An Alternative Two-Step Mixed-Mode Approach to a Monoclonal Antibody (mAb) Purification Process

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Abstract

Reducing the number of steps in a purification workflow can lead to higher yields, shorter processing times and, in turn, improved process economics. The purification of a monoclonal antibody (mAb) typically consists of a three-step process beginning with an initial capture step using Protein A chromatography, followed by intermediate and polishing steps that can include various resins, such as ion exchange (IEX) and hydrophobic interactions. Strategies that combine mechanisms of interaction can reduce the number of chromatography steps, making the overall process more efficient. To demonstrate this, two post–Protein A capture two-step mAb processes were developed and compared to an archetypical three-step mAb purification process using two IEX steps after Protein A capture. The first process used Nuvia aPrime 4A, a hydrophobic anion exchange (AEX) mixed-mode resin, and the second used CHT™ Ceramic Hydroxyapatite XT Media, a calcium affinity cation exchange (CEX) mixed-mode media. The results demonstrate the ability to achieve a high-purity product with fewer units, and therefore, fewer processing steps, using mixed-mode chromatography.

Introduction

The US and EU patents of several biologic drugs are set to expire by 2040 (https://purplebooksearch.fda.gov/patent-list), paving the way for the development and approval of additional biosimilars. As a result, there is high demand for solutions that will deliver efficient, flexible, and cost-effective mAb purification and biosimilar production. Downstream purification platforms commonly include a Protein A–based capture step followed by two polishing steps to remove the remaining impurities. Mixed-mode chromatography (also known as multimodal chromatography) combines, for example, the properties of AEX media and hydrophobic interaction media. Mixed-mode resins are increasingly important tools for downstream process purification, since the media have a large design space for binding and elution. This broad canvas allows for the development of highly robust methods in a commercial manufacturing setting. In this study, multiple two-step purification processes were developed for the purification of a trastuzumab biosimilar that are alternatives to the generic three-step process, with the aim of obtaining comparable yields and purity while decreasing the processing costs and time. All three processes began with a capture purification step using a Protein A affinity resin. In the three-step process, this was followed by an intermediate polishing step and a final polishing step (Figure 1, Workflow 1). In the two-step processes, the capture purification step was followed by a polish purification step with either an AEX resin (Figure 1, Workflow 2) or a CEX resin (Figure 1, Workflow 3). A complete overview of the three workflows that were evaluated is represented in Figure 1.

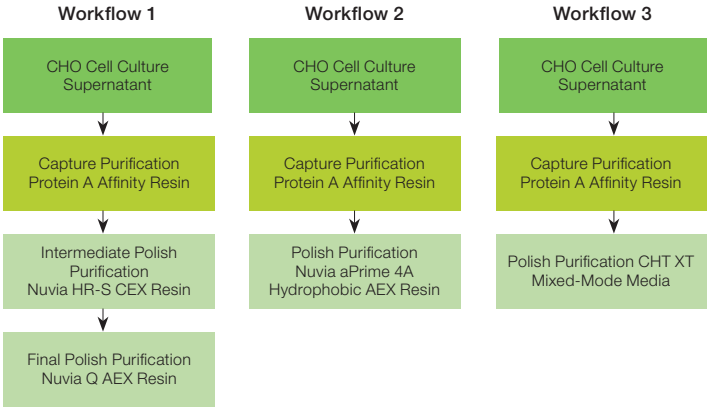

Fig. 1. An overview of the three workflows for the purification of a trastuzumab biosimilar that are alternatives to the generic three-step process. Workflow 1 is the standard three-step process with Protein A capture followed by two ion exchange-polishing steps using Nuvia Ion Exchange Resins. Workflow 2 is a two-step process with a mixed-mode chromatography step following Protein A capture, using Nuvia aPrime 4A Hydrophobic Anion Exchange (AEX) Resin. Workflow 3 is a two-step process with a mixed-mode chromatography step following Protein A capture, using a calcium affinity cation exchange (CEX) mixed-mode resin, CHT Ceramic Hydroxyapatite XT Media. CHO, Chinese hamster ovary.

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Table 1. Overview of the types of materials, conditions, modes of operation, corresponding buffers, and flow rates in each of the workflows for the purification of a trastuzumab biosimilar. Workflows 1–3 include the Protein A capture step. Workflow 1 includes both the intermediate and final polish steps. Workflow 2 includes the Nuvia aPrime 4A polish step. Workflow 3 includes the CHT XT polish step.

