



Development of an automated mid-scale parallel protein purification system for antibody purification and affinity chromatography



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ABSTRACT

Protein purification is often a bottleneck during protein generation for large molecule drug discovery. Therapeutic antibody campaigns typically require the purification of hundreds of monoclonal antibodies (mAbs) during the hybridoma process and lead optimization. With the increase in high-throughput cloning, faster DNA sequencing, and the use of parallel protein expression systems, a need for high-throughput purification approaches has evolved, particularly in the midsize range between 20 ml and 100 ml. To address this we modified a four channel Gilson solid phase extraction system (referred to as MG-SPE) with switching valves and sample holding loops to be able to perform standard affinity purification using commercially available columns and micro-titer format deep well blocks. By running 4 samples in parallel, the MG-SPE has the capacity to purify up to 24 samples of greater than 50 ml each using a single-step affinity purification protocol or a two-step protocol consisting of affinity chromatography followed by desalting/buffer exchange overnight (~12 h run time). Our evaluation of affinity purification using mAbs and Fc-fusion proteins from mammalian cell supernatants demonstrates that the MG-SPE compared favorably with industry standard systems for both protein quality and yield. Overall the system is simple to operate and fills a void in purification processes where a simple, efficient, automated system is needed for affinity purification of midsize research samples.

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1. Introduction

In large molecule drug discovery, protein purification has become a bottleneck for protein and antibody production. During a typical therapeutic antibody campaign tens to hundreds of antibodies need to be purified from hybridoma cell supernatants for early characterization [1]. Additionally, as programs progress many lead variants that are generated as secreted recombinant proteins will also need purification. Because these samples are often expressed in volumes ranging between 20 and 100 ml and require only affinity purification by Protein A [2] for initial assessment, we determined that a simple, easy to use, high-throughput midscale parallel purification system capable of single-step affinity chromatography would be of significant value in early stage large molecule drug discovery efforts.

Currently, the industry standards for antibody and protein

purification are the ÄKTA FPLC systems (GE Life Sciences). These systems are capable of mid-to large-scale multi-sample purification in series and can perform many complicated purification tasks such as salt gradients and size exclusion chromatography (SEC) precisely and reliably. However, standard ÄKTA FPLCs don't allow parallel purification and they are overly-complex and expensive in cases when only single-step affinity purification is required for antibodies and other affinity purification. Because of this we felt that ÄKTAs should remain the system of choice for complex multi-step purifications and large-scale lab preparations, but a simpler more cost-effective system that allowed parallel sample processing could increase efficiency when sample numbers are high and the purification required is simple on/off affinity chromatography. Over the years, many protein purification methods and systems have been developed for handling multiple samples [3–8]. However, most of these methods and systems were designed for small-scale affinity purification of 1 ml or less. Other systems have been developed that enable mid-to large-scale purification of samples in parallel. These systems do not run in series and they require a number of columns

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equal to the number of proteins being purified which can become costly and time consuming to set up [9]. Additionally, these systems are not setup to use micro-titer plates requiring an extensive network of tubing for sample loading and collection. Recently, an auto-sampler coupled to an ÄKTA FPLC has been reported to work very efficiently for automated two-step purification of antibodies [10].

We set out to develop a system that could run >20 mAb (or other standard affinity purifications) overnight unattended and deliver the same quality and yield as a standard single-step ÄKTA purification. The design criteria included running multiple samples in parallel to save time and using micro-titer plates to simplify sample loading and collection while facilitating compatibility with upstream and downstream automation processes. Additionally, we wanted to be able to use standard commercially available columns and have an interface that was simple and easy to use. To make the system cost-effective we determined that a UV detector was not required because of the predictability of affinity chromatography and that a gradient maker for ion exchange was not needed. After considering multiple systems we chose to modify a Gilson SPE system (GX-274) with switching valves and holding loops to accommodate GE Hi-trap affinity columns and added a unique bed layout to hold 8 micro-titer plates. In this paper we describe how the system was designed and evaluated and we present data demonstrating that it can perform single-step affinity purification with comparable quality and yield to industry standard systems in less time.

