Protein Expression and Purification 165 (2020) 105497

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Development of BioRad NGC and GE ÄKTA pure systems for highly automated three column protein purification employing tandem affinity, buffer exchange and size exclusion chromatography

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ARTICLE INFO

Keywords:
High throughput purification
Automated chromatography
ÄKTA
NGC
Multi-dimensional chromatography

ABSTRACT

Affinity purification, such as Protein A (ProA) followed by size exclusion chromatography (SEC) remains a popular method to obtain research scale proteins. With the need for higher throughput protein production increasing for discovery research, there is substantial interest in the automation of complex protein purification processes, which often start with a ProA step followed by SEC. However, the harsh elution conditions from ProA based chromatography can destabilize some proteins resulting in particulates, which in turn can cause column fouling and potential cross-contamination of subsequent purifications. We modified both Bio Rad NGC and ÄKTA Pure systems to run a three-column process (ProA to buffer exchange to SEC) enabling automated tandem affinity to SEC purification while minimizing the risk of SEC column fouling and subsequent cross-contamination. The intervening buffer exchange column, unlike the final SEC column, can be rapidly regenerated using harsh methods between runs, and these automated systems are capable of processing up to six samples per day without user intervention.

1. Introduction

Efficient, automated purification of proteins at a preparative scale is of increasing interest and is the subject of a recent review [1]. Commercially available chromatography instruments such as the NGC Chromatography systems (BioRad, Hercules, CA) and the ÄKTA systems (Pure, Avant and Express, GE Life Sciences, Piscataway, NJ) have been utilized for automated lab scale multi-step protein purification. For example, an ÄKTA Avant has been employed to automate a protein workflow that included IMAC affinity capture, enzymatic modification, buffer exchange, and SEC polishing [2]. The ÄKTAxpress and NGC Chromatography instruments are both capable of automating the purification processes with four sequential steps, including IMAC and SEC [3,4] and an NGC instrument has been designed for automated affinity, buffer exchange and SEC [5]. Often these automated methods require holding vessels or sample loops; which can add time to the setup and be scale limiting. However, these can be eliminated by loading the first column eluate directly onto the second column [6,7]. Additional pumps and valves can be added to an ÄKTA to accommodate up to five sequential loadings followed by tandem two column purification [8]. For large numbers of molecules at small scale (4–100 mL), an ÄKTA equipped with an autosampler can automate affinity chromatography with a subsequent buffer exchange step [6]. For larger scale purifications with more stringent purity requirements, an ÄKTA system can be configured for automated two column purification by using an in-line mixer to condition the affinity column eluate for binding to an ion exchange column [9]. However, for some proteins, where there is lack of prior knowledge of effective ion exchange conditions, or the primary contaminants are high- and/or low-molecular weight species, or the protein is marginally stable at low pH and requires rapid buffer exchange, we found it preferable to employ SEC as the polishing step immediately following the affinity purification step.

Use of affinity steps that require low pH conditions for elution, such as protein A (ProA), can expose molecules to destabilizing conditions resulting in unwanted aggregation and particulation [10–14]. Furthermore, direct loading of these particulates onto an SEC column can lead to premature fouling, carry over contamination or failed runs. Cleaning of SEC columns between runs with an aggregate solubilizing buffer such as 6 M guanidine HCl would be prohibitively time consuming due to the low flow rates required for these columns. In

Abbreviations: ProA, protein A; SEC, size exclusion chromatography; PBS, phosphate buffered saline; CM, conditioned media; TT, triple tandem; BX, buffer exchange; OV, outlet valve; SV, sample valve

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https://doi.org/10.1016/j.pep.2019.105497
Received 15 August 2019; Received in revised form 30 August 2019; Accepted 6 September 2019
Available online 07 September 2019
1046-5928/

addition, frequent exposure to these harsh conditions can damage the fragile SEC column, resulting in a shorter useable lifespan. Insertion of an inexpensive, more robust column to immediately buffer exchange the ProA eluate prior to loading onto the SEC column can minimize the risk of introducing precipitates downstream, and an in-line filter placed immediately upstream of the SEC column during the load can offer added protection by capturing any remaining particulates that escape the buffer exchange column. Furthermore, both the buffer exchange column and in-line filter can be operated at much faster flow rates than SEC columns enabling rapid harsh regeneration and re-equilibration between samples.

Here we present both ÄKTA Pure and NGC Chromatography systems capable of automated direct tandem purification using ProA, buffer exchange and SEC columns run in series, which we refer to as the Triple Tandem (TT) system. The TT system is capable of purifying up to seven samples for the ÄKTA Pure and six samples for the NGC without user intervention, significantly decreasing the amount of labor required to obtain highly purified material at high yield. These TT systems can operate at scales from 2.5 to 500 mg protein load with no limits on volume and are potentially scalable beyond these parameters.

2. Material and methods

2.1. ÄKTA modifications

An ÄKTA Pure 150 L (GE Life Sciences, Piscataway, NJ), dedicated to the Triple Tandem system was configured with three Versatile Valves (V9H-V1-3) as described in Fig. 1A and Fig. 2. Port 1 of V9H-V1 is connected to port 10 of the outlet valve V9H-O, while port 2 of V9H-V1 is connected to port 4 of V9H-V2 and port 3 of V9H-V1 is connected to the column valve outlet (V9H-C). Port 3 of V9H-V2 is connected to port 4 of V9H-V3. Port 1 of V9H-V3 is connected to a waste container while port 3 of V9H-V3 is connected to the UV flow cell, conductivity meter followed by the input port of the outlet valve. Two-tandem 5 mL HiTrap MabSelect SuRe columns (ProA) (GE Life Sciences, Piscataway, NJ) are in the number 1 position of V9H-C. Flow to the 53 mL HiPrep 26/10 G-25 Buffer exchange column (BX) (GE Life Sciences, Piscataway, NJ) is from the connection of port 4 of V9H-V1, while flow from the column proceeded to a filter unit with biocompatible housing with an inexpensive 2 µm mega frit (Phenomenex, Torrance, CA) to port 2 of V9H-V2. The flow to the HiLoad 26/600 Superdex 200 pg (GE Life Sciences, Piscataway, NJ) column (SEC) is from port 1 of V9H-V2 and from the column to port 2 of V9H-V3.

2.2. Unicorn control of the triple tandem system

The use of the Unicorn scouting method allowed for the sample valve to direct the flow through the first column from the appropriate loading vessel at 10 mL/min with the flow going through V9H-V1 ports 2–3 and ports 3–4 for both V9H-V2 and V9H-V3 and then the outlet valve is used to collect the flow through in the appropriate vessel (Fig. 2A). When air is detected by the air sensor located just downstream from the sample valve, the sample pump inlet is switched to Dulbecco's PBS (Gibco Life Technologies, Grand Island NY) and the media remaining in the pump is loaded on the ProA column. The column is then switched to bypass using the column valve and the tubing and pump are flushed with PBS. Using the system pump, PBS from line A1 is used to wash the ProA column at 5 mL/min. The column is then eluted at 2.5 mL/min with 100 mM sodium acetate pH 3.6 from line B1 using the system pump. When the UV exceeds 50 mAU the flow is diverted using V9H-O outlet 10 to V9H-V1 ports 1–4 and then to the BX column (Fig. 2B). The loading of the BX column with ProA eluate continues until the UV is less than 25 mAU or until 15 mL is loaded on the BX column. Formulation buffer from Line A2, also PBS, is used to elute the BX column with V9H-C in bypass and V9H-V1 set for flow from ports 3–4, the BX eluate flowed through the in-line filter and V9H-V2 ports 2–3 and then through V9H-V3 ports 3–4 to the UV and conductivity monitors (Fig. 2C). When the eluate from the BX column exceeded 25 mAU the flow is diverted using V9H-O outlet 10 to load the SEC column. The BX column is eluted until the UV mAU is less than 25, or a maximum of 19 mL is reached, whichever occurs first. The SEC column is eluted with PBS loaded on the column through V9H-V1 ports 2–3 (which by-passes the BX column) and V9H-V2 ports 1–4, the eluate then is directed to the UV and conductivity monitors through V9H-V3 ports 2-3 and V9H-O fraction collector port (Fig. 2D). Fractions of a maximum of 6 mL are taken when the mAU exceeded 5. To ready the system for the next run in the scouting series, the ProA and BX columns