Column Type	Resin Type	Mode of Operation	Workflow Position	Buffer A, Binding Buffer	Buffer B, Elution Buffer	Flow Rate, cm/hr	Catalog #
Protein A, 5 ml	UNOsphere SUPrA	Bind and elute	Capture	50 mM sodium phosphate, 150 mM NaCl, pH 7.4	100 mM sodium acetate, pH 3.0; step elution	300	7324749
Nuvia HR-S, 1 ml	Strong CEX	Bind and elute	Intermediate polish	50 mM sodium acetate, pH 5.0	50 mM sodium acetate; 1 M NaCl, pH 5.0	150	7324723
Nuvia Q, 1 ml	Strong AEX	Flowthrough	Final polish	50 mM Tris, 50 mM NaCl, pH 8.0	Flowthrough	150	7324721
Nuvia aPrime 4A, 5 ml	Hydrophobic AEX	Flowthrough	Polish	50 mM Tris, 50 mM NaCl, pH 8.0	Flowthrough	150	12007393
CHT XT, 5 ml	Metal affinity CEX	Bind and elute	Polish	10 mM sodium phosphate, pH 7.0	10 mM sodium phosphate; 1 M NaCl, pH 7.0	150	12003149

CHT, ceramic hydroxyapatite; CEX, cation exchange; AEX, anion exchange.

Materials and Methods

Column chromatography was conducted on an NGC Discover 10 Pro Chromatography System (Bio-Rad Laboratories, Inc., catalog #7880011). Protein fractions were analyzed by SDS-PAGE using Mini-PROTEAN™ TGX Stain-Free Protein Gels, 4–20% linear gradient (Bio-Rad, #4568096), which were imaged on the ChemiDoc™ MP Imaging System (Bio-Rad, #12003154). Analytical size exclusion chromatography (SEC) of column fractions was performed using a MabPac SEC-1 HPLC Column (5 µm, 4 x 150 mm) (Thermo Fisher Scientific Inc., #075592) on a Dionex UltiMATE 3000 HPLC/UHPLC System (Thermo Scientific, #IQLAAAGABHFAPBMBFB).

Capture with Protein A

All three workflows began with the Protein A capture step. Clarified Chinese hamster ovary (CHO) cell culture supernatant expressing a trastuzumab biosimilar (pH 8.6) was obtained from collaborators at the Agency for Science, Technology and Research (A*STAR), and was loaded onto a 5 ml Foresight™ UNOsphere SUPrA Protein A Column (Bio-Rad, #7324749). The process conditions are described in Table 1. Ten runs of Protein A were performed, with approximately 125 mg loaded per run. The Protein A pools from the ten runs were combined and this material was used for the downstream purification workflows.

Intermediate Polishing with Nuvia HR-S Resin and Final Polish with Nuvia Q Resin

As described in Figure 1 (Workflow 1), 50 mg of Protein A eluate material was adjusted and loaded in 50 mM sodium acetate (pH 5.0) and a 0–100% linear gradient to 1 M NaCl was performed (Table 1) on a prepacked 1 ml Foresight Nuvia HR-S Column (Bio-Rad, #7324723). The elution pool from the column was adjusted to pH 8.0, with 160 µl 1.0 M Tris, then 35.5 mg of the sample was loaded directly onto a 1 ml Foresight Nuvia Q Column (Bio-Rad, #7324721). The binding buffer was 50 mM Tris, 50 mM NaCl, pH 8.0, and the column was operated in flow-through mode.

Two-Step Workflows

In Workflow 2, 181.7 mg of Protein A eluate was adjusted to pH 8.0 with 1.0 M Tris and the conductivity was adjusted to match that of the 50 mM Tris/50 mM NaCl (pH 8.0) buffer solution. The Foresight Nuvia aPrime 4A Column (5 ml) (Bio-Rad, #12007393) was operated in flow-through mode, and the flow-through fraction was collected. Operating conditions are described in Table 1. In Workflow 3, 125 mg of post–Protein A eluate was loaded in 10 mM sodium phosphate (pH 7.0) on a 5 ml Foresight CHT XT Column (Bio-Rad, #12003149) and was then eluted using a 0–100% gradient up to 1 M NaCl. Host cell protein (HCP) content in the elution fractions and product pools, as well as Protein A content, were analyzed using a commercially available HCP ELISA Kit (Cygnus Technologies, R1120422-3) and Protein A ELISA Kit (Cygnus, R1120422-3).