2. Materials and methods

2.1. Expression of purification of recombinant antibodies and Fc-fusions

Recombinant expression of mAbs was performed using 2936E Human Embryonic Kidney (HEK) cells in F17 media supplemented with 1X GlutaMAX, 25 µg/ml Geneticin, and 0.1% Pluronic F68 (all reagents from Thermo-Fisher, Life Technologies). Cells were grown in non-baffled shake flasks (Corning) at 150 rpm at 37 °C supplemented with 5% CO₂ and transfected at a cell density of 1.2×10^6 /ml using 25 kDa linear polyethyleneimine (PEI). DNA was diluted into pre-warmed media then added to 1.5 mg/ml PEI (DNA:PEI = 1:3). Transfection complex was incubated at room temperature for 20 min prior to transfection. Following transfection cells were grown for 7 days and cell supernatants were collected and filtered through a 0.45 µm filter. For some experiments, antibodies were purified directly from supernatant as indicated.

For experiments using purified mAb added back to conditioned media, IgGs or Fc-Fusions were purified by loading supernatants on to a recombinant protein A column (MabSelect Sure; GE Life Sciences) using an ÄKTA FPLC (GE Life Sciences). Columns were washed with 10 CV of PBS followed by elution with 100 mM acetic acid. Elution was concentrated and run on a Hi-Load 26/60 Superdex size exclusion chromatography column (SEC) developed with 10 mM sodium acetate pH 5.2, 150 mM NaCl to any remove aggregate. Antibodies were quantified using Nanodrop A280 and added at indicated concentrations to F17 media.

2.2. One-step purification

For developing the general protocol to perform affinity chromatography purification, four samples were purified by the MG-SPE in a single run. One milliliter mAbSelect Sure columns (GE Life Sciences) were used to perform purification with a flow rate of 2 ml/min. Prior to loading samples, the system was primed with system fluid (10% glycerol, 0.02% NaN₃) and sanitized using 0.2 N

NaOH. Then the system was equilibrated with PBS buffer (pH 7.4). Forty-four milliliter cell supernatant containing a recombinant expressed mAb was loaded on to the column for each channel. After loading the samples, the system was washed with 10 ml PBS buffer (pH 7.4) and eluted with 3 ml of 100 mM acetic acid. The eluates were analyzed by non-reducing 4%–12% SDS-PAGE (Thermo-Fisher Life Technologies). Ten microliters of each eluate was loaded on to the gels shown. Gels were stained with Safe Stain (Thermo-Fisher). The quality of the purified protein was also analyzed by analytical SEC. A Superose 6 10/300 GL Column (GE Life Sciences) was used to perform the analysis. Fifty microliters of the elution was loaded onto the column and run isocratically with PBS.

The purified yield was measured by purifying antibody of known amount using MG-SPE. Seven samples containing different amounts of antibody (100 µg, 200 µg, 500 µg, 1000 µg, 2000 µg, 4000 µg) were purified and the recovery was calculated based on UV absorbance at 280 nm obtained on Nano-drop (Thermo Scientific). In order to make the comparison, same experiments were run on an ÄKTA FPLC (GE Life Sciences).

In order to test the cross contamination carry-over from previous runs, 12 samples were purified in three runs sequentially. Cell supernatant containing recombinant expressed mAb was purified in first and third runs and cell supernatant containing a recombinant expressed Fc-fusion protein was purified in second run. After purifying the first four samples, the system was cleaned with 25 ml 0.2 N NaOH and equilibrated with 25 ml PBS. Then samples 5–8 were purified by the same protocol as samples 1–4. The cleaning step was repeated prior to purification of samples 9–12. The eluates were analyzed by reducing and non-reducing SDS-PAGE.

