Implementation of a Fluorescence Based Process Analytical Technology Control for Fouling of Protein A Chromatography Resin

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Abstract

BACKGROUND: Protein A chromatography fouling is accompanied by two major events, one is the loss of protein A ligands and other is fouling due to non-specific, irreversible interactions of foulants with resin particles. This paper presents implementation of process analytical technology based control for fouling of protein A chromatography resin using a novel, fluorescence based approach. This approach enables direct, in-situ measurement of protein A ligand density as well as monitoring of resin fouling during resin reuse.

RESULTS: A novel, fluorescence based PAT tool has been designed and used for screening a variety of cleaning protocols. A two-step cleaning protocol was created using this methodology. The above mentioned fluorescence based approach was successfully used to monitor fouling and

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take a decision on when to initiate cleaning. This resulted in effective maintenance of dynamic binding capacity and step yield at 50 cycles (DBC: 97% of the original value vs. 65% with conventional protocols and yield: 93% of the original value vs. 73% with conventional protocols) and even at 200 cycles (> 90% of the original value).

CONCLUSIONS: The proposed fluorescence based approach has been effectively used for monitoring and control of resin fouling upon reuse, thereby resulting in a substantial increase in resin lifetime.

Key words: Protein A chromatography, resin lifetime, column reuse, monoclonal antibodies, fluorescence, LC-MS/MS, Cleaning in place (CIP), process control.
1. Introduction

Chromatographic separations continue to be the backbone for purification of biotherapeutic products. Their popularity and ubiquity is a result of the unparalleled selectivity, resolution, scalability, and ruggedness that they offer when compared to other process options. However, process chromatography steps are known to account for a significant portion of the processing cost, often the majority of that of downstream processing.\(^1\) The usable lifetime of media is anywhere from 50 cycle to 200 cycles.\(^2\) The number of times the resin is used has a significant impact on process performance and quality attributes of the resulting product. As per regulatory guidance, it is critical that performance of the chromatographic resin remain consistent so that predetermined quality and safety attributes can be achieved throughout the resin lifecycle. However, performance attributes like product recovery and impurity clearance capacity of the resin reduce with resin cycling. This is particularly true in the case of Protein A chromatography, which has become the \textit{de facto} standard for purification of monoclonal antibody and Fc fusion protein products.\(^3,4\)

As per the present approach, the biotherapeutic manufacturer first optimizes the chromatography process, including the cleaning and sanitization steps.\(^5,6\) Next, cycling studies are performed at laboratory scale using scale-down models of the process and resin lifetime is established.\(^7,8\) Finally, this target is verified at scale by collecting appropriate data during commercial manufacturing. As is evident, this is quite a resource and time intensive approach. Moreover, a clear determination of fouling is lacking and multiple attributes including step yield, product quality, impurity levels, and binding capacity are monitored to assess deterioration in resin performance over cycling. Finally, presently used controls are likely to result in varying product quality as the quality of incoming feedstock itself is known to vary due to the complexity
associated with biological processes. In the Process Analytical Technology (PAT) and Quality by Design (QbD) paradigm, an approach that is based on mechanistic understanding of resin fouling would be desirable. Such an approach would be expected to involve use of dynamic controls that can significantly reduce or even eliminate the incoming variability from various sources and ensure consistency in product quality.