The Vericheck ddPCR™ CHO Residual DNA Quantification Kit (Bio-Rad, #17000031) was used to quantify residual host cell DNA (HCD), following the manufacturer's instructions. To prepare samples for testing, 5 µl of test sample per reaction was digested with Proteinase K, resulting in a 10.4-fold dilution of the sample. In an initial test of samples 1 and 2 in batch 2, the samples were above the dynamic range of the CHO kit. Therefore, sample 1 was further diluted 1,000-fold and 10,000-fold and sample 2 was further diluted 100-fold prior to testing. Tenfold serial dilutions from 0.005–5 pg/µl of a CHO genomic DNA reference standard (United States Pharmacopeia [USP], #1130710) were prepared as a standard curve for converting from copies/µl to pg/µl. The samples, CHO DNA standard dilutions, and no template water control (NTC) were each tested with n = 3 replicate wells. ddPCR reactions were set up according to the kit user guide. DTT was not included in the ddPCR reactions. For CHO DNA standard dilutions, samples 1–10 from batch 1 and 1–2 from batch 2, 1 µl per reaction was tested. For samples 3–10 from batch 2, 5 µl per reaction were tested. The QX200™ AutoDG™ Droplet Digital™ (ddPCR™) PCR System (Bio-Rad, #1864100) was used for ddPCR reactions. Batch 1 data were collected and analyzed using QX Manager Software, Regulatory Edition, version 1.2 (Bio-Rad, #12012172). Batch 2 data were collected using QuantaSoft™ Software, version 1.7 (Bio-Rad, #1864011), and analyzed using QX Manager Software, Standard Edition, version 1.2 (Bio-Rad). Thresholds were set manually based on the NTC wells and the three replicate wells were merged in the software for downstream data processing.

The concentration of CHO DNA in pg/µl was calculated following the kit user guide. The concentration was multiplied by 20, then by the dilution factor to determine the concentration of CHO DNA in the original sample. For sample 1 from batch 2, the concentration of CHO DNA was determined by averaging the concentration from the 1,000-fold and 10,000-fold dilutions.

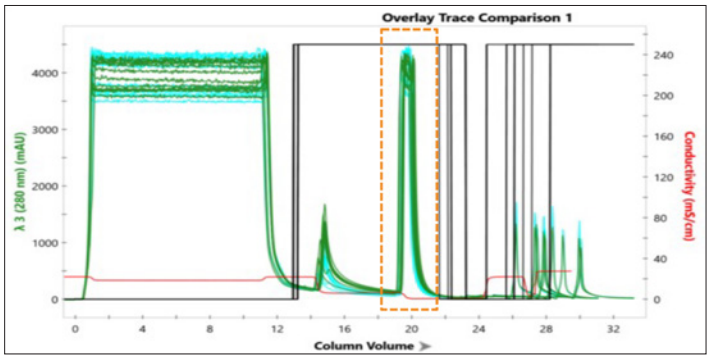
Results and Discussion

Capture with Protein A Chromatography Across All Workflows

The starting material generated for the two- and three-step purification workflows began with the processing of the trastuzumab cell culture via Protein A chromatography. Ten runs were performed and the elution from each run was pooled to generate enough material for each of the three workflows described.

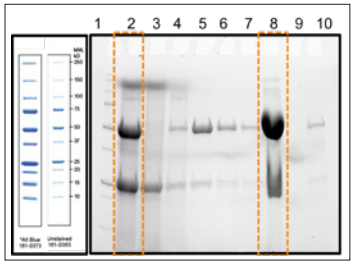
Figure 2A shows the chromatogram of ten runs performed using the CHO cell culture supernatant. The overlays show consistency among all the samples. The two peaks shown are the intermediate wash peak and the sodium acetate (pH 3.0) elution peak (outlined in the orange box).

A



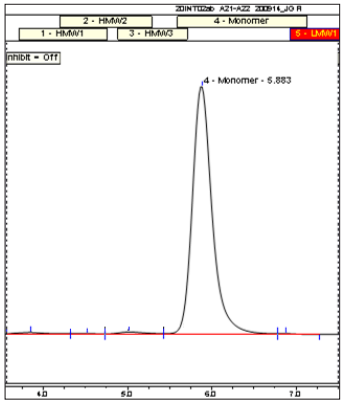
Elution: 0.1 M sodium acetate, pH 3.0.
10-run overlay.

