3M Science. Applied to Life.™

Tech Note 02

# Identification and tracking of problematic host cell proteins when using 3M™ Emphaze™ AEX Hybrid Purifier in mAb manufacturing processes.

*Adapted from S. Gilgunn, H. El-Sabbahy, S. Albrecht, M. Gaikwad, K. Corrigan, L. Deakin, G. Jellum, J. Bones, Identification and tracking of problematic host cell proteins removed by a synthetic, highly functionalized nonwoven media in downstream bioprocessing of monoclonal antibodies, J. Chromatography. A., 1595 (2019), pp. 28-38.*


## Summary

During the purification of a monoclonal antibody (mAb), host cell proteins (HCPs) were identified and tracked following clarification and Protein A chromatography. This study explored the impact of implementing Emphaze™ AEX Hybrid Purifier during clarification on the removal of problematic HCPs in downstream mAb purification. HCPs in the Protein A eluate generated from material clarified using 3M depth filtration with and without Emphaze™ AEX Hybrid Purifier were identified and quantified over multiple chromatography cycles using advanced proteomic methods. Chromatographic clarification with Emphaze™ AEX Hybrid Purifier coupled to Protein A chromatography enabled the removal of commonly occurring and problematic HCPs, including: serine protease HTRA1, matrix metalloproteinase-19 and protein S100-A6 among others. Additionally, when compared to 3M depth filtration alone, the implementation of Emphaze™ AEX Hybrid Purifier followed by Protein A chromatography provided an average 38-fold higher reduction in HCP levels and a 2.3 LRV lower concentration in host cell DNA (HCDNA).


## Introduction

Monoclonal antibodies are typically produced in cells leading to the generation of impurities such as the cells themselves, cell debris, HCDNA, and HCPs. These contaminants are present in the clarified cell culture fluid (CCCF) in addition to the product. Large, insoluble debris is mostly removed during clarification while soluble species including HCDNA and HCPs are commonly cleared during the downstream processing. FDA guidelines limit the amount of HCDNA in the final drug product to 10 ng/dose or less.[1] A maximum HCP limit of 100 ppm has been adopted across the industry, while processes in practice are designed to target lower level.[2,3]

The removal of HCPs during purification is challenging, as they constitute a wide range of proteins exhibiting different properties and characteristics such as isoelectric point, molecular weight, hydrophobicity and structure.[4] In addition, HCPs pose a threat to final drug product quality. A number of HCPs have the potential to degrade or modify the product. Other HCPs have the potential to induce an undesired immune response affecting the efficacy and safety of the therapeutic.[5,6]

Table 1 summarizes several problematic HCPs that were the focus of this study and are commonly encountered during mAb production using CHO-based cell culture.

Total HCP concentration is most commonly measured using Enzyme Linked Immunosorbent Assay (ELISAs). This technique often has low sensitivity and can preferentially detect certain HCPs, such those eliciting high immunogenicity, over other species. This study made use of sensitive liquid chromatography coupled to mass spectrometry (LC-MS) quantitation to identify HCPs and track their removal during clarification and across Protein A chromatography over multiple cycles to determine the effect of clarification strategy on the removal of problematic HCPs. The clarification approaches considered were 3M depth filtration and 3M depth filtration followed by chromatographic clarification with 3M™ Emphaze™ AEX Hybrid Purifier. Emphaze™ AEX Hybrid Purifier is a synthetic, multi-mechanism clarifying product containing both a Q-functional anion exchange nonwoven, and a microporous membrane for fine particle removal. The quaternary amine functional nonwoven provides mechanical sieving of particles, as well as a high anion exchange capacity for reduction of negatively charged DNA, HCP, endotoxin, and cell debris.

**Table 1.** *Common problematic HCPs present in mAb's derived from CHO-based cell culture*

| HCP Type | HCP Name | Gene | Location |
|---|---|---|---|
| **Degradative Enzymes** | Cathepsin B/D[7,8,9,10] | CTSB/D | Secreted, extracellular space |
| | Matrix Metalloproteinase-19[7,8] | MMP19 | Secreted, extracellular space |
| | Serine Protease HTRA1 [7,8,9] | HTRA1 | Secreted |
| | Protein disulfide-isomerase A6[8] | PD1A6 | Endoplasmic reticulum lumen |
| | Protein S100-A6[7,8] | S100A6 | Cell membrane/Nuclear Envelope |
| **Potentially Immunogenic HCPs** | 60s ribosomal protein L30[7] | RPL30 | Cytoplasm |
| | Annexin A5[7] | ANXAS | Cytoplasm |
| | C-X-C motif chemokine 3[7] | CXCL3 | Secreted |
| | Histone H3[7] | HIST1H3A | Nucleus |
| | Putative phospholipase B-like 2[7,8] | PLBD2 | Lysosome |

## Materials and Methods

**Feed Material.** Tocilizumab, a recombinant IgG1 biosimilar, was expressed using a CHO cell line to a titer of ~ 3.5 g/L. The feed stock was clarified through two clarification systems. As shown in Figure 1, the clarification approaches were: 3M depth filtration and a combination of 3M depth filtration and Emphaze™ AEX Hybrid Purifier. Following clarification the material was aliquoted and stored at -80 °C.