Protein A chromatography has firmly established its place as the capture step in purification platforms used for production of monoclonal antibody therapeutics. This mode of chromatography has been successfully employed over the decades to purify antibodies from a variety of sources including mammalian cell culture and transgenic plants. One of the long recognized challenges with the Protein A ligand has been its limited stability under strongly alkaline conditions that are routinely used in clean-in-place (CIP) procedures for chromatography columns. As a result, regeneration of Protein A chromatography columns is typically carried out using high concentrations of chaotrope agents such as urea or guanidine hydrochloride, sometimes at acidic pH. Researchers have reported that limited exposure to mild alkaline conditions can be successfully employed for regeneration of Protein A resins. More recently, resin manufacturers have attempted to engineer the Protein A ligand so as to improve its alkaline tolerance and this has resulted in launch of products such as MabSelect SuRe™. Despite the significant advancements that the industry has made in design of Protein A resins, what has remained unchanged is that Protein A resins are the largest contributors to the cost of manufacturing. As a result, typical manufacturing practice involves reuse of resin. As per a recent survey of users of Protein A chromatography, it has been reported that while most manufacturers desire to obtain 100-200 reuses for this resin, typical usage ranges from 50-100 cycles. In a protein A chromatography process, the resin is subjected to a strip
condition post product elution to remove any residual product and impurities from the column prior to its regeneration (CIP). There are numerous sources which can result in variability of performance of a Protein A chromatography step, including variation in the elution, strip, and regeneration (CIP) procedures and in the composition of the feed material (concentration of product, lipids, host cell proteins, nucleic acids, and cell culture media constituents). The latter is particularly significant as the Protein A column is used as a capture step and as a result the feed contains a myriad of impurities. In view of the complexity of this step, an improved understanding of fouling of Protein A resin is critical for improving resin lifetime. As the incoming feed material from upstream manufacturing consists of a complex mixture of components including target product, impurities, and unwanted cell metabolites, feed composition is likely to significantly affect resin clearance capacity by reducing binding capacities and altering process kinetics. In extreme cases, it may lead to failure of batches, and likely impact product safety and efficacy. For this reason, on-line monitoring and control is critical to ensure that the process operates within defined limits and that product quality consistently meets the specifications.

The PAT approach emphasizes the need for real time monitoring of process parameters and raw material attributes by application of various in-situ and/or at-line analytical tools to ensure that any deviations during processing are identified and immediately dealt with. The use of such a scheme ensures robust process performance over the entire lifecycle of the product and consistent product quality at the end of the manufacturing process. There have been various case studies reported where PAT has been successfully implemented for various downstream unit operations like protein refolding, filtration, inline mixing, protein pegylation, viral inactivation, and process chromatography. Currently, various techniques such as UV spectroscopy, HPLC,
and mass spectroscopy are available to monitor effluent of column and provide useful information about product purity and impurity clearance.\textsuperscript{20,23} However, these are all indirect measures of performance monitoring. Often, by the time significant change is observed in these measures, the resin may have been irreversibly damaged. Researchers have also used microscopy to examine resin surface after fouling.\textsuperscript{27,28} While quite effective, this approach suffers from several key disadvantages. Firstly, microscopic approaches offer qualitative information at best. Secondly, such examinations require a representative sample to be taken from the column and this in turn would involve unpacking of the column and mixing of the resin prior to sampling. This can only be done after the column has reached the end of its lifetime and hence these measurements cannot be used for monitoring during column reuse.

This paper presents application of a novel, fluorescence based approach for direct, in-situ measurement of protein A ligand density and of resin fouling for effective monitoring and process control during resin reuse.\textsuperscript{18} The proposed approach aligns with the PAT methodology and has been successfully used for development of a two-step cleaning protocol that has been demonstrated to be significantly more effective than the conventionally used cleaning regimens both with respect to observed loss of protein A ligand, dynamic binding capacity, as well as product yield. We think this paper would be of great interest to manufacturers of biopharmaceutical products.

2. Materials and methods

2.1 Materials and equipment

A human IgG 4 monoclonal antibody (mAb) with isoelectric point 6.4 and molecular weight ~150KD, expressed in Chinese Hamster Ovary (CHO) cells, was donated by a major
manufacturer of biosimilar products. The feed material consisted of clarified harvest with product concentration of 4-6 mg.ml⁻¹.

All Protein A resin cycling studies were performed on an Äkta Purifier™ (GE Healthcare, Uppsala Sweden) chromatographic system. This system has built-in UV, pH, and conductivity detectors to monitor the effluent from the chromatographic experiments. Empty Tricorn glass columns were procured from GE Healthcare Life Sciences (Uppsala, Sweden). MabSelect SuRe™ resin was also procured from GE Healthcare (Uppsala, Sweden). Octet® RED 96 system (Pall Life Sciences, California, USA) was used for impurity detection and Agilent (California, USA) HPLC 1200 system was utilized for aggregate analysis.

Sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate buffer salts were purchased from Merck, India. For preparative chromatography, analytical grade chemicals were used. For analytical chromatography, HPLC grade chemicals were used.