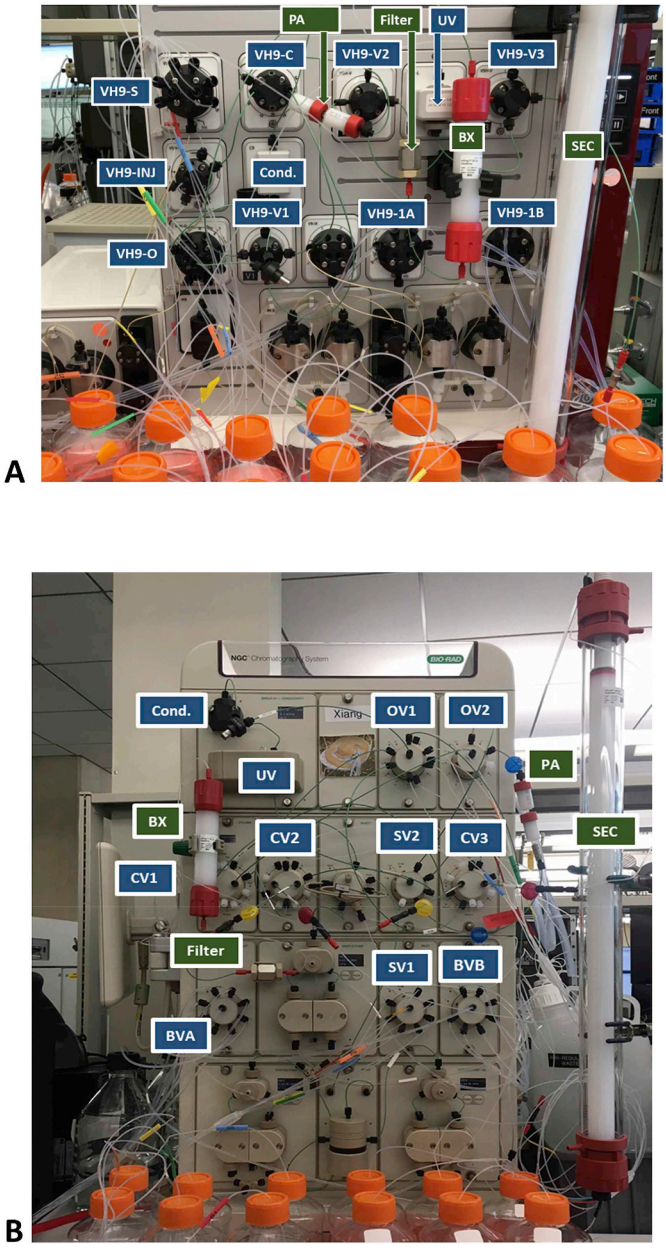

Fig. 1. A. The ÄKTA PURE system was configured for three-column tandem capability. The buffer exchange column (BX) exchanges the buffer of the ProA elution to that of the running buffer of the size exclusion column (SEC). Three versatile valves were used to select the flow to accommodate the automated three column purification. B. Similarly the NGC instrument was configured with two pump inlet valves, two sample valves, an injection valve, three column valves and two outlet valves. For both systems the in-line filter prevented any precipitated protein which may have been present in the BX eluate from being loaded on the SEC column, reducing the risk of run to run contamination.

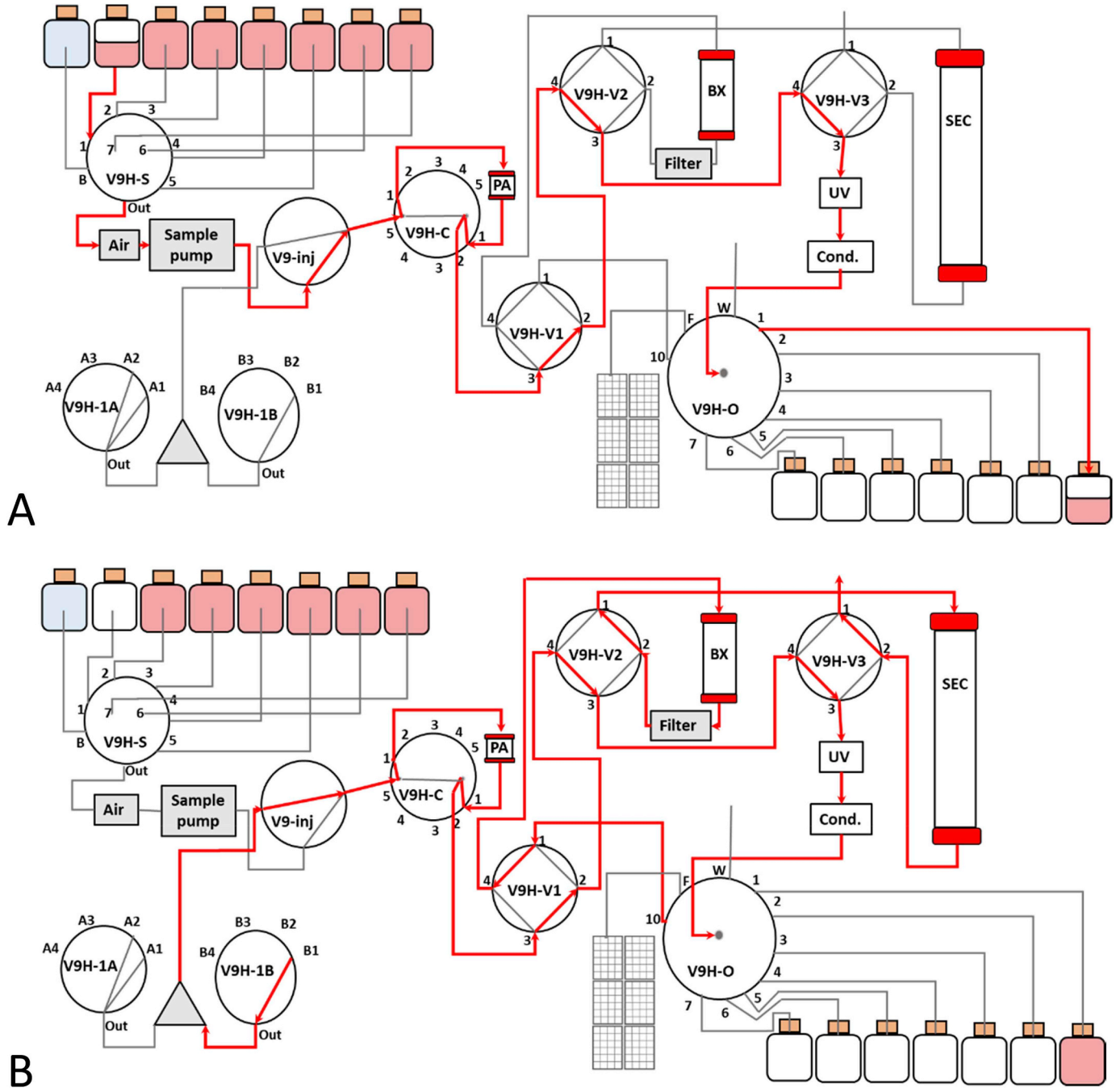

Fig. 2. Flow diagram for the ÄKTA Pure Triple Tandem system. The base system includes a sample valve, injection valve, column valve and outlet valve. The customized flow pattern was accomplished using three versatile valves. (A) The flow for the loading of the Mab Select SuRe column (ProA) is indicated by red arrows; the ProA flow through was collected in a separate vessel. (B) The red arrows indicate the flow path used for the ProA elution and the tandem loading of the Buffer exchange column (BX). (C) The elution of the BX column and the tandem loading of the SEC column is indicated by red arrows. (D) The elution of the SEC column is indicated by the red arrows with fractions collected when the UV absorbance threshold was exceeded.

are regenerated with 6 M guanidine HCl and the columns and all the lines are re-equilibrated. The first step is to flush V9H-O outlet 10 with 5 mL of A2 buffer PBS with V9H-V1 ports 1–4, V9H-V2 ports 1–2 and V9H-V3 ports 3–4. The ProA is regenerated with 3 CV of 6 M guanidine HCl with the flow for V9H-C through col. 1 and V9H-V1 set at port 2–3 and the flow through V9H-V2 ports 3-4 and V9H-V3 ports 3–4. The BX column and in-line filter are then regenerated with 1 CV of 6 M guanidine HCl the flow of which went from V9H-C in by-pass, V9H-V1 ports 3-4, V9H-V2 ports 1-2 and V9H-V3 ports 3–4. The BX is re-equilibrated with 3 CV of PBS by changing buffer input to A2. The ProA column is re-equilibrated with 3 CV of PBS from buffer line A1 with V9H-C set at column position 1 and V9H-V1 ports 2-3 and V9H-V2 ports 3-4 and V9H-V3 ports 3–4. The system is then ready to begin the next purification using the scouting method. Once the scouting run has completed, another run is initiated after the sample leads are flushed with PBS. The Unicorn script employed for this method is presented in Supplemental Fig. 1.