**Protein A Chromatography.** 1 mL MabSelect SuRe™ HiTrap Protein A columns coupled to an AKTA Avant were used for chromatography experiments. The steps performed, flow rates, and volumes for each feed material are shown in Table 2. The elution peak was automatically collected into tubes containing 250 µL and 300 µL of neutralization buffer (1 M Tris–HCl, pH 9) for Emphaze™ AEX Hybrid Purifier clarified material and 3M depth filter clarified material, respectively.

A preliminary Protein A chromatography study over 20 cycles was carry out to purify 3M depth filtered CCCF and Emphaze™ AEX Hybrid Purifier CCCF. After the 20th cycle, the Protein A columns were sanitized using 0.1 M NaOH. Chromatography runs were continued with these two feed streams for 100 additional cycles without any sanitization.

For Protein A purification of the 3M depth filtered CCCF, additional sanitization strategies were evaluated during the cycling study: mild sanitization with 0.1 M NaOH after every 5th cycle; and harsh sanitization using 0.5 M NaOH after every 3rd cycle.

**Quantification Techniques.** Antibody titer was measured using a 1 mL HiTrap Protein G column attached to an Agilent™ 1100 HPLC system. HCDNA recovered from Protein A eluates after every 20th cycle was quantified using qPCR. HCPs in the Protein A eluate were quantified using a ProteinSEQ™ CHO HCP Quantitation Kit, after every 20th Protein A cycles.

**HCP Identification and Analysis.** After every 20th Protein A cycle, aliquots of the eluate were collected, concentrated, and subjected to tryptic digestion. Digested samples were analyzed using data dependent LC-MS/MS. Proteomic data analysis of the resulting mass spectra was performed using Progenesis™ QI for Proteomics V 3.2 software after completing a database search using PEAKS software against the *Cricetus griseus* (Chinese Hamster) NCBI FASTA database.