2.2 Methods

2.2.1 Protein A chromatography

Protein A chromatography was performed using Mabselect SuRe™ resin. Fouled resin samples were generated on a XK-16 column (with bed height of 10cm) by performing resin cycling till 50 cycles without intermittent cleaning. The fouled resin generated in this case was used to evaluate cleaning reagents in 96 well predictor plates packed with 50µL of fouled resin. MabSelect SuRe™ has an alkali-tolerant rProtein A ligand attached to highly crosslinked agarose base matrix. For process control experiments, 1mL Mabselect SuRe™ column (5mm ID) was used for the cycling studies. To perform cycling studies, the column was equilibrated with 25mM phosphate, 150mM NaCl, pH 5.5 buffer. This was followed by loading of the clarified cell
culture broth (CCCB) at a loading capacity of 15 mg.ml\(^{-1}\) of resin. Post loading, a minimum of five CV of equilibration buffer was passed through the column. Elution was performed using 100 mM acetate, pH 3.5 buffer, and was followed by regeneration with 2M NaCl. The mobile phase velocity was 200 cm/h during elution.

2.2.2 Estimation of dynamic binding capacity (DBC)

DBC at 10% of the breakthrough curve was determined using fresh resin (before starting cycling studies on the column) and in between cycling studies using purified mAb. DBC was evaluated at 200cm/hr. The calculation for calculating the dynamic binding capacity was as follows:

\[
D_{10\%} = \frac{(V_{10\%} - V_0)C_0}{V_c}
\]  

(1)

Where, \(C_0\) is the antibody concentration (mg/ml), \(V_c\) is the geometric total volume (ml), and \(V_0\) is the void volume (ml).

2.2.3 Impurity analysis

Histone HCP content was estimated with EpiQuik Total Histone Quantitation Kit (Epigentek Inc., NY, USA). Samples were extracted for 16 h (overnight) in 200 mM hydrochloric acid, pH 1.0 before centrifugation at 13,000 rpm for 10 min at 4\(^\circ\)C. Supernatants were applied to histone ELISA plates in the same buffer and ELISA was performed as per manufacturer’s recommendations.

2.2.4 Screening of CIP conditions

The CIP buffer screening were carried out on fouled resin using the empty PreDictor\(^{TM}\) plates (GE Healthcare, Sweden). Approximately 50 \(\mu\)L of fouled resin sample (generated after 50 cycles on XK-16 column) was packed in empty 96 well predictor plates. Firstly, the fouled resin samples in the predictor plate were washed with 200 \(\mu\)L ultrapure water. The bottom of the PreDictor plate was blotted with a soft paper tissue and the plate was placed on a 96-well
collection plate with 500 µL V-shaped bottom (Code no. 28-4039-43, GE Healthcare, Sweden). Next, 300 µL CIP solutions were added to the wells and the top of the predictor plate was covered with a microplate foil (96-well). The PreDictor plate placed on the collection plate was secured with rubber bands on a microplate shaker (MTS 2/4 digital, IKA, Germany, 3 mm circular centripetal movement) and incubation was done for 15 min at 1,100 rpm. Finally, 300 µL PBS (20 mM phosphate, 0.15 M NaCl, pH 7.4) was added followed by wash with 300 µL ultrapure water. This procedure (incubation in CIP solutions and washes) was repeated depending on the number of CIP steps in the sequence. After the final CIP step, the fouled and cleaned resin in the wells were washed with 300 µL PBS followed by wash with 300 µL ultrapure water. The % ligand degradation and % foulant clearance were calculated using the following equations.

\[
\text{% Ligand degradation} = \frac{\text{Intensity at 303nm (before cleaning) \, \text{−} \, \text{after cleaning)}}}{\text{Intensity at 303nm before cleaning}} \times 100 \quad (2)
\]

\[
\text{% Foulant clearance} = \frac{\text{Intensity at 340nm (before cleaning) \, \text{−} \, \text{after cleaning)}}}{\text{Intensity at 340nm before cleaning}} \times 100 \quad (3)
\]