B



Lane	Protein A Resin, 5 ml Column
1	Precision Plus Protein Unstained Standards (Bio-Rad, #1610363)
2	Cell culture supernatant
3	Flowthrough
4	Wash 1 pool
5	Wash 2 fraction
6	Wash 2 pool
7	Elution fraction 1
8	Elution pool
9	Blank
10	Strip

C



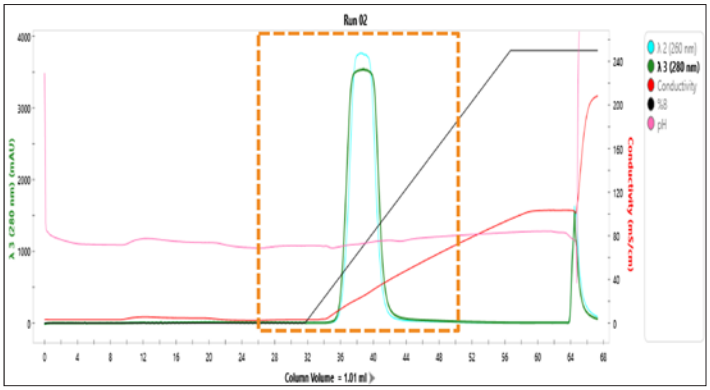
Lane	Protein A Resin, 5 ml Column
Yield, %	100
Purity, %	96.1
Load, mg	125.0

Fig. 2. Protein A capture using the Chinese hamster ovary (CHO) cell culture supernatant. A, chromatogram of ten pooled runs. A_{280} (—); A_{260} (—); conductivity (—); % elution buffer (—). Orange box indicates pooled fractions. **B**, SDS-PAGE analysis. **C**, size exclusion chromatography (SEC) analysis. Yield was 100% and purity was 96.1%.

All ten runs were combined and SDS-PAGE was run to measure the success of the purification step (Figure 2B). SEC was performed on the pool to assess purity in terms of percentage of the monomer (Figure 2C). Substantial cleanup from the supernatant to the elution pool is visible and SEC revealed a yield of 100% and purity of 96.1%. The total amount of mAb loaded was 125 mg.

Workflow 1: Three-Step Standard Purification Approach

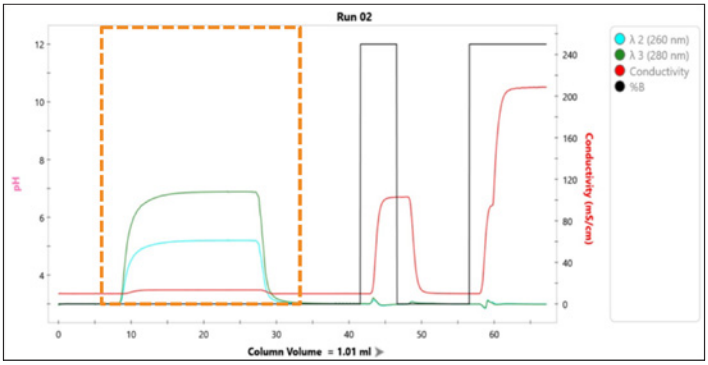
Following intermediate purification on Foresight Nuvia HR-S Columns (Figure 3), final polishing chromatography was performed on Nuvia Q in flow-through mode. Figure 4A shows the chromatogram of the Foresight Nuvia Q Column run. SDS-PAGE was run on the pooled fraction of the final polishing Nuvia Q step to measure the success of the purification workflow (Figure 4B). SDS-PAGE shows the absence of impurity bands. SEC was performed on the pool to assess purity in terms of percent monomer (Figure 4C). This revealed a yield of 100% and purity of 97.0%.



Elution: 50 mM sodium acetate, 1.0 M NaCl, pH 5.0.

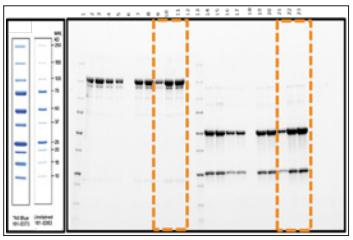
Fig. 3. Chromatogram of the intermediate polishing step of the three-step chromatography process using Nuvia HR-S. The elution peak during the gradient is outlined. A_{280} (—); A_{260} (—); conductivity (—); % elution buffer (—). Orange box indicates pooled fractions.