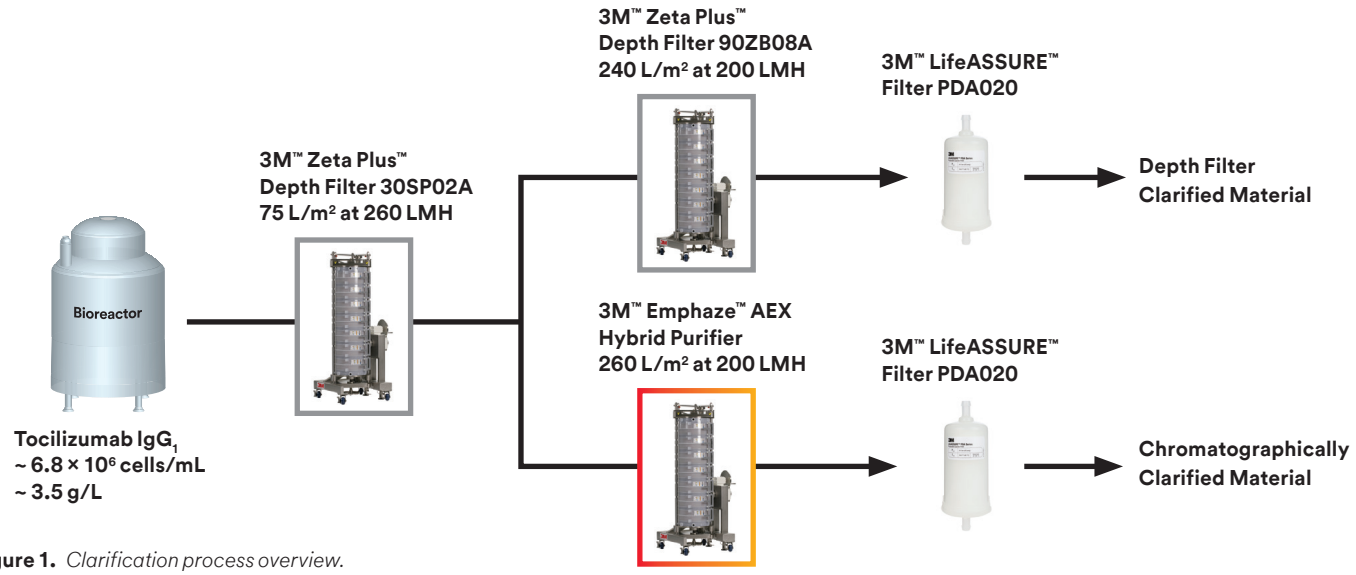


**Figure 1.** *Clarification process overview.*

**Table 2.** *Process steps, buffers, flow rates and volumes for the Protein A chromatography runs.*

| Step | Buffer/Sample | Flow rate (mL/min) | Volume (mL) |
|---|---|---|---|
| Equilibration | 20 mM sodium phosphate, 0.15 M NaCl, pH 7.0 | 0.5 | 10 |
| Feed | 3M™ Emphaze™ AEX Hybrid Purifier or 3M depth filter CCCF | 0.25 | 8 |
| Post-load wash 1 | Equilibration buffer | 0.25 | 1 |
| Post-load wash 2 | Equilibration buffer | 0.5 | 9 |
| Elution | 0.1 M sodium citrate, pH 3.2 | 0.5 | 8 |
| Acid strip | 500 mM acetic acid | 0.5 | 2 |
| Equilibration | Equilibration buffer | 0.5 | 10 |
| Sanitization | 100 mM NaOH/0.5M NaOH | 0.3 | 5 |
| Equilibration | Equilibration buffer | 0.5 | 10 |

## Results and Discussion

### *Quantification of HCPs and HCDNA levels.*

As presented in Figure 2, the use of Emphaze™ AEX Hybrid Purifier during clarification resulted in a significant improvement in HCP and HCDNA clearance as well as more consistent clearance across Protein A chromatography compared to material clarified by 3M depth filtration alone.

Over multiple Protein A cycles, chromatographic clarification with Emphaze™ AEX Hybrid Purifier provided a consistent HCP reduction to 100 ppm or less. In contrast, Protein A eluate from purification of 3M depth filtered material had HCP concentrations ranging from ~2000 to ~8000 ppm. For the 3M depth filter material, HCP concentration was observed to decrease as the number of Protein A cycles increased. This decrease may have been due to fouling of the Protein A resin leading to less nonspecific binding with increased cycle number and, therefore, less coelution of contaminants from the column.

The implementation of NaOH sanitization between Protein A cycles for purification of 3M depth filtered CCCF slightly improved HCP clearance, however, the eluate was still found to contain significantly higher HCP levels than eluate generated from material clarified with 3M™ Emphaze™ AEX Hybrid Purifier. For instance, harsh sanitization conditions between Protein A cycles during purification of 3M depth filtered material reduced the total HCP concentration but still had, on average, a 38x higher HCP levels than the eluate from material clarified using Emphaze™ AEX Hybrid Purifier.

Incorporation of Emphaze™ AEX Hybrid Purifier during clarification provided increased HCDNA clearance across Protein A chromatography. Presented in Figure 2B, the HCDNA concentration in Protein A eluates generated from Emphaze™ AEX Hybrid Purifier were on average 2.3 LRV lower than material clarified by 3M depth filtration alone.

When Emphaze™ AEX Hybrid Purifier was implemented during clarification, Protein A chromatography performance was maintained over multiple cycles: HCDNA concentration in the Protein A eluate remained low and, taking into account assay error due to the low DNA concentration, was consistently around 10 ppb over 100 cycles without sanitization. In contrast, HCDNA concentration in eluates generated from material clarified by 3M depth filtration alone appeared to follow a downward trend as the number of Protein A cycles increased. This trend was most pronounced when sanitization of the Protein A resin was not performed, but was still present for the mild and harsh sanitization regimes and is thought to be due to resin fouling.