2.3. NGC modifications

The NGC Quest 100 instrument (BioRad, Hercules, CA) was extensively modified to accommodate all the required flow paths of the

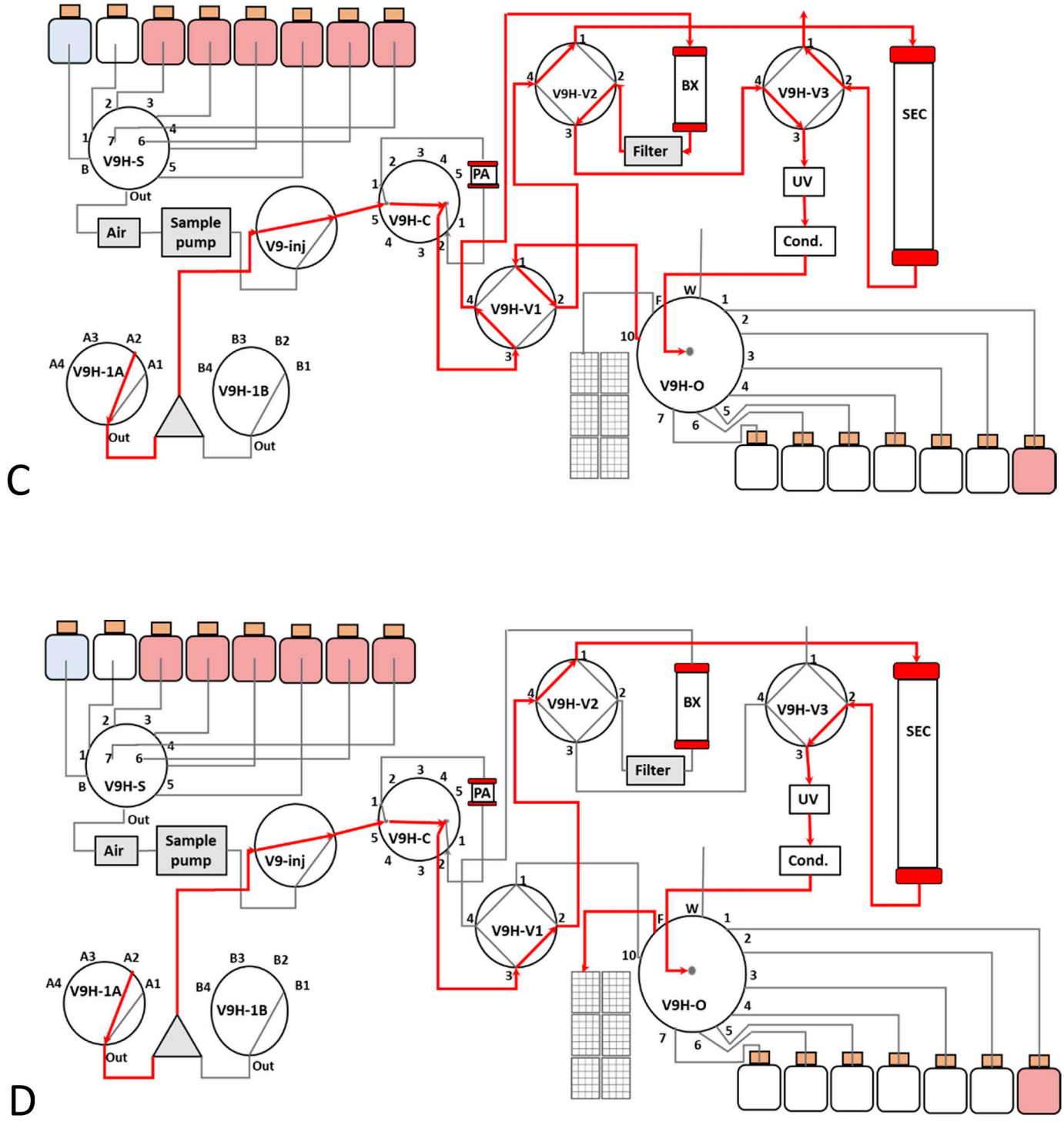

Fig. 2. (*continued*)

automated Triple Tandem method. The instrument is configured with two buffer inlet valves, two sample valves, an injection valve, three column valves and two outlet valves, as shown in Figs. 1B and 3. The sample valve 1 (SV1) and the injection valves are plumbed in the conventional manner. For the column valve 1 (CV1); the "in" port is connected to the column port of the injection valve, the "out" port is connected to the common port of outlet valve 2 (OV2), the top port 1 is connected to the bottom port 3 of the CV2 and the top port 2 is connected to the bottom port 1 of the column valve 3. For the column valve 2 (CV2); the "in" port is connected to the bottom of the HiLoad 26/600 Superdex 200 pg column (SEC), the "out" port is connected to the top of the 53 mL HiPrep 26/10 G-25 Buffer exchange column (BX), the top port 1 is attached to the port 3 of SV2, the top ports 3 and 5 are waste lines, the bottom port 1 is connected to port 11 of outlet valve 1 (OV1). For column valve 3; the "in" port is connected to the filter (connected to the bottom of the BX), the "out" port is attached to the top of the SEC column, the top port 1 is a waste line, the top port 2 is connected to port 2 of sample valve 2 (SV2), the bottom port 2 is connected to port 9 of the outlet valve 1. For SV2, the common port is connected to the UV and conductivity monitors, port 1 is attached to port 1 of OV2, port 4 is connected to the bottom of the two-tandem 5 mL HiTrap MabSelect SuRe columns (ProA). Port 3 of outlet valve 2 is connected to the top of

the ProA columns. OV1 common port is attached to the UV and conductivity monitors, with lines to the fraction collector and six lines to collect flow through material.