A



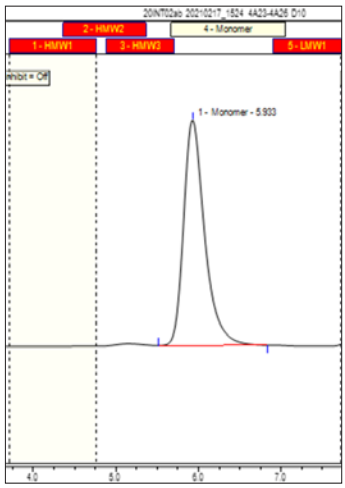
Buffer conditions: 50 mM Tris; 50 mM NaCl, pH 8.0.

B



Lane	Protein A Resin, 5 ml Column
9	Load nonreduced
10	Flowthrough/wash nonreduced
11	Strip nonreduced
21	Load reduced
22	Flowthrough/wash reduced
23	Strip reduced

C



Sample	Nuvia Q Resin, 1 ml Column
Yield, %	97.1
Purity, %	100.0
Load, mg	35.5

Fig. 4. Analysis of the final polishing step of the three-step purification process using Nuvia Q AEX Resin. A, chromatogram. A_{280} (—); A_{260} (—); conductivity (—); % elution buffer (—). Orange box indicates pooled fractions. **B**, SDS-PAGE analysis of the pooled fractions of the final polishing Nuvia Q step to measure the success of the purification workflow. **C**, size exclusion chromatography (SEC) analysis of the pooled fractions to measure the success of the purification workflow. Yield was 97.1% and purity was 100%.

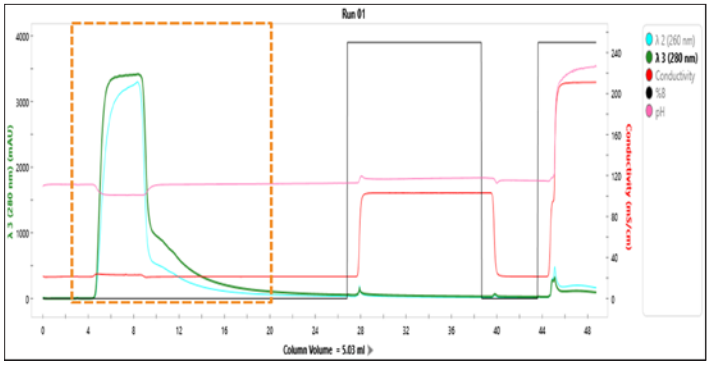
Workflow 2: Two-Step Approach Using AEX Nuvia aPrime 4A Resin

Unlike single-mode traditional resins, the synergistic effects of the ligand structure of mixed-mode resins combine modes of interaction into one operational step, refining selectivity and enabling a streamlining of the process. With mixed-mode resins, target proteins that interact via alternative modes in response to different buffer conditions can be selectively isolated by manipulating the buffer conductivity and pH at binding and elution. Similarly, columns can be run in bind-elute or flow-through modes, depending on the purification requirements.

The two-step mAb purification procedure using the hydrophobic AEX Nuvia aPrime 4A Resin isolates target proteins through IEX and hydrophobic interaction pairing. The Nuvia aPrime 4A Resin ligand has a quaternary amine with a net positive charge. As a result, basic species, such as high-isoelectric point (pI) target proteins and host cell proteins experience electrostatic repulsion, while acidic species, including low-pI proteins and HCPs, endotoxins, and viruses, experience electrostatic interaction. After Protein A capture, the single polishing step using Nuvia aPrime 4A Resin can be performed in either flow-through or bind-elute modes.

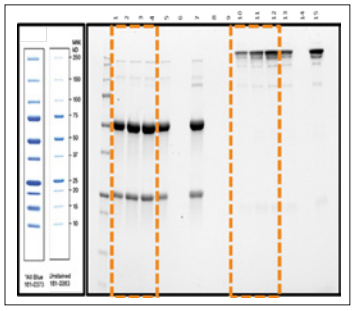
In the study, the pH and conductivity of the post–Protein A capture eluate were adjusted, and the material was run in flow-through mode. Analysis was conducted via chromatography, SDS-PAGE, and SEC (Figure 5 A–C, respectively). SEC revealed a yield of 92.4% and 99.0% purity.