2.3. Two-step purification including buffer exchange

After purification by affinity chromatography, the 3 ml eluates were buffer-exchanged to 10 mM sodium acetate pH 5.2, 150 mM NaCl by passing them through 8.3 ml PD-10 desalting columns by gravity flow with the addition of 4 ml additional formulation buffer. Columns (GE Life Sciences) were set up on a collection block shown in Fig. 5 and placed on the plate deck in the elution block spot. The product from a single step Protein A purification was dispensed directly on top of the column and allowed to pass into the column by gravity. To have full elution, 4 ml of additional formulation buffer was dispensed on top of the column and the ~7 ml of eluate was collected as purified product and analyzed. The product was analyzed by non-reducing SDS-PAGE. The recovery after two-step purification was evaluated using the same method described in Section 4.

3. Results and discussion

3.1. Gilson setup and modifications

The standard Gilson GX-274 SPE (Gilson Inc.) system is composed of three main parts (Fig. 1): four syringe pumps (A), a robotic arm with four channels (B), and a plate deck (C). The syringe pumps connect to the robotic arm which can transfer liquid samples on the plate deck. In order to perform on/off affinity chromatography, modifications to the standard SPE system were as follows (Fig. 1): 1) a 25-ml holding loop was installed upstream of each syringe pump to permit sample and buffer loading without entry into the pump (E); 2) a 6-port switching valve that accepts standard chromatography cartridges (including Hi-Trap columns) was placed between the robotic arm and the holding loop for each channel (D); 3) a new plate deck was added to hold eight micro-titer plates and 4–500 ml buffer reservoirs (C). Detailed plans are available from Gilson Inc. Additionally, sample blocks with standard micro-titer

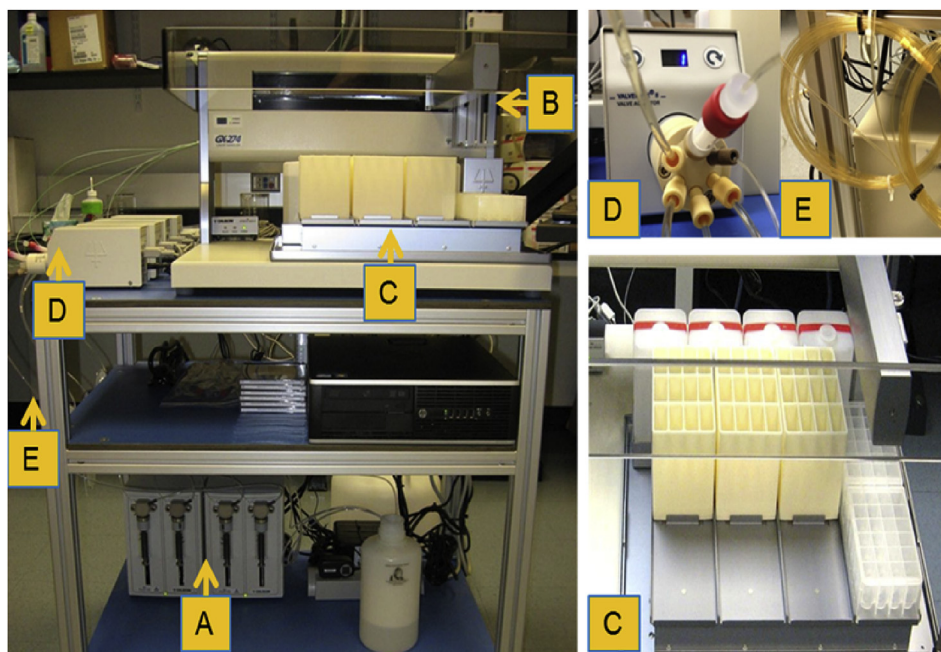


Fig. 1. MG-SPE system configuration: A) Syringe pumps B) 4 channel robotic arm C) Modified plate deck with custom sample blocks D) Switching valves (added to system) E) 25-ml holding loops (added to system).

footprint containing 12 wells of 75 ml capacity each were manufactured by a 3D printer (in order to perform purifications of samples in the 50 ml range) and the system was mounted on a mobile cart.