2.4. ChromLab control of the triple tandem system

The use of the ChromLab scouting method allowed for the SV1 to direct the flow via the injection valve, from the appropriate loading vessel at 10 mL/min with the flow going through the OV2 common port to port 3 through the first column and then the OV1 is used to collect the flow through in the appropriate vessel (Fig. 3A). When air is detected by the air sensor located just downstream from the SV1, the sample pump inlet is switched to Dulbecco's PBS (Gibco Life Technologies, Grand Island NY) and the media remaining in the pump is loaded on the ProA column. The column is then taken off line using OV2 port 1 and the tubing and pump are flushed with PBS. Using the system pump, PBS from line A1 is used to wash the ProA column at 5 mL/min through OV2 port 3. The column is then eluted at 2.5 mL/min with 100 mM sodium acetate pH 3.6 from line B1 using the system pump (Fig. 3B). When the UV exceeded 50 mAU the flow is diverted using OV1 port 11 and CV2 bottom port 1 to the “out” port and loading the BX column. The flow through of the BX load flows through the in-line filter to the column valve 3 “in” port and collected in a waste vessel from the top port 1. The loading of the BX column with ProA eluate continues until the UV is less than 25 mAU or until 15 mL is loaded on the BX column. Formulation buffer from line A2, also PBS, is used to elute the BX column with CV1 port 1 through CV2 bottom port 3, to the BX column from CV2 “out” (Fig. 3C). The BX eluate flows through the in-line filter to the “in” port of CV3 through top port 2, to port 2 of the SV2 to the UV and conductivity monitors. When the eluate from the BX column exceeds 25 mAU the flow is diverted using OV1 port 9, bottom port 2 of CV3 to load the SEC column from the “out” port. The flow through of the SEC load proceeds to the “in” port of CV2 to a waste container from top port 3. The BX column is eluted until the UV mAU is less than 25, or for a maximum of 20 mL. The SEC column is eluted with PBS loaded on the column through CV1 top port 2 to bottom port 1 of CV3 to the “out” port which bypasses the BX column (Fig. 3D). The eluate then is taken to the UV and conductivity monitors through CV2 port 1 and SV2 port 3 to the fraction collector port using OV1. Fractions of a maximum of 6 mL are taken when the mAU exceeded 5. To ready the system for the next run in the scouting series, the ProA and BX columns are regenerated with 6 M guanidine HCl, and the columns and all the lines are re-equilibrated using the following protocol. A 6 M guanidine HCl, 50 mM Tris pH 8.0 buffer and then NaOH are applied to the system using the flow paths indicated by the following script commands; for ProA CV1, CV2 and CV3 are switched to by-pass and SV2 to port 4, OV2 to port 3 and OV1 to the waste line in port 1 for 30 mL. The CV3 is changed to port 3, CV1 in by-pass, CV2 to port 1 and SV2 port 4 and outlet port 1 for 1 mL. The BX column and in-line filter are then addressed with CV1 to port 1, CV3 to port 2, CV2 port 3 and OV1 to port 1 waste for 60 mL. The ProA column is then equilibrated with the PBS wash buffer, column valve 1, 2 and 3 in by-pass, SV2 port 4, OV2 port 3 and OV1 port 1 for 30 mL. The appropriate lines are then equilibrated with the ProA wash buffer (PBS) by switching SV2 to port 1, OV2 to port 1. The buffer is then switched to BX and SEC running buffer and the lines are cleared with 40 mL with CV1 on bypass, CV3 port 3, CV2 port 1, SV2 port 1, OV2 port 1 and OV1 port 11. The BX is then equilibrated with 170 mL with the path of CV1 port 2, CV2 port 3, CV3 port 2 and OV1 and OV2 to port 1. Tubing is then flushed with 2 mL following the flow path directed with these commands; CV1 port 2, CV3 port 1, CV2 port 1. The flow path is then equilibrated with ProA wash buffer with 15 mL using these commands; column valve 1, 2 and 3 in bypass, and SV2, OV1 and 2 to port 1. The ChromLab script employed for this method is presented in Supplemental Fig. 2.

2.5. Capacity studies using purified human antibody

Capacity studies of the Triple Tandem systems were performed by diluting 2.5, 5, 12.5, 25, 100, 200, 300 and 500 mg of purified human IgG1 (10 mg/mL) to 400 mL with FreeStyle F17 expression medium (Life Technologies, Grand Island, NY). This series of samples was applied in triplicate to Triple Tandem systems for both the ÄKTA Pure and NGC. To calculate yield, all SEC elution fractions were pooled, and the amount of antibody recovered was compared to the amount loaded. To convert absorbance to protein concentration, the following formula was used (1 mg/mL = 1.472 OD at 280 nM). Absorbance was determined by analyzing 2 µl of sample on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), and the pool volumes were measured in appropriately sized graduated cylinders.

2.6. Resolution studies using human antibody containing aggregate and human Fc

The resolution of the SEC column when used in the Triple Tandem mode was evaluated with the use of partially purified recombinant human antibody to which purified Hu Fc was added. The antibody was obtained from a large-scale cation exchange fraction that contained aggregated material. Purified Hu Fc was added to this protein to produce a mixture that was about 5% Hu Fc. The resulting Triple Tandem load material was evaluated using analytical SEC with a BEH C-200 column (1.8 µm particle size, 4.6 mm × 150 mm), the running buffer was 100 mM NaPO4, 50 mM NaCl, pH 6.8 in HPLC water + 7.5% reagent ethanol at flow rate of 0.4 mL/min. The load purity was measured at 89.6% main peak monomeric antibody, 5.6% aggregate and 4.8% low molecular weight (hu Fc). The load material at 12.9 mg/mL was aliquoted into 12.5, 100 and 200 mg aliquots and diluted to 400 mL with FreeStyle F17 expression medium (Life Technologies, Grand Island, NY). Each diluted aliquot was loaded on the ÄKTA and NGC Triple Tandem systems. The Triple Tandem pools of the main peak were made based on the SEC chromatogram; yield and purity (analytical SEC) were determined using the assays previously described.

3. Results

To enable the automation of a three-column process (ProA to buffer exchange to SEC), a set of auxiliary valves were added to both systems to accommodate the required flow paths (Fig. 1). An ÄKTA Pure 150 L was modified to enable TT purification by adding additional valves: one sample inlet, one column, two buffer inlets, one outlet, and three versatile. Similarly, an NGC Quest 100 system was modified by adding two sample, three column, two buffer inlet, and two outlet valves. Both systems were equipped with air sensors prior to the sample pump to enable flexible sample size by detecting the end of the load to trigger the next chromatographic step (Not pictured).

The ÄKTA Pure flow path for the ProA load and wash, ProA elution with tandem BX load, BX elution with tandem SEC load, and SEC elution with peak detection-based fractionation are shown in Fig. 2. Conditioned media (CM) samples were loaded onto the ProA column via the corresponding V9H-S sample inlet, while the flow-through bypasses the BX and SEC columns and was directed to the appropriate flow-through collection vessel via V9H-O (Fig. 2A). After washing the ProA column, the ProA elution buffer was delivered to the ProA column via the pump B valve. When the user defined UV threshold from the ProA was exceeded, the V9H-O directed the ProA elution to the BX column through V9H-V1, with the BX flow directed by V9H-V2 to the SEC column and then to a waste container via V9H-V3 (Fig. 2B). The tandem BX elution and SEC loading is described in Fig. 2C. Briefly, buffer valve V9H-1A directed the buffer exchange/SEC running buffer (A2) to the BX column via the 3-4 positions of V9H-V1, by-passing the ProA column (V9H-C). The flow from the BX column then proceeded through an in-line filter, to remove any precipitated material emerging