A



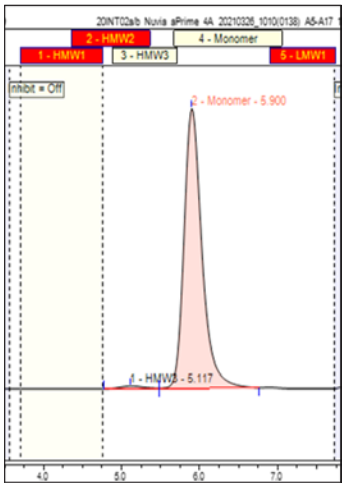
Buffer conditions: 50 mM Tris; 50 mM NaCl, pH 8.0. Orange box is the flow-through collection.

B



Lane	Nuvia aPrime 4A Resin, 5 ml Column
2	Load reduced
3	FT reduced
4	Wash reduced
10	Load nonreduced
11	FT nonreduced
12	Wash nonreduced

C



Sample	Nuvia aPrime 4A, 5 ml Column
Yield, %	92.4
Purity, %	99.0
Load, mg	181.7

Fig. 5. Analysis of the polishing step of the two-step purification process using Nuvia aPrime 4A Resin. A, chromatogram. A_{280} (—); A_{260} (—); conductivity (—); % elution buffer (—). Orange box indicates pooled fractions. **B**, SDS-PAGE analysis. FT, flowthrough. **C**, size exclusion chromatography (SEC) analysis. Yield was 92.4% and purity was 99%.

Workflow 3: Two-Step Approach Using CHT Ceramic Hydroxyapatite XT Media

CHT Ceramic Hydroxyapatite XT Media is a calcium affinity CEX mixed-mode media. The amino residues will interact with phosphate sites in a classical CEX mode, which are dissociated with NaCl and other buffering salts, such as phosphate. Alternatively, the interaction can be weakened or dissociated by increasing pH. The carboxyl residues will interact with calcium sites forming calcium chelation. This interaction is several times stronger than the ionic interactions and, therefore, requires phosphate to dissociate from the calcium sites. Similarly, the phosphoryl groups will also interact with calcium chelation via the phosphoryl oxygens. This calcium chelation through the carboxyl and phosphoryl groups is also several times stronger than the ionic interactions; therefore, they also require phosphate for dissociation. The combined mechanisms of action consisting of IEX and metal affinity pairing enables mAb purification in a singular step after Protein A capture.

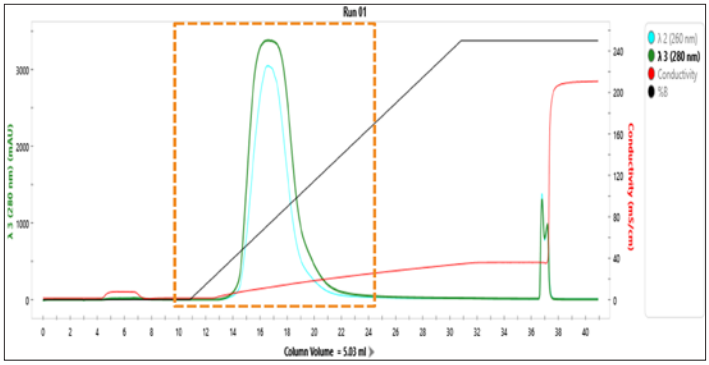
In the study, post–Protein A capture was run in flow-through mode, and analysis by chromatography, SDS-PAGE, and SEC (Figure 6A–C, respectively). SEC revealed a yield of 99.0% and 100.0% purity.

Comparison of the Two- and Three-Step Workflows

HCP and residual Protein A were measured using the aforementioned ELISA kits in all pooled samples. These results serve as indicators of purity. Additionally, ddPCR assays were used to evaluate the removal of host cell DNA (HCD) as a measure of purity.

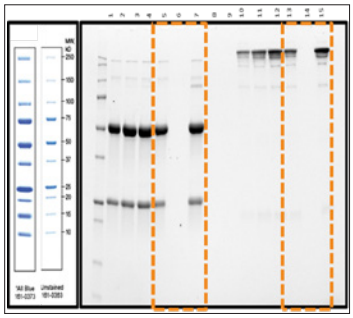
Table 2 shows the final purity and yields across all three workflows. An overall comparison of the yield, purity, and amount of material loaded from the three-step and two-step purification strategies reveals comparable purity, HCP, and HCD removal. While both the two- and three-step processes effectively purify the target mAb, the two-step processes can obtain a slightly higher yield with one less operational step.