2.2.5 Fluorescence analysis

Fluorescence analysis of resin samples used for evaluation of cleaning buffers was carried out in 96 well plate using Spectra Max M2e Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) in Greiner Cellstar 96-well black bottom plate\(^{31}\). This analysis was performed in atline mode. The resin was monitored online for foulant deposition during chromatographic run after every two cycles. The column excitation and emission was recorded at 250nm to 500 nm as a spectra. The light emitted by the light source was transferred to the slit.
and polarizer through the excitation monochromator. The slit and polarizer transmit light of a predefined wavelength to the emission monochromator. The predefined wavelength emitted by the emission monochromator was passed through the black sheet of the column packed with resin thereby resulting in the recording of fluorescence intensity by the detector. Specifically, the fluorescence intensity was recorded at every cycle and the deposition of foulants was estimated by subtracting the reading obtained for the freshly packed column with fresh chromatographic resin. The increase in the amount of fluorescence intensity at the end of respective cycles estimated the deposition of foulants on the resin over reuse.\textsuperscript{30,31}

2.2.6 Total protein determination using Bradford assay

Total protein content was measured by Bradford assay using bovine serum albumin as a standard and absorbance of 595 nm (Bradford, 1976).\textsuperscript{32}

3. Results and discussion

As per a recent survey, industry users have indicated that the desired lifetime of the Protein A resin is anywhere from 100-200 cycles while the actual number of reuses varies from 50-100 cycles.\textsuperscript{2} Fouling can occur due to a variety of sources including ligand leaching, ligand occlusion due to deposition of foulant on resin surface and pores, and/or irreversible binding of the product and other host cell impurities to the resin surface. The contribution of different sources has been examined in a recent publication.\textsuperscript{27} Decrease in binding capacity of the protein A resin at the end of 100\textsuperscript{th} cycle when feed material was used was found to be five times greater than when feed material was not used. Compared to the fresh resin, the cycled resin samples exhibited a 24% reduction in particle porosity and a 51% reduction in pore mass transfer coefficient. The major event in fouling was identified as the non-specific adsorption of the feed material components on...
the resin, signaling that pore diffusion is the rate limiting step. In another recently published study, it has been shown that the composition of the protein cohort that remains on the resin surface evolves. As the resin is reused, a decreasing proportion of mAb and a concomitant increase in HCPs is observed. Further, it has been reported that cytoskeletal, protein synthesis, and metabolism related proteins are a major proportion of the carried-over HCPs.\textsuperscript{28} Direct measurement of the foulants present on the resin can facilitate use of appropriate cleaning conditions at the opportune time to clear the foulants deposited on the resin. Use of a very harsh cleaning regime very frequently is likely to result in chemical degradation of the resin and result in a short lifetime. On the contrary, inadequate cleaning will result in continued build-up of deposited foulants on the resin surface. This will either result in significant deterioration of the resin and a short lifetime or eventually require use of very harsh conditions to clean and in the process resulting in irreversible damage to the resin ligands. Recombinant protein A has tyrosine and phenylalanine residue in its sequence but lacks tryptophan. On the other hand, the foulants (host cell proteins and mAb) have tryptophan together with tyrosine and phenylalanine (detected using LC-MS/MS). Thus, we would expect the fluorescence intensity at 340 nm to be due to foulants present on the resin and the fluorescence intensity at 303 nm to be due to protein A ligand present on the resin.\textsuperscript{30,31} Figure 1 describes the approach for implementation of PAT based process control strategy to avoid resin fouling upon protein A chromatography reuse. In the following sub-sections, we discuss the results in more detail.

3.1 Screening of Cleaning in Place (CIP) buffers

CIP of chromatography columns is performed to achieve removal of very tightly bound, precipitated, or denatured substances from the chromatography resin. These contaminants might
affect the chromatographic properties of the column, result in a decrease in binding capacity and come off in subsequent runs thereby resulting in carryover of contaminants or product across cycles. The above stated fluorescence tool was used to screen cleaning conditions. For this, the fouled resin after 50 cycles was incubated under different cleaning regimens using a HTPD predictor plate, while mixing on a microtiter plate shaker. An incubation time of 15 min was used and this corresponded to the CIP contact time in a column. Protocol for operating the HTPD chromatography platform was as has been suggested in literature. Both single step and multi-step CIP regimens were evaluated (Table 1). All experiments were performed in triplicates. After cleaning, the resin samples were monitored for ligand degradation and foulant clearance using fluorescence based analysis as described earlier. Total protein in the CIP elute was evaluated using the Bradford assay.

