed. This indicates that endotoxin bound by hydrophobic interactions can be depleted efficiently, while endotoxin bound by ionic interactions cannot. In case an ultra-low endotoxin content is required or the endotoxin selectively binds to the protein through ionic interactions, the iED wash-step can be complemented by another method for endotoxin removal that is based on an arginine wash-step [11].

The iED wash-step was characterized by a DoE-approach. We identified variables influencing the endotoxin depletion and quantified their impact on the endotoxin reduction efficiency and the chromatography yield. For endotoxin depletion, the most influential variables are the concentration of TX114 in the feed and in the iED wash-step buffer. Compared to that, the wash volume and flow-rate have minor effects. The yields were generally high but can be lowered by prolonged or slow washing. Surprisingly, we found that adding TX114 only to the feed depletes most endotoxin, can increase yields and leave the chromatography's method and run-time unchanged. Thus, adding TX114 to the feed offers multiple upsides while it has no apparent downside.

With the iED wash-step characterization, we identified its optimal conditions for three application scenarios relevant to academia and industry. Endotoxin depletion is very efficient when TX114 is added to the feed solution. If stronger endotoxin depletion is required, an iED wash-step can be introduced that can be optimized for maximum endotoxin depletion or highest cost-effectiveness by varying its TX114 concentration, flow-rate and wash-volumes.

The iED wash-step can thereby contribute to a higher quality of protein preparations in academia and industry alike. In academic research labs, the iED wash-step can consistently ensure protein preparations with low endotoxin levels and thereby prevent any artefacts in *in vitro* and *in vivo* assays. In industrial GMP manufacturing, the iED method can reduce costs significantly where downstream processes require additional endotoxin depletion steps. In these cases, the iED wash-step can integrate fast and efficient endotoxin depletion into an established downstream process, thereby avoiding higher costs, reduction of yield and further stress of the protein caused by additional membrane absorbers, extra chromatography steps or crossflow filtrations. Thus, the iED wash-step leads to fast and efficient endotoxin depletion that replaces the need for any additional endotoxin depleting downstream steps.

5. Conclusion

We demonstrate that the iED wash-step is broadly applicable to deplete endotoxin during protein purification. We believe that its characterization and optimization for different application scenarios enables widespread adoption in academia and industry for routine, efficient and cost-effective depletion of endotoxin at any scale.

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Author contribution

TK and DK performed the experiments, TK and DK conceived the experiments and analyzed the data, TK wrote the manuscript and UM, DK, TK critically revised the manuscript. All authors read and approved the final manuscript.

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Declarations of interest

None.

Author information

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2018.05.029>.

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