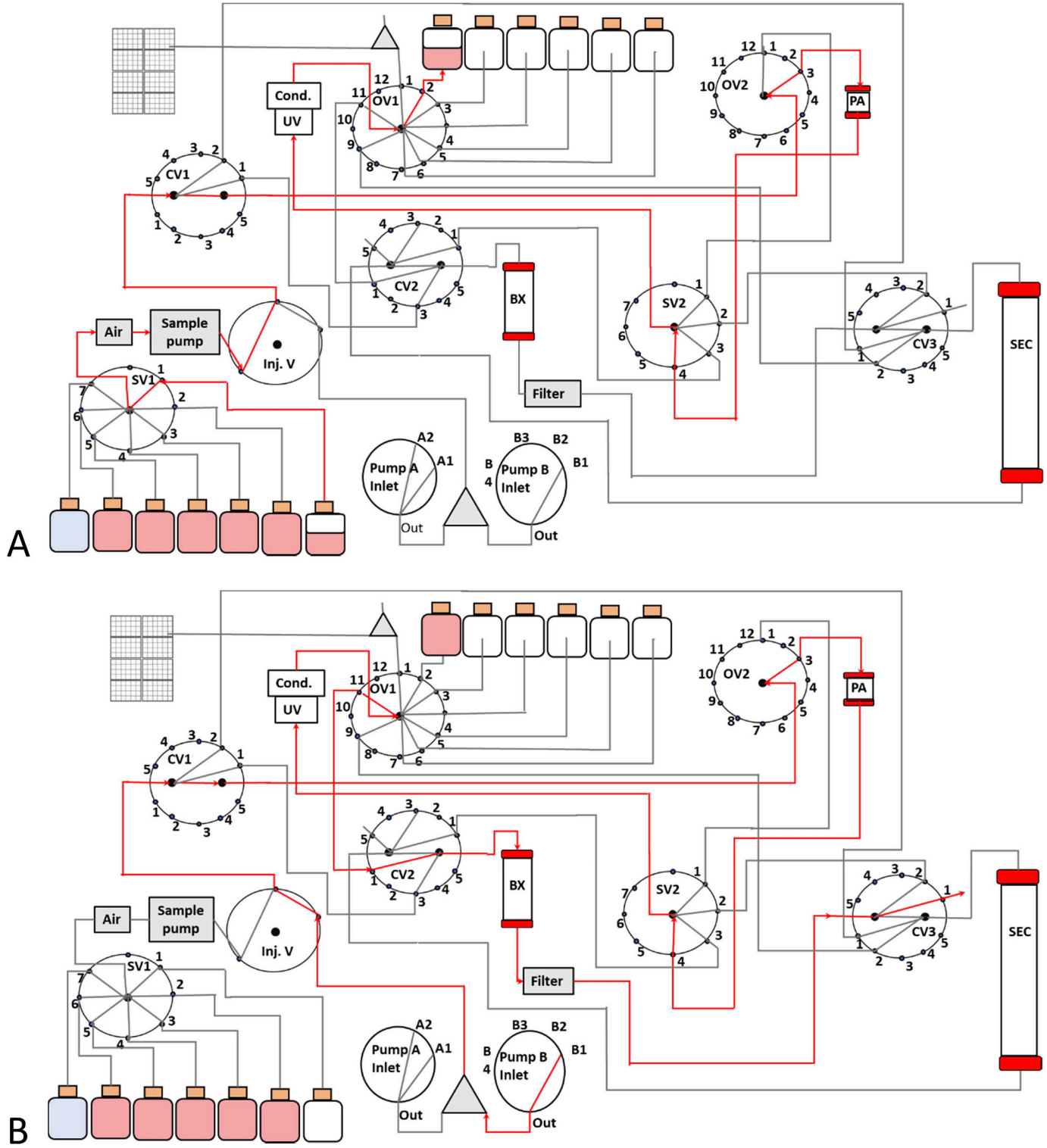

Fig. 3. Flow diagram for the NGC Triple Tandem system. The system includes two sample valves, an injection valve, 3 column valves and 2 outlet valves. The customized flow pattern was accomplished by the unique plumbing of the column valves to accommodate all the required flow paths. (A) The flow for the loading of the Mab Select SuRe column (ProA) is indicated by red arrows with the flow through of the ProA collected in a separate vessel. (B) The red arrows indicate the flow used for the elution of the ProA column and the simultaneous loading of the Buffer exchange column (BX). (C) The elution of the BX column and the concurrent loading of the SEC column is indicated by red arrows. (D) The elution of the SEC column is indicated by the red arrows with fractions collected when the UV absorbance threshold was exceeded.

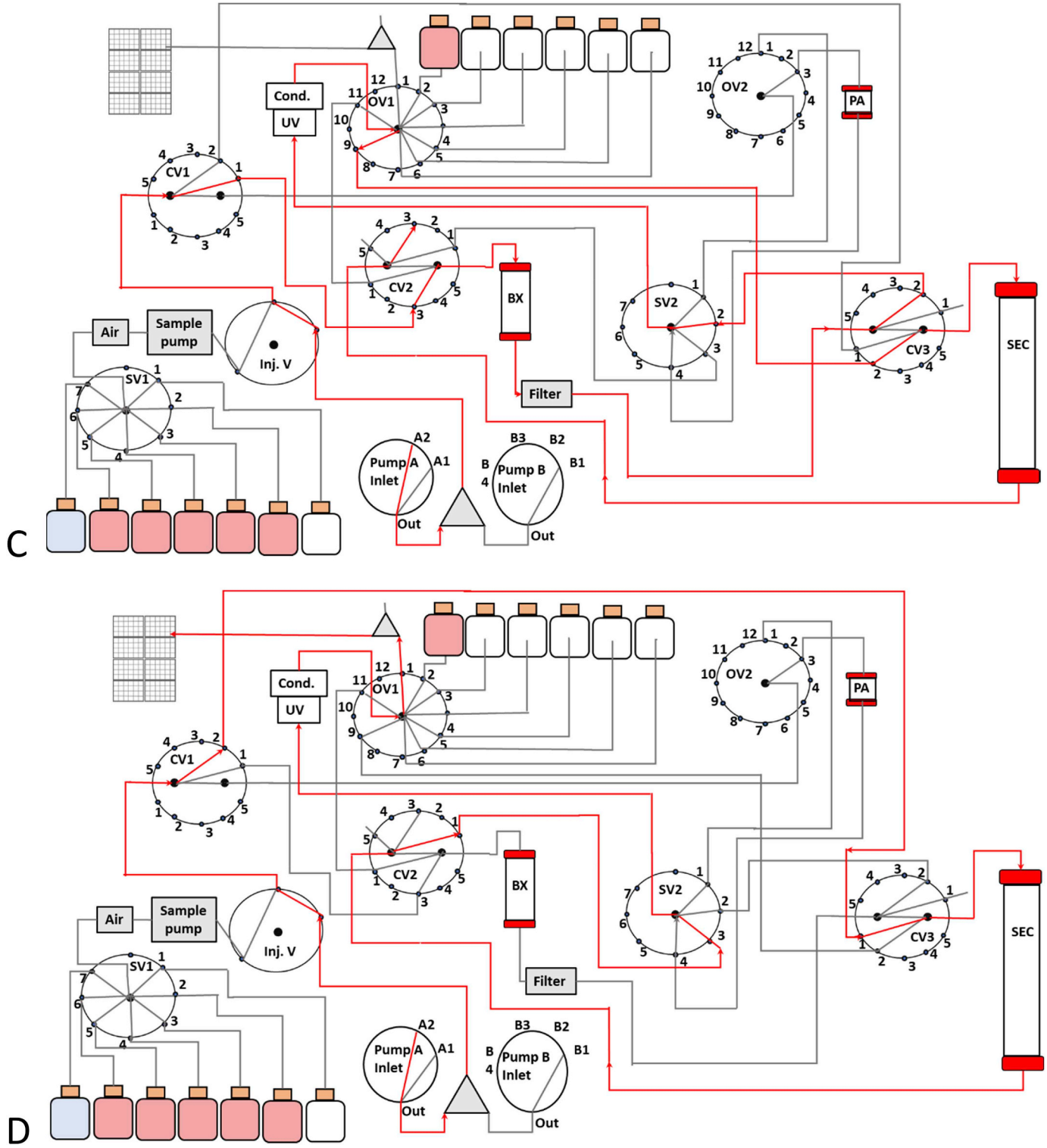

Fig. 3. (*continued*)

from the BX column, then to V9H-V2 ports 2-3 and V9H-V3 ports 3–4 which directed flow to the UV and conductivity monitors. When the user defined UV threshold was achieved, V9H-O directed the flow to the SEC column via V9H-V1 ports 1-2 and V9H-V2 ports 1–4. The SEC flow was then collected in a waste container as directed by V9H-V3 ports 1–2. The SEC elution buffer flow path is described in Fig. 2D, where V9H-C directed the flow to V9H-V1 ports 2–3, then V9H-V2 ports 1–4. The SEC column flow was directed to the UV and conductivity monitors by V9H-V3 ports 2–3, where V9H-O directed the flow to the fraction collector when the user defined UV threshold was exceeded.

The NGC flow diagram is presented in Fig. 3 showing the ProA load, followed by tandem ProA elution and BX load, then tandem BX elution and SEC load, followed by SEC elution. The conditioned media (CM) was loaded onto the ProA column via the corresponding port on SV1, which was directed through the by-pass setting on CV1 to the ProA column via port 3 of OV2. The flow-through from the ProA column was sent through port 4 of SV2 to the UV and conductivity monitor, then directed to the appropriate collection vessel by OV1. (Fig. 3A). The

ProA elution buffer was delivered via the pump B inlet valve to the ProA column, and when the user selected threshold was exceeded, OV1port 11 directed the elution to the BX column through the bottom port 1 to the outlet of CV2, while the flow during this BX load was directed by top port 1 to a waste container from the inlet of CV3 (Fig. 3B). The elution of the BX columns, and simultaneous loading of the SEC column, is described in Fig. 3C. Briefly, buffer inlet valve A directed the buffer exchange/SEC running buffer through CV1 top port 1 to CV2 bottom port 3 to the BX column. The flow from the BX column then proceeded through an in-line filter, which removed any precipitated material emerging from the BX column, then to CV3 top port 2 and SV2 through port 2 of SV2 to the UV and conductivity monitors. When the user selected threshold was achieved, OV1 directed the flow to the SEC column via port 9 and CV3 bottom port 2. The flow from the SEC column during the SEC load was collected in a waste container as directed by CV2 top port 3. The SEC elution buffer flow path is shown in Fig. 3D, where flow to the SEC column was directed by CV1 top port 2 to CV3 bottom port 1. The SEC column flow was then directed to the UV and conductivity monitors by the CV2 top port 1 to SV2 port 3, where OV1 directs the flow to the fraction collector when the user defied UV threshold was exceeded.