A schematic of MG-SPE is shown in Fig. 2. The robotic arm can transfer liquid between any of the blocks on the plate deck and the switching valve has two positions. In position 1 samples and buffers bypass the column when being drawn into the holding loop. In position 2, the contents of the holding loop are pushed over the column.

The methods are designed in Trilution® software which allows users to design each step and build up methods based on the steps. Parameters including loading volume, result volume, flow rate and channel number can be set up before runs or modified during runs. The user interface and bed layout window are shown in Fig. 3. To perform affinity chromatography, four 500 ml reservoirs on the top of the deck were designated as buffer containers (Wash, Elution, Sanitization, Formulation) and the micro-titer blocks were designated for sample handling and collection (Crude sample, Flow-through Collection, Column Wash, Elution as indicated). Although we used a similar setup for all purifications described, the positions on the plate deck are flexible and various purification methods can be set up using the software. For example, flow-through and column wash can be discarded to waste to allow more blocks for crude sample and eluate collection to increase capacity.

3.2. One-step parallel affinity purification

As described above, MG-SPE was modified with switching valves, buffer holding loops, and a new bed tray to perform mid-scale parallel protein purification. The system consists of four parallel channels that allow six sequential runs of ~44 ml each to be performed in an automated fashion allowing purification of 24 samples without intervention. To test the system's core function of on/off affinity purification capabilities, we chose to use mAb purification by protein A resin as a model system [2].

In order to establish a single-step protein A purification protocol

that could be used for 24 samples, we first ran only 4 samples in parallel to test the feasibility and optimize conditions. Four samples of 44 ml each of conditioned media with previously purified human mAb added were purified in parallel by MG-SPE. The flow rate was 2 ml/min and the Protein A resin used was 1-ml mAbSelect Sure

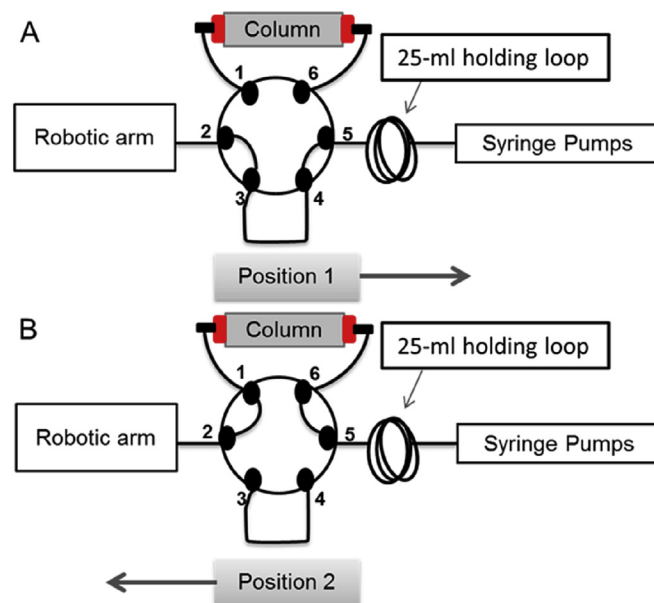


Fig. 2. Schematic of MG-SPE system. (A) Loop Filling - Valve at position 1; column out of line for loading sample or buffer into the holding loops. Port 2, 3, 4 and 5 are connected to the system. Sample is aspirated from the robotic arm into port 2 and out port 5 to the holding loop. Port 3 and port 5 are connected via a bypass loop through port 3 and 4. (B) Column Loading - Valve at position 2; column in line for passing sample or buffer over the column from the holding loop. Sample is pushed from the holding loop into port 5 and out port 6 to the column. Sample leaving the column reenters the valve at port 1 and is directed to the robotic arm for dispensing on the plate deck through port 2. At no point does the sample enter the syringe pump or mix with the system fluid due to an air gap.