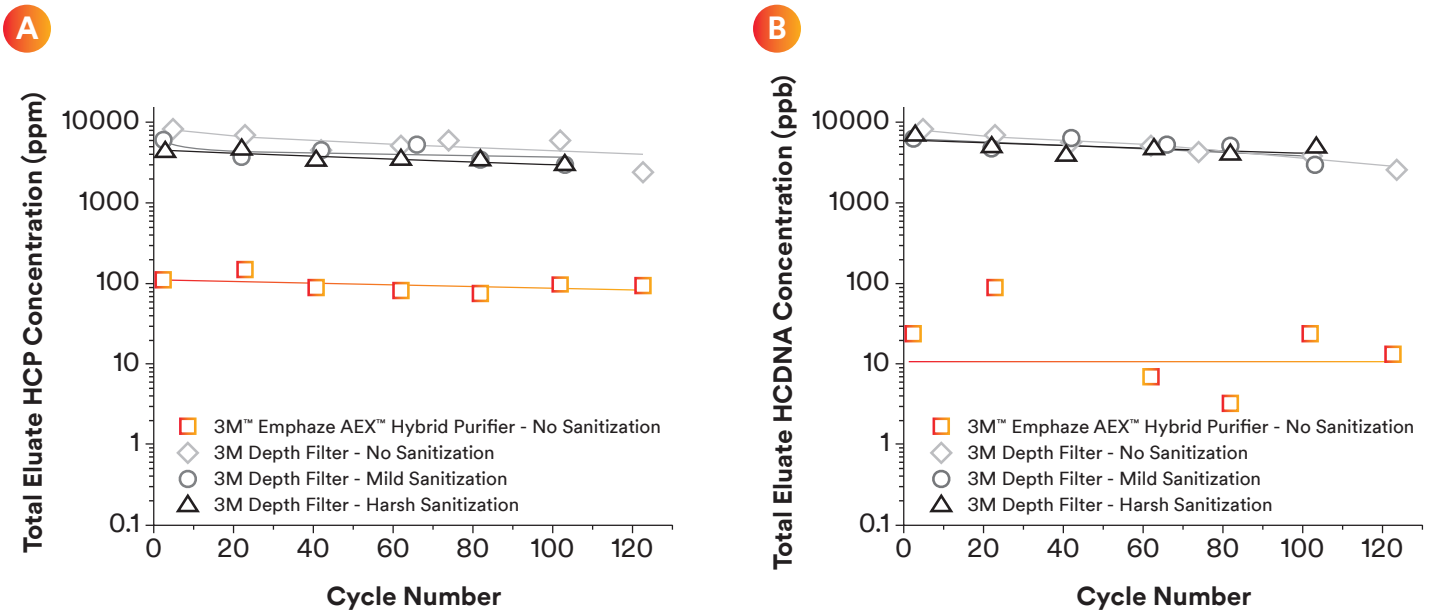


**Figure 2.** *(A) HCP concentration in Protein A eluate over multiple chromatography cycles. (B) HCDNA concentration in Protein A eluate over multiple chromatography cycles.*

## *Analysis and Categorization of Problematic HCPs.*

Proteins difficult to remove through the purification process or that affect the safety and efficacy of the final drug are commonly termed problematic HCPs.[9] Failure to reduce these proteins has potential impact on product quality, as HCPs can cause aggregate formation or catalyze fragmentation and degradation of the product.[11] HCPs not only have the potential to impact product quality, but also to negatively impact patient safety. Problematic HCPs identified in this study were divided into three categories: (1) histones, (2) degradative proteins or (3) immunogenic proteins. Figure 3 shows the concentration of select challenging HCPs from each of these categories following clarification and after Protein A chromatography.

Histones are a component of chromatin. Nonspecific binding of chromatin heteroaggregates (consisting of DNA, histones, non-histone proteins, and aberrant forms of IgG) to Protein A has been shown to affect both Protein A column capacity and eluate purity. Removal of chromatin during clarification, as demonstrated by the removal of histones, has the potential to improve Protein A performance.[11] Following 3M depth filtration, histones H2B and H3 were still present in the filtrate and persisted through Protein A. In contrast, clarification with Emphaze™ AEX Hybrid Purifier removed these histones below the limit of detection. The level of histones in the Protein A eluate generated from 3M Emphaze AEX Hybrid Purifier pool remained very low and close to the limit of detection.

*Proteolytic HCPs* are enzymatically active proteins that have the potential to impact the quality of the target mAb through degradation, aggregation and/or fragmentation. 3M™ Emphaze™ AEX Hybrid Purifier resulted in lower proteolytic HCP levels during clarification compared to 3M depth filtered CCCF. Further, the use of 3M Emphaze AEX Hybrid Purifier together with Protein A removed the identified degradative HCPs.

*Immunogenic HCPs* are impurities that have the potential to elicit an immune response in patients. The three immunogenic proteins presented in Figure 3 have a high immunogenicity score derived from CHOPPI, a web tool specifically developed for determination of immunogenicity risk of HCPs in CHO-based protein therapeutics.

C-X-C Motif Chemokine 3 was removed by 3M Emphaze AEX Hybrid Purifier during primary clarification while 3M depth filtration alone was not able to clear it. Protein S100-A6 was present following both depth filtration and chromatographic clarification with Emphaze™ AEX Hybrid Purifier. This protein was detected in the Protein A eluate generated from the 3M depth filtered material, however, it was below the limit of detection in Protein A eluate produced from material clarified using Emphaze™ AEX Hybrid Purifier. The third immunogenic HCP presented in Figure 3, PLBL2, has the potential to bind to humanized mAbs making clearance of this HCP challenging. Regardless of clarification approach, PLBL2 was not found in the Protein A eluate.