To demonstrate the effective range of the Triple Tandem systems, 2.5–500 mg of antibody was purified using both the ÄKTA pure and the NGC systems (Fig. 4). A test sample was made by diluting purified antibody (10 mg/mL) to produce samples from 2.5 to 500 mg total protein diluted with 400 mL F17 cell culture media. The yield was calculated by comparing total protein recovery from the SEC fractions to the amount loaded. The yield of the Triple Tandem was consistent for challenges between 12.5 and 100 mg, with recoveries ranging from 82 to 92% for both instruments. The recovered yield for the 2.5 mg challenge was 58% for the Pure and 55% for the NGC, while the 5 mg challenge was 67% for both systems. For challenge amounts of 200, 300 and 500 mg, the yield was higher for the NGC Triple Tandem (89%, 82% and 65%, respectively) compared to the ÄKTA Pure (82%, 64% and 44%, respectively).

To evaluate the ability of the TT to separate desired products from high- and low-molecular weight contaminants, test material was prepared by combining a partially purified, aggregate-containing antibody with purified Fc, diluted into fresh cell culture media. The test material was purified using both ÄKTA Pure and NGC Triple Tandem systems, and the chromatograms are shown in Fig. 5. The test material was first loaded on the ProA column, and non-binding UV absorbing material was observed as ProA flow-through. The sample pump was flushed with the PBS wash buffer to chase the remaining test material through the system to maximize yield and rinse the sample pump. The ProA column was then washed with PBS to remove unbound material from the column (P1). The protein was then eluted from the ProA column, and when the elution peak (P2) was detected, it was redirected to the BX column. The flow path was then changed to detect the eluent from the BX column. When the BX elution peak (P3) was detected, the flow was redirected to the SEC column. The SEC column was then developed, and the chromatograms showed resolution of the three components in the test material (P4 is the high molecular weight material, P5 is the monomeric antibody and P6 is the Fc). The chromatogram also shows the guanidine HCl strip, NaOH strip and re-equilibration of ProA and BX columns. Stripping, sanitization and re-equilibration of the SEC column is not required, since only soluble, filtered material reaches this column, and it was maintained in the running buffer throughout the process.

The resolution capability of the ÄKTA Pure and NGC Triple Tandem systems are shown in Figs. 6 and 7. The test material was a purified aggregate-containing antibody to which purified Fc protein was added, resulting in a mixture with 89.6% monomeric antibody by analytical SEC. The system was challenged at 12.5 mg, 100 mg and 200 mg total protein, and the pooling was based on the SEC chromatograms shown in Fig. 6. The chromatograms for the 200 mg challenge show asymmetry with a more prominent right shoulder present in the NGC profile. Similar chromatograms with clear right shoulders of the main peak were observed when challenging the Pure system at 300 mg or greater. The entire main peak was included in the pool, even in cases of asymmetry, and as shown on Fig. 7, the analytical SEC purity of the

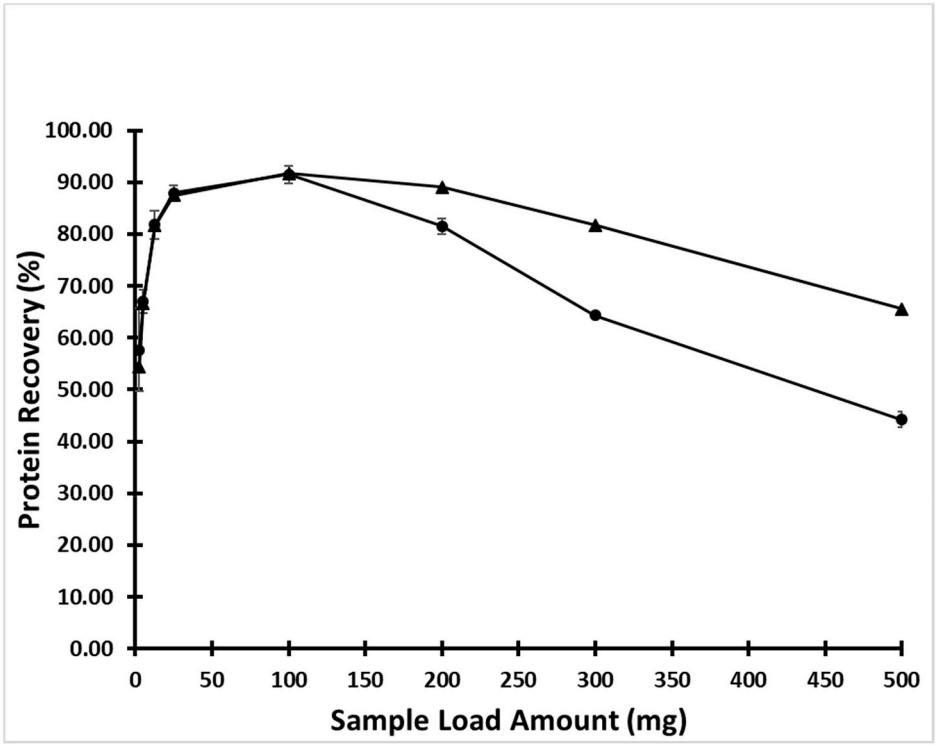

Fig. 4. Total capacity of the ÄKTA (●) and the NGC (▲) triple tandem systems. Protein loads between 2.5 and 500 mg of an IgG1 antibody were tested on a tandem system using a 10 mL ProA, 53 mL buffer exchange column and a 320 mL Superdex 200 column. The yield was determined by the total protein eluted from the SEC and the total protein load. All values shown are averages of three independent experiments and the error bars are the standard error of the mean.