For single step cleaning regimen, resin samples were incubated with different samples of NaOH, urea, and DTT. Effect of salt concentration in combination with NaOH was also evaluated. Sodium hydroxide is a widely used CIP buffer and is known to prevent contaminant build-up. It has been reported that NaOH efficiently removes precipitated proteins, lipids, and nucleic acids while inactivating bacteria, viruses, yeast and endotoxins. High pH conditions during CIP also inactivate microbes while removing contaminants that could be otherwise carryover into subsequent purification cycles. Figure 2A represents the impact of increasing NaOH concentration in the CIP buffer on ligand degradation and foulant clearance. With increase in NaOH concentration from 50mM to 500mM, the foulant clearance increased from 20% to 75% whereas ligand degradation increased from 20% to 50%. Addition of salt in the NaOH solution reduced ligand degradation, but also reduced foulant clearance. Using chaotropes as a CIP buffer has been reported to aid in removal of foulants by breaking non-covalent
interactions.\textsuperscript{30} With increase in urea concentration, ligand degradation increased from 10% to 18% while foulant clearance remained at \textasciitilde 50%. Reducing agents aid in removal of impurities by breaking the disulfide bonds.\textsuperscript{33} Use of DTT resulted in a foulant clearance of \textasciitilde 60% with negligible ligand degradation. Among the three cleaning reagents examined in this study (NaOH, chaotropes and DTT), DTT was found to have a comparable foulant clearance capacity as compared to NaOH, however with negligible ligand degradation.

Two step cleaning regimens were also examined using different concentrations of chaotrope, reducing agent, and combinations of chaoptropes and reducing agents in the first step followed by different NaOH concentrations in the second step (Table 1). Urea and DTT followed by NaOH were found to improve foulant clearance. Further, the ligand degradation was observed to be higher with NaOH followed by urea followed by DTT (Figure 3). Two step cleaning resulted in foulant clearance of \textasciitilde 80% in all cases (Figure 2B). Cleaning with reducing agent followed by NaOH resulted in maximum foulant clearance with low ligand degradation (conditions highlighted in Figure 2B). This finding also aligned with the total protein estimation using Bradford assay (Figure 4).

While the proposed fluorescence based monitoring approach can monitor fouling and deposition, it cannot identify the nature of foulants. To get more insight into the foulants, ELISA was performed by digesting the resin samples cleaned with NaOH, 100mM DTT and 3M Urea as described above. It was observed that the order for cleaning reagents with respect to their capacity of histone clearance was as follows: 100 mM DTT \equiv 3M Urea > NaOH. Our results demonstrate that use of chaotropes and reducing agents assist in removing histones, while NaOH is more efficient in removal of non-histone proteins. Thus, use of a two-step cleaning protocol is likely to yield a better foulant clearance as compared to single step cleaning.
MabSelect SuRe™ consists of an engineered Protein A ligand with a homotetramer-Z domain, where a number of asparagine residues have been replaced to eliminate interactions with the variable region, thereby reducing the binding heterogeneity between antibodies and allowing the resin to be able to withstand stronger alkaline conditions. The Protein A ligand does not contain any disulfide bonds, and is not impaired by reducing agents in the cleaning solutions.30

3.2 Establishing correlation between fluorescence intensity, yield and DBC

When subjecting the chromatographic resin to numerous reuses, there can be a gradual buildup of contaminants on the chromatography resin. To evaluate this, resin samples were withdrawn from the column every 10 cycles (100-200µL of resin was withdrawn at 10th, 20th, 30th and 40th cycle using a 20mL column) and fluorescence intensity was monitored. The performance loss during chromatographic runs was measured in terms of yield and DBC10%. Figure 5 illustrates the variation in yield, DBC10%, and fluorescence as a function of cycle numbers. It is observed that as the fluorescence intensity at 340 nm increases, the yield and DBC decrease thereby indicating a buildup of foulants on the resin surface upon reuse. No significant change was observed in the Protein A ligand intensity. This may be due to the fact that the cycling studies were performed without intermittent cleaning.