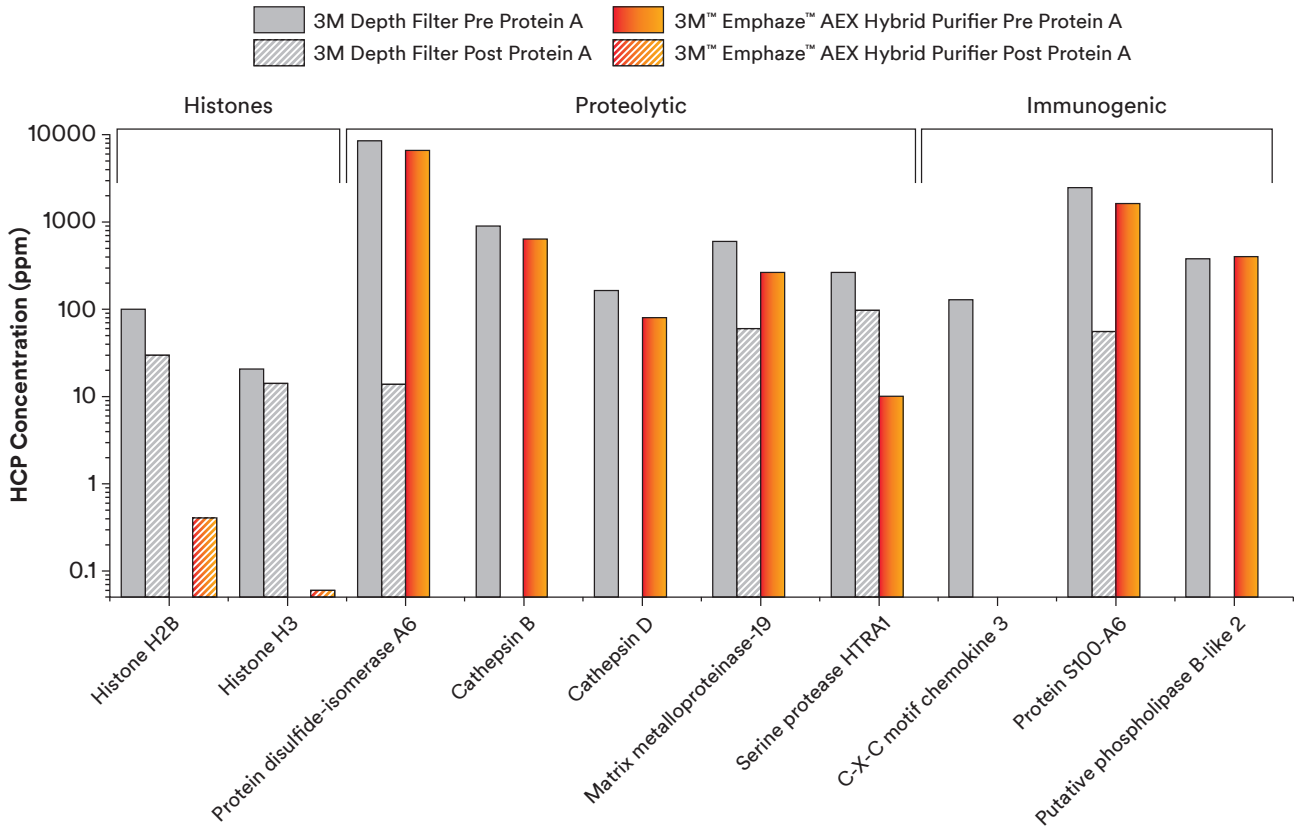


**Figure 3.** *Removal of select problematic HCPs during primary clarification through Protein A chromatography.*

## *Tracking Common and Problematic HCPs in Protein A Eluate.*

Several of the more commonly observed HCPs, as well as, select problematic HCPs were tracked in the Protein A eluate over 100 chromatography cycles (see Figure 4). The use of different sanitization conditions between Protein A cycles for purification of 3M depth filtered CCCF was found to impact the level and number of distinct HCPs present in the eluate.

Relative to no sanitization, mild sanitization of the Protein A resin was found to reduce the concentration of HCPs in the Protein A eluate. When harsh sanitization conditions were applied, the highest concentrations and greatest variety of HCPs were present in the Protein A eluate.

Protein A eluate from 3M™ Emphaze™ AEX Hybrid Purifier clarified material showed good clearance of all the problematic HCPs considered and significant reduction of a majority of the commonly observed HCPs. The remaining commonly observed HCPs not cleared by the synergetic effects of Emphaze™ AEX Hybrid Purifier and Protein A chromatography were present in the eluate at concentrations lower than those in the eluate generated from 3M depth filter clarified material.