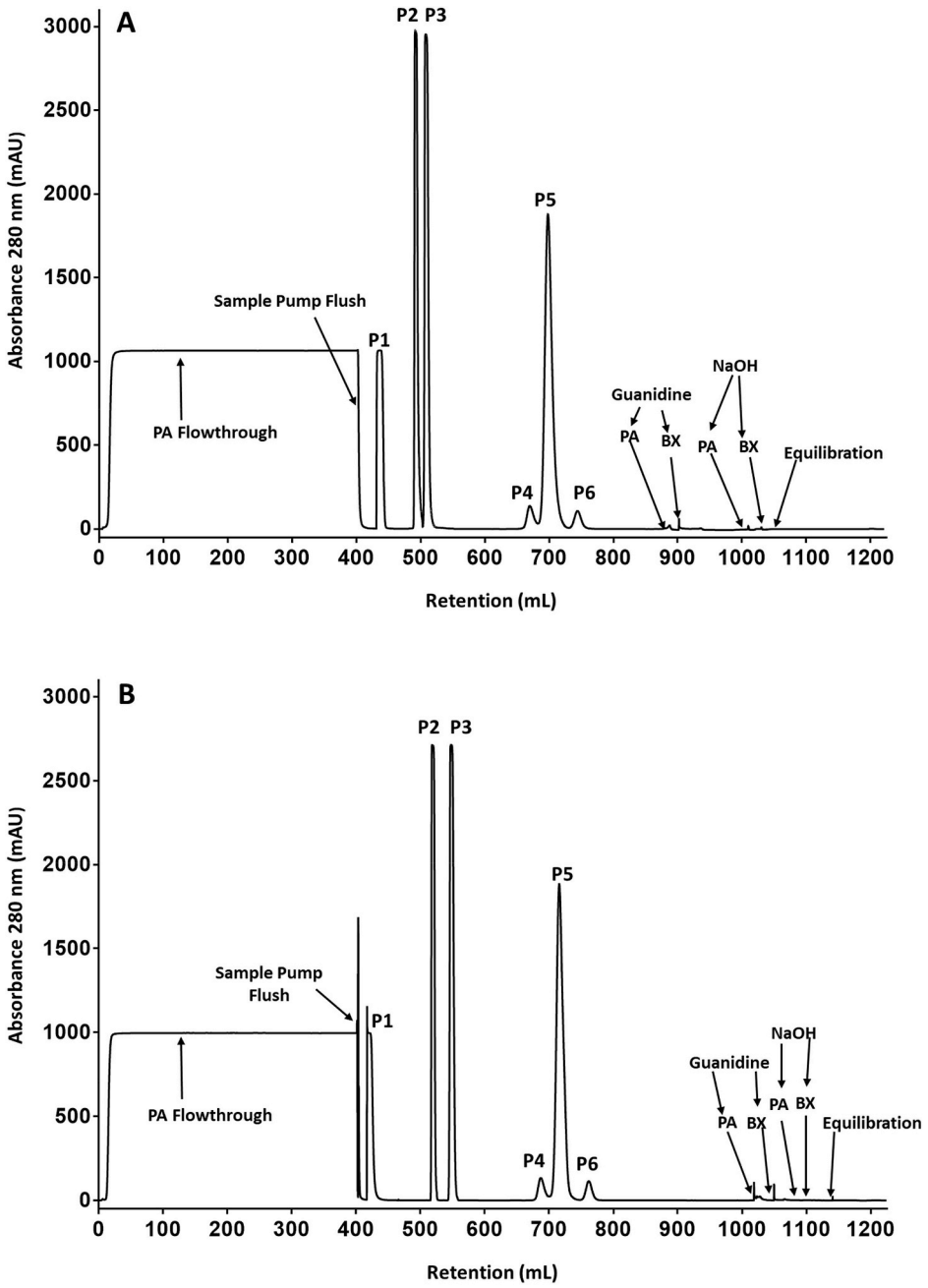

Fig. 5. Representative chromatograms of the Triple Tandem system purification of culture media to which purified antibody containing both aggregate and lower molecular weight protein (hu Fc) was added. The chromatogram of the ÄKTA system is A and the NGC is B. P1 is the ProA wash, P2 is the ProA elution and P3 is the BX elution. P4, P5 and P6 are the SEC elution peaks, with P4 is the aggregate of the antibody, while P5 is the antibody and P6 is Hu Fc.

main peak pools for both the ÄKTA Pure and NGC was 99.8% or greater at all challenge levels. Representative analytical SEC profiles from this study are shown in Supplemental Fig. 3. The recovered yield was calculated from the amount of protein in the main peak pool divided by the total protein in the load. The yields for the ÄKTA Pure at the 12.5, 100 and 200 mg challenges were 77%, 81% and 78%, respectively, and the NGC at the same challenge levels was similar at 74%, 83% and 79%, respectively. The maximum expected recovery would be 89.6% based on the sample preparation.

Table 1 summarizes the cycle time for Triple Tandem purification for both systems, including regeneration. For example, a 50 mL load has a 245 min cycle time, while a 1000 mL load has a 340 min cycle time, corresponding to 5.9 and 4.2 purification cycles per day, per system, respectively.

4. Discussion

While high-throughput affinity purification often suffices for initial molecule screens, larger amounts of higher quality material is frequently required for more comprehensive assessments and candidate selection. Thus, the ability to simultaneously increase throughput and decrease labor to produce larger amounts of more pure protein products can be a major advantage. Currently, there are systems capable of carrying out multidimensional chromatography at laboratory scale, typically using affinity capture followed by ion exchange (9) or SEC (3, 4, 8), covering a broad range of applications. However, existing tandem affinity-SEC methods are vulnerable to post-affinity column precipitation events (10–14) that can foul the SEC column resulting in reduced service life and possible cross-contamination between samples

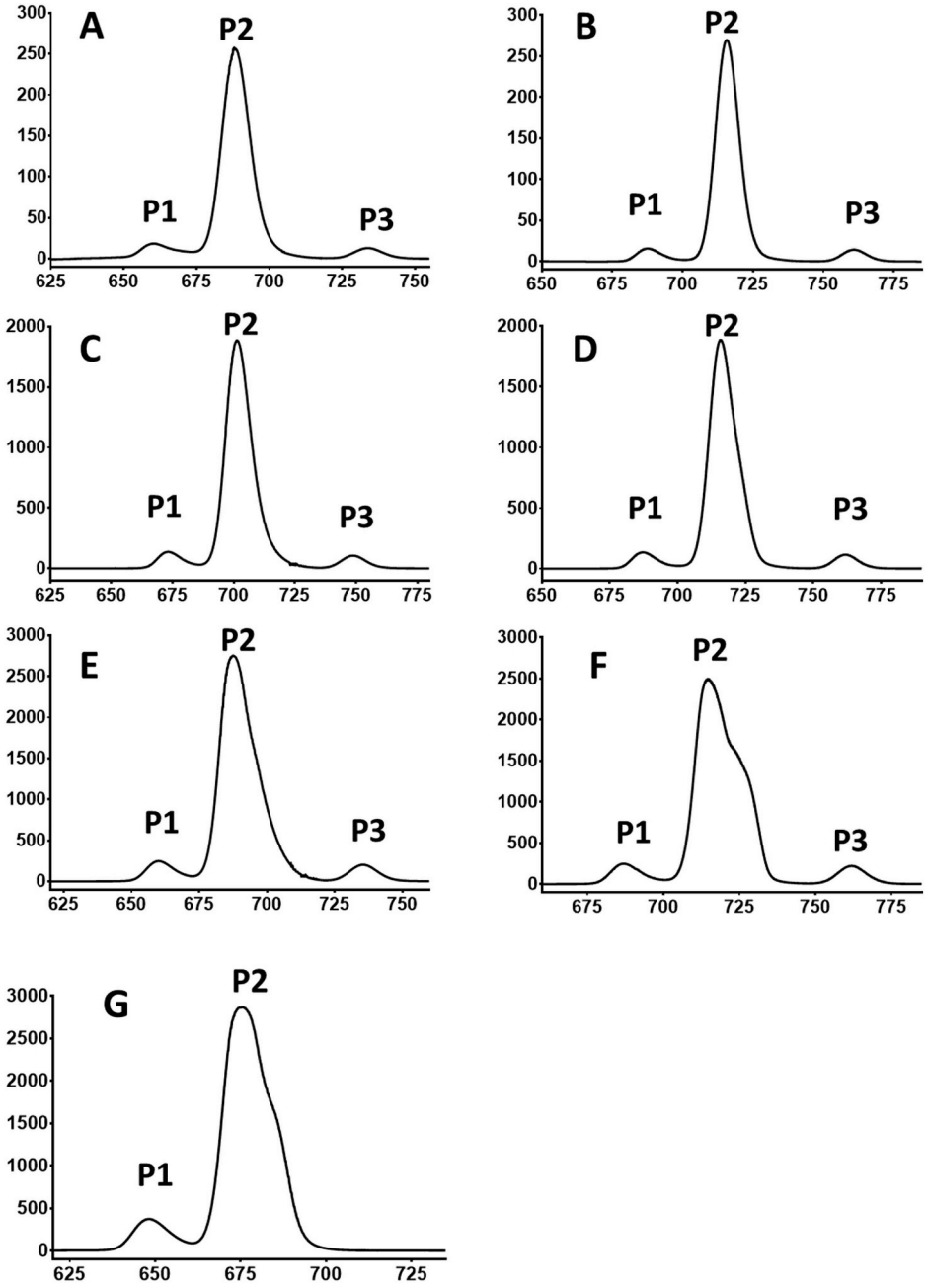

Fig. 6. The SEC elution profiles of the ÄKTA triple tandem system (y-axis is mAU and x-axis are mL). The protein load of the system was an antibody containing aggregate and Fc protein. The antibody monomer peak of the load is 89.6% by analytical SEC. The elution profile for a 12.5 mg challenge is presented for the ÄKTA (A) and the NGC (B), the profile of a 100 mg challenge is shown for the ÄKTA (C) and the NGC (D), a profile of a 200 mg total protein challenge for the ÄKTA (E) and the NGC (F). P1 is the antibody aggregate, P2 is the antibody peak, P3 is the Hu Fc protein. The ÄKTA chromatogram of a 300 mg challenge of the antibody containing aggregate without the Fc addition (P3) is depicted in G showing a similar profile of main peak to that of the NGC at 200 mg challenge.

detectable by mass spectroscopy. These precipitation events can occur when the harsh affinity column elution conditions are shifted to the mobile phase conditions of the SEC column, which usually includes a large jump in pH. Due to the low flow requirements and relative fragility of SEC columns it is generally not practical to run harsh cleaning cycles on these columns between every sample to clear aggregated material. To minimize the risk of lot to lot carryover, we designed a Triple Tandem system that added a robust buffer exchange column between the affinity and SEC columns to rapidly transfer the affinity eluate into the mobile phase of the SEC column and capture any precipitate that may occur with a post column in-line filter. Since the buffer exchange column and in-line filter are robust and can accommodate high flow rates, it is possible to rapidly clean them between cycles with harsh reagents. The affinity column selected for this study was Pro A; however, the system is flexible and can be applied to other affinity chromatography methods. For cases where the affinity elution peak is broad a relative reduction in affinity column size may be required to ensure most of the eluate will be loaded on the buffer exchange column.