A



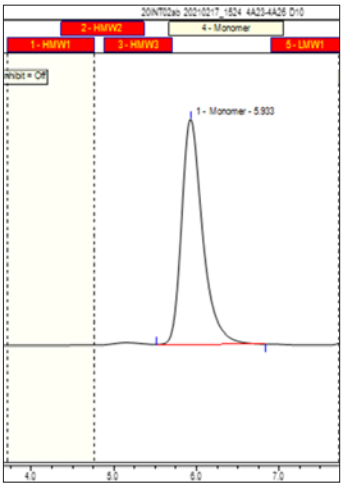
Elution: 0–100% Buffer B gradient using 10 mM sodium phosphate, 1 M NaCl, pH 7.0. Orange box is the collected fraction pool.

B



Lane	Protein A Resin, 5 ml Column
5	Load reduced
6	Pre-peak fraction reduced
7	Main fractions reduced
13	Load nonreduced
14	Pre-peak nonreduced
15	Main nonreduced

C



Sample	CHT XT, 5 ml Column
Yield, %	100.0
Purity, %	99.0
Load, mg	125.0

Fig. 6. Analysis of the polishing step of the two-step purification process using CHT XT Media. A, chromatogram. A_{280} (—); A_{260} (—); conductivity (—); % elution buffer (—). Orange box indicates pooled fractions. **B**, SDS-PAGE analysis. **C**, size exclusion chromatography (SEC) analysis. Yield was 100% and purity was 99%.

Table 2. Comparison of the yields and purities associated with the two- and three-step procedures for the purification of a trastuzumab biosimilar. Workflows 1–3 all include the Protein A capture step (cell culture feed). Workflow 1 includes both the intermediate and final polish steps (steps 2 and 3). Workflow 2 includes the Nuvia aPrime 4A polish step (step 2). Workflow 3 includes the CHT XT polish step (step 2).

Workflow	Step	Mode	HCP, ng/ml	Protein A, ng/ml	HCD, pg/µl	Monomer, %	Yield, %
All	Cell culture feed	–	4,252.0	–	90,348.9	N/A	N/A
1	1	Bind and elute	196.6	10.5	1,879.5	96.1	>95
1	2	Bind and elute	23.7	*	BDL	98.1	94.3
1	3	Flowthrough	3.0	4.1	BDL	99.2	97.1
2	2	Flowthrough	BDL	0.2	BDL	99.0	92.4
3	2	Bind and elute	3.5	0.1	0.15	99.0	>95

BDL, below detection limit; HCD, host cell DNA; HCP, host cell protein.
* Each workflow's leached Protein A concentration was determined only after the Protein A capture and final chromatography steps.

Conclusions

The results of this study demonstrate that a mAb can be effectively purified with high yield and purity via two-step workflows with different types of mixed-mode resins. Overall, mAb purity and yield were comparable across the two- and three-step processes. Additionally, the two-step purification strategy using the Nuvia aPrime 4A Resin can be operated in either flow-through mode, which simplifies the purification process, or bind-elute mode. Both two-step workflows provide excellent aggregates, leached Protein A, HCP, and HCD removal. In mixed-mode chromatography, contaminant removal can be optimized by adjusting the buffer pH and conductivity to impact the ionic and hydrophobic interactions, enabling the selectivity and recovery of target molecules to be fine-tuned. Unlike traditional three-step processes, this creates unique selectivity in the purification process, allowing impurities such as viruses, HCP, aggregates, and nucleic acids to be removed in a single chromatography step. Additional benefits arising from fewer processing steps include a reduction in quality control testing, regulatory and documentation burden, suite time, and costs of operational units, labor, and capital equipment. Overall, a mixed-mode chromatography approach provides an effective alternative to a traditional three-step purification process. Reducing the processing steps by one operational unit allows for higher productivity and lower costs, while also achieving comparable or higher yields with equivalent purity.

Acknowledgments

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Visit bio-rad.com/MixedMode and explore Nuvia aPrime 4A Resin and CHT XT Media.

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