Some HCPs including lipoprotein lipase and nidogen are considered particularly difficult to remove as they associate with many different mAbs and coelute over several different types of polishing chromatography. Nidogen 1 was cleared by the combination of chromatographic clarification and Protein A chromatography. The concentration of lipoprotein lipase was reduced by approximately 1 order of magnitude.

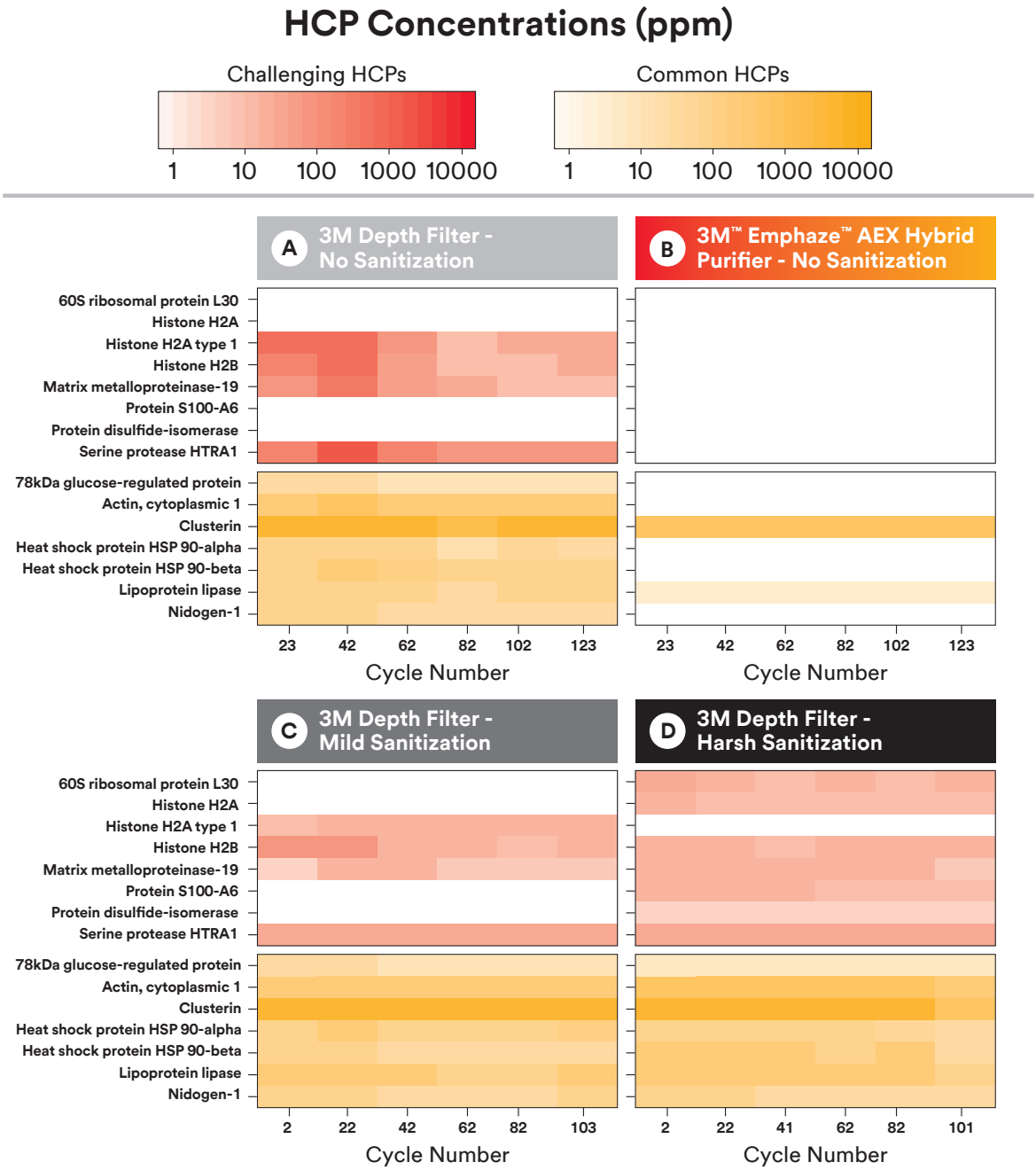


**Figure 4.** *Heat maps representing problematic (red) and commonly (orange) occurring HCPs concentration in the Protein A eluates generated during the cycling experiments. Graph (A) shows cycling experiments with 3M depth filter clarified material with no sanitization during 100 cycles, (B) shows 100 cycles with Emphaze™ AEX Hybrid Purifier clarified material and no sanitization, (C) shows 100 cycles with mild sanitization conditions, and (D) shows 100 cycles with harsh sanitization regime. Colour intensity corresponds to concentration: the darker colour the greater the HCP concentration.*