The design of the Triple Tandem systems required unique plumbing and unconventional utilization of the available flow valves. Monitoring the UV and conductivity of the column eluants required redirection of the monitored flow to concurrently load the next column, which eliminated the need for holding vessels or loops. This novel flow requirement required the repurposing of waste lines for the BX and SEC load eluent. Also, regeneration, sanitization and equilibration of the ProA, BX and system required flow paths that bypassed the SEC during this process.

The ÄKTA Pure, equipped with standard buffer, injection, column and outlet valves, also required the addition of three versatile valves to accomplish the required flow paths. The resultant design has the capability of running seven independent purifications through Triple Tandem purification without user intervention. The limitation on the number of runs is dictated by the available lines on the sample pump, as

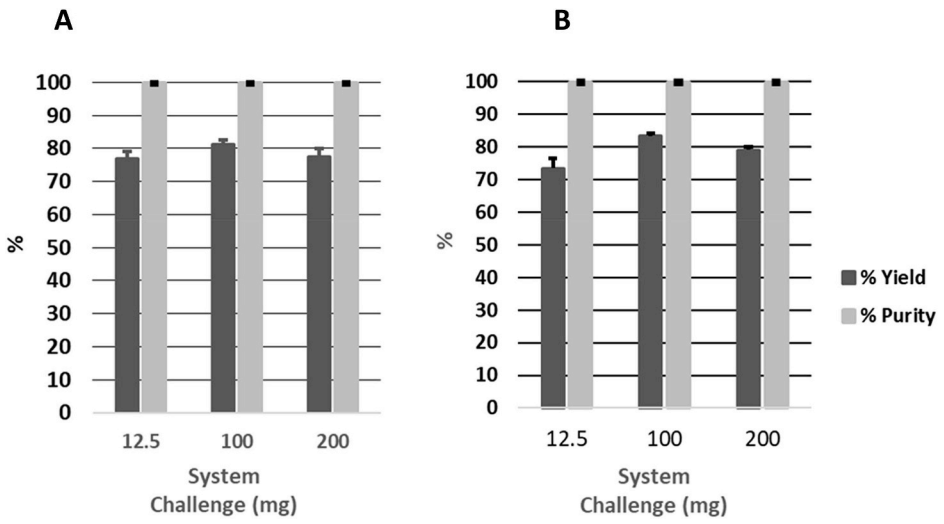

Fig. 7. The resolution of the ÄKTA (A) and NGC (B) triple tandem systems. The protein load was an antibody containing aggregate to which Fc protein is added. The main peak of the load is 89.6% by analytical SEC. The system was challenged at 12.5 mg, 100 mg and 200 mg of total protein. The pooling was based on the SEC chromatogram. The yield was calculated from the amount of protein in the pool and the total protein load. Purity was determined by analytical SEC. The error bars are the standard deviation of three independent experiments of each challenge level.

Table 1
The total cycle time of the ÄKTA and NGC Triple Tandem system when the volume load ranges from 50 to 1000 mL using a 10 mL ProA, 53 mL BX and a 320 mL SEC column.

Volume Load (ml)	Cycle Time (min)	Samples/Day
50	245	5.9
100	250	5.8
200	260	5.5
500	290	5.0
1000	340	4.2

well as the limit of 144 fractions in the fraction collector when using six 24 deep well blocks. The Triple Tandem system could also be used for basic single column chromatography without plumbing changes; however, the method would require modification compared to a standard system, since the versatile valves at the default settings in the Triple Tandem configuration do not allow for conventional flow paths.

The NGC system, in addition to the injection and buffer valves required three column, two sample and two outlet valves for Triple Tandem capability. The resultant design has the capability of running six independent consecutive purifications without user intervention. This limitation is due to the number of available lines on the sample pump valve as well as the limit of 192 fractions in the fraction collector equipped with eight 24 deep well blocks. The NGC Triple Tandem system can also be used for conventional single column purification without plumbing changes if the column is placed in the CV1 port 3; however, unlike the ÄKTA Pure, no modification of the method script is required.

The Triple Tandem system is designed to produce high quality material for more comprehensive testing and to meet the increased material requirements frequently needed for later phase discovery research assessments. In addition, since expression levels of proteins can vary unpredictably, and it is often of interest to obtain purified proteins for both low and highly expressed constructs, we also sought to design a system capable of a broad range of capacities. Both the ÄKTA Pure and NGC Triple Tandem systems are capable of producing purified product over a wide range of protein loads (2.5 mg–500 mg) while maintaining good recovery yields. For example, both systems gave yields > 50% at relatively low 2.5 mg load conditions, and the yield increased rapidly to > 80% at moderate load levels of 12.5 mg. Both Triple Tandem systems were also effective when the ProA column was overloaded with 500 mg protein. When challenged at the high end of the range, the NGC modestly outperformed the ÄKTA Pure with regard to yield. For example, at a 500 mg protein load, the NGC resulted in a yield of 65% while the ÄKTA Pure resulted in a 44% yield. This unexpected difference in yield could not be attributed to column age but may be attributed to plumbing and configuration differences allowing for increased binding and elution from the ProA column during overloading conditions. It should also be noted that a shifted range of load levels and recovery yield may be possible by employing columns sets of different sizes and dynamic binding capacities than used in this study.

Both Triple Tandem systems produced high purity material greater than 99.8% with protein loads between 12.5 and 200 mg. At these load levels the yields of the products were essentially equivalent, ranging from 74 to 83% (89.6% maximum possible), demonstrating that both instruments can produce similar results from a panel across a wide range of titers. The main peak profiles from the SEC chromatograms exhibited asymmetry when challenged at a high, overloaded levels. Although the main peak purity was maintained throughout, the main peak asymmetry may be due to a transient protein conformation change due to instability induced during the concentrated low pH ProA elution conditions.

Processing time is a critical parameter for overall productivity. Due to the low flow rates of SEC, rapid processing is not possible; however, by enabling a system to couple affinity capture and SEC in an automated fashion, as well as process multiple samples without user intervention, efficiency can be gained by enabling hands-off operation 24 h per day. The automation of the process increases instrument utilization efficiency by eliminating scientist time spent pooling, clarifying ProA elutions, and setting up SEC columns, which when automated can occur during non-working hours. In addition, the risk of human error during pooling and column loading is eliminated using this coupled method.

Both the ÄKTA Pure and NGC systems can be customized as Triple Tandem purification systems. There are only minor capability differences between the systems. For example, the AKTA Pure can process seven separate purifications without intervention, while the NGC can process six; however, the NGC can be used as a conventional system in single column mode without requiring custom scripts to accommodate non-standard plumbing. It is not entirely clear why the NGC produced higher yields than the AKTA Pure at very high protein loading conditions, but this may be due to some nuances of the plumbing required, UV triggers or scripting. Overall, these systems perform very similarly with this complex and advanced configuration. The Triple Tandem is a flexible and robust system that implements novel plumbing configurations and enables the purification of high-quality proteins, across a wide range of scales. The TT is a very robust automated system that can significantly increase the efficiency and throughput of multi-dimensional purifications without user intervention and reduce potential user errors. Although the design of the system reduces the risk of cross contamination, the proteins produced using this system should be

verified using analytical techniques such as mass spectroscopy to verify that cross contamination did not occur. Furthermore, the Triple Tandem configuration shown here could likely be adapted to accommodate other automated triple column chromatography process by changing the column types and size and making modest changes to the scripting.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2019.105497.

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