3.3 Process control of resin fouling

By integrating the evaluated cleaning condition and the mathematical correlation, a process control scheme was created and tested for its efficacy in avoiding performance loss. As seen in Figure 5, with increase in fluorescence intensity to >300 RFU, the yield is <90%. In view of this observation, the process control strategy that was undertaken involved online monitoring31 of the fluorescence intensity at 340 nm and once the fluorescence intensity exceeded 250 RFU (corresponds to yield of 90%), cleaning with 100mM DTT followed with 50 mM NaOH was
initiated. Figure 6A represents the comparison of yield and fluorescence intensity for the case where this control scheme was implemented to the case where cleaning was not performed. Upon cleaning with 100mM DTT, the fluorescence intensity dropped back to < 50RFU (Figure 6A: red bar at cycle 14\textsuperscript{th}, 24\textsuperscript{th} and 40\textsuperscript{th} cycle). If fluorescence intensity did not drop down to <50RFU with single cleaning step then the cleaning with 100mM DTT was followed by cleaning with 50mM NaOH (Figure 6A: red and yellow bar at cycle 28\textsuperscript{th} cycle). Use of this process control strategy resulted in maintenance of yield at >90% up to 50 cycles. The PAT cleaning strategy was further extended to 200 cycles and no significant loss in yield was observed (Figure 6C). Figure 6B shows the comparison of yield and DBC at 0\textsuperscript{th} cycle and 50\textsuperscript{th} cycle for the case without CIP, a traditional cleaning protocol (50mM NaOH + 1M NaCl), and with PAT cleaning. The yield and DBC at 50\textsuperscript{th} cycle in the case of PAT cleaning was found to be comparable to that of fresh resin. Thus, real time monitoring and execution of prompt control action (via cleaning protocols) can facilitate achieving of yield and DBC\textsubscript{10\%}>90% till 200 cycles without any buildup of HCP, HCD and protein A leachate (all <100 ppm).

The PAT based cleaning strategy reported in this paper would be of great value to the biopharmaceutical industry as the strategy allows the operator to apply the appropriate cleaning measures as per the extent of fouling with the latter monitored in real time. As demonstrated by the results obtained, a significant enhancement in lifetime of the protein A resin can be achieved together with a likely reduction in repacking of chromatography columns. A greater plant productivity as well as reduced risk of contamination can result by implementation of this PAT based cleaning.

3. Conclusions

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Repeated reuse of resin and the use of traditional cleaning methodology causes degradation of the Protein A ligand and deposition of contaminants over it thereby reducing IgG binding. This work presents use of fluorescence based approach for real time, direct monitoring of protein A resin fouling and creation of a PAT based control strategy that facilitates optimal use of the cleaning regimen. This has been demonstrated to result in a significant increase in resin life with minimal loss in yield and column performance. Use of this methodology allows measurement of both ligand density and the amount of adsorbed protein on the beads following cleaning-in-place (CIP), thereby resulting in a significant increase in resin lifetime.

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References


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Table 1: List of Different CIP buffers used for single and two step cleaning (Grönberg et. al. 2011; Lute et. al. 2008; Lund et. al. 2012; McCaw et. al. 2014; Winters et. al. 2015)

<table>
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<tr>
<th>Exp No.</th>
<th>CIP 1</th>
<th>CIP 2</th>
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<tbody>
<tr>
<td>1</td>
<td>PBS buffer (Control)</td>
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<tr>
<td>2</td>
<td>50mM NaOH</td>
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<tr>
<td>3</td>
<td>50mM NaOH+0.5M NaCl</td>
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<td>4</td>
<td>50mM NaOH+1M NaCl</td>
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<td>5</td>
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<td>6</td>
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Figure 1. Illustration of PAT based process control strategy to avoid protein A chromatography performance loss.

Figure 2. Percent ligand degradation and fouling clearance using (A) single step cleaning and (B) two step cleaning procedure.
Figure 3. Statistical analysis showing effect of NaOH, Urea and DTT on (A & B) foulant clearance and (C & D) on ligand degradation.

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Figure 4. Total protein concentration in the CIP elute determined by Bradford assay for different cleaning reagents.
Figure 5. Yield, DBC at 10% breakthrough and fluorescence intensity at 340 nm for 50 cycles.
Figure 6. (A) Plot for yield and fluorescence intensity in case of controlled cleaning and without cleaning. (B) Comparison of yield and DBC for fresh resin, 50th cycle fouled resin using controlled cleaning approach, traditional cleaning and without cleaning. (C) Yield and fluorescence intensity data for 200 cycles carried out using controlled cleaning approach.