### *Resin Fouling*

In this study reduced Protein A fouling was also noted. A column sanitization in the final cycle for all the cleaning strategies was carried out using 0.1 M NaOH for the no sanitization and mild sanitization regimes, and 0.5 M NaOH for the harsh sanitization condition. As shown in Figure 5, LC/MS-MS analysis of each sanitization fraction showed a difference in the number of HCPs identified for the 3M depth filter clarified material that underwent no sanitization (74 HCPs), mild sanitization (61 HCPs), and harsh sanitization (96 HCPs). More notably, the number of HCPs present in the final sanitization fraction for the material clarified with 3M™ Emphaze™ AEX Hybrid Purifier was 3.5 times less than the mild sanitization for the 3M depth filtered material, indicating there was less overall fouling of the Protein A column over 100 cycles.

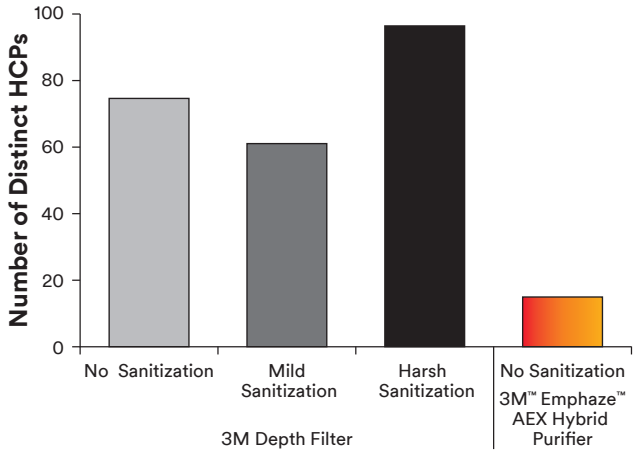


**Figure 5.** *Number of HCPs present in samples taken from sanitization following 100 cycles of Protein A chromatography under the different conditions evaluated.*

## Conclusions

Implementation of Emphaze™ AEX Hybrid Purifier in conjunction with Protein A chromatography cleared to less than the limit of detection all problematic HCPs tracked in this study and the majority of commonly occurring HCPs with consistent clearance over 100 cycles of Protein A. In contrast, 3M depth filtration in conjunction with Protein A chromatography was not able to clear many of these HCPs.

On average, the total levels of HCP and HCDNA following Protein A were 38 fold and 2.3 log lower, respectively, for chromatographically clarified CCCF compared to depth filter CCCF. The Protein A eluate HCP and HCDNA concentrations were also consistent over 100 chromatography cycles without sanitization of the chromatography column for Emphaze™ AEX Hybrid Purifier Clarified material. This was not the case for 3M depth filter clarified material.

Implementation of Emphaze™ AEX Hybrid Purifier during mAb clarification process can substantially enhance purification performance of the Protein A column by significantly reducing DNA and HCP load. In addition, clarification with 3M Emphaze AEX Hybrid Purifier in conjunction with Protein A chromatography may reduce HCPs that are known to persist through the polishing train. Integration of Emphaze™ AEX Hybrid Purifier in the clarification stage may simplify mAb purification by reducing multiple types of impurities in a single process step, leading to better process economics.

## References


1 H. Yang, "Establishing Acceptable Limits of Residual DNA," *PDA, J. Pharm. Sci. Technol,* no. 67, pp. 155-163, 2013.

2 C. de Zafra, V. Quarmby, K. Francissen, M. Vanderlaan and J. Zhu-Shimoni, "Host cell proteins in biotechnology-derived products: A risk assessment framework," *Biotechnol. Bioeng.,* no. 112, p. 2284–2291., 2015.

3 K. N. Valente, N. E. N.E. Levy, K. H. K.H. Lee and A. M. Lenhoff, "Applications of proteomic methods for CHO host cell protein characterization in biopharmaceutical manufacturing.," *Curr. Opin. Biotechnol.,* no. 53, pp. 144-150, 2018.

4 A. Tscheliessnig, J. Konrath, R. Bates and A. Junbauer, "Host cell protein analysis in therapeutic bioprocessing - methods and applications.," *Biotechnol. J.,* no. 8, pp. 655-670, 2013.

5 Y. H. Kao, D. Hewitt, M. Trexler- Schmidt and M. Laird, "Mechanism of antibodyreduction in cell culture production processes.," *Biotechnol. Bioeng.,* no. 107, pp. 622-632, 2010.

6 M. Trexler-Schmidt, S. Sargis, J. Chiu, S. Sze-Khoo, M. Mun, Y. Kao and M. Laird, "Identification and prevention of antibody disulfide bond reduction during cell culture manufacturing.," *Biotechnol. Bioeng.,* no. 106, pp. 452-461, 2010.

7 A. Farrell, S. Mittermayr, B. Morrisey, N. Mc Loughlin, N. Navas Iglesias, I. Marison and J. Bones, "Quantitative Host Cell Protein Analysis Using Two Dimensional Data Independent LC–MSE.," *Anal. Chem,* no. 87, p. 9186–9193, 2015.

8 N. Aboulaich, W. K. Chung, J. Thompson, C. Larkin, D. Robbins and M. Zhu, "A Novel Approach to Monitor Clearance of Host Cell Proteins Associated With Monoclonal Antibodies," *Biotechnol. Prog.,* no. 30, pp. 1114-1124, 2014.

9 N. Levy, K. Valente, L. Choe, K. Lee and A. Lenhoff, "Identification and characterization of host cell protein product-associated impurities in monoclonal antibody bioprocessing: HCP-mAb Product-Associated Impurities," *Biotechnol. Bioeng.,* no. 111, pp. 904-912, 2014.

10 J. Bee, L. Tie, D. Johnson, M. Dimitrova, K. Jusino and C. Afdahl, "Trace levels of the CHO host cell protease cathepsin D caused particle formation in a monoclonal antibody product," *Biotechnol. Prog.,* no. 31, pp. 1360-1369, 2015.

11 C. Goey, S. Alhuthali and C. Kontoravdi, "Host cell protein removal from biopharmaceutical preparations: Towards the implementation of quality by design.," *Biotechnol. Adv.,* no. 36, pp. 1223-1237, 2019.

**Intended Use:** Single-use processing of aqueous based biological pharmaceuticals (drugs) and vaccines strictly following the product operating instructions and cGMP requirements, where applicable.

**Prohibited Use:** As a component in a medical device that is regulated by any agency, and/or globally exemplary agencies, including but not limited to: a) FDA, b) European Medical Device Directive (MDD), c) Japan Pharmaceuticals and Medical Devices Agency (PMDA); Applications involving permanent implantation into the body; Life-sustaining medical applications; Applications requiring food contact compliance.

**Technical Information:** The technical information, guidance, and other statements contained in this document or otherwise provided by 3M are based upon records, tests, or experience that 3M believes to be reliable, but the accuracy, completeness, and representative nature of such information is not guaranteed. Such information is intended for people with knowledge and technical skills sufficient to assess and apply their own informed judgment to the information. No license under any 3M or third party intellectual property rights is granted or implied with this information.

**Product Selection and Use:** Many factors beyond 3M's control and uniquely within user's knowledge and control can affect the use and performance of a 3M product in a particular application. As a result, end-user is solely responsible for evaluating the product and determining whether it is appropriate and suitable for end-user's application, including completing a risk assessment that considers the product leachable characteristics and its impact on drug safety conducting a workplace hazard assessment and reviewing all applicable regulations and standards (e.g., OSHA, ANSI, etc.). Failure to properly evaluate, select, and use a 3M product and appropriate safety products, or to meet all applicable safety regulations, may result in injury, sickness, death, and/or harm to property.

**Warranty, Limited Remedy, and Disclaimer:** Unless a different warranty is specifically stated on the applicable 3M product packaging or product literature (in which case such warranty governs), 3M warrants that each 3M product meets the applicable 3M product specification at the time 3M ships the product. 3M MAKES NO OTHER WARRANTIES OR CONDITIONS, EXPRESS OR IMPLIED, INCLUDING, BUT NOT LIMITED TO, ANY IMPLIED WARRANTY OR CONDITION OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR ARISING OUT OF A COURSE OF DEALING, CUSTOM, OR USAGE OF TRADE. If a 3M product does not conform to this warranty, then the sole and exclusive remedy is, at 3M's option, replacement of the 3M product or refund of the purchase price.

**Limitation of Liability:** Except for the limited remedy stated above, and except to the extent prohibited by law, 3M will not be liable for any loss or damage arising from or related to the 3M product, whether direct, indirect, special, incidental, or consequential (including, but not limited to, lost profits or business opportunity), regardless of the legal or equitable theory asserted, including, but not limited to, warranty, contract, negligence, or strict liability.

**3M Separation and Purification Sciences Division**
**3M Canada**
300 Tartan Drive
London, Ontario N5V 4M9
Canada
1-800-443-1661
3M.ca/bioprocessing