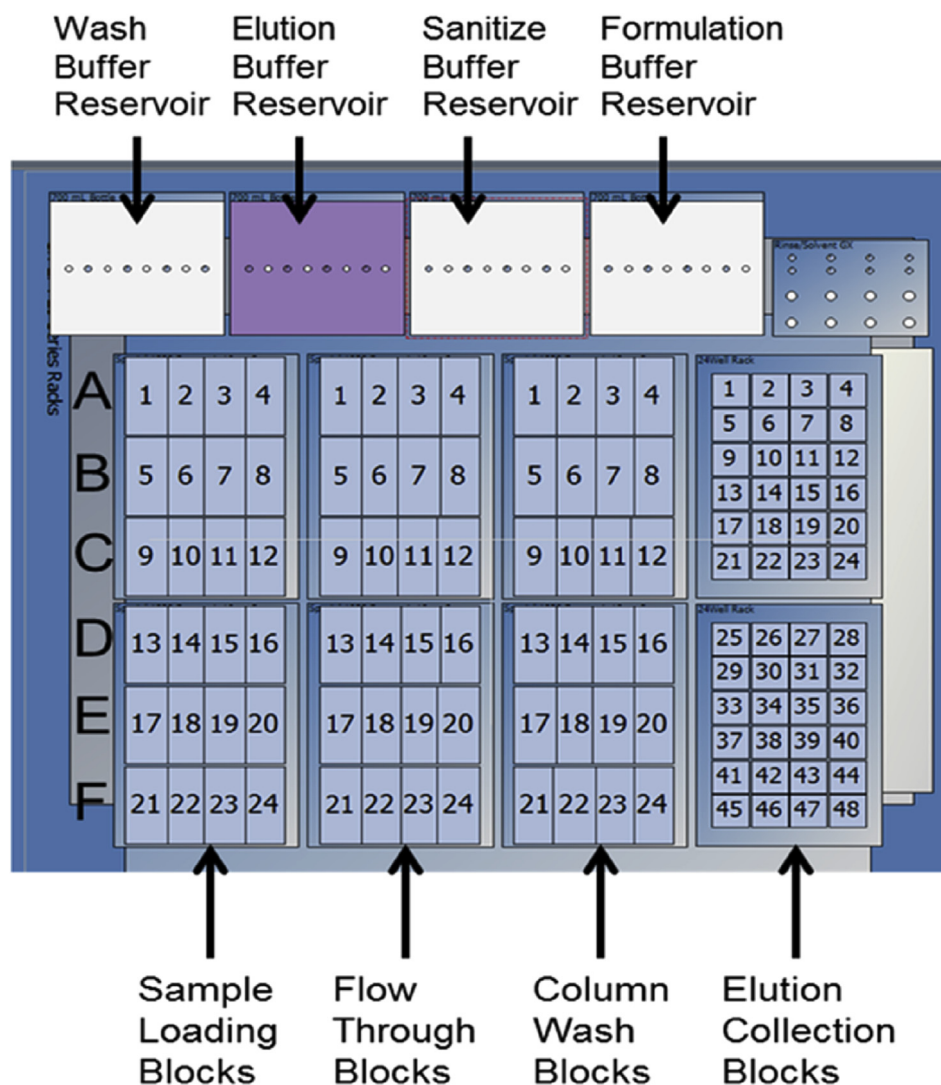


Fig. 3. Bed layout for protein A purification. User interface for Trilution® software shown. The four blocks on the top side of the plate deck were used as buffer reservoirs and all the other blocks with well numbers were designed for sample loading, flow through collection, column wash collection and elution collection. The bed layout is flexible and can be changed to fit the application. In this example the second elution block will not be used for elution and can be used for something else. Alternatively, the user can choose not to collect Flow through and/or wash and use those blocks as additional sample loading blocks to run 48 samples requiring two elution blocks.

columns (GE Life Sciences). The general protocol developed for single-step affinity purification using Protein A is shown in Table 1. Prior to loading the sample, the 4 pumps were primed with system fluid (10% glycerol, 0.02% NaN_3) to prevent air bubbles in a step called “prime loops” followed by a system/column wash with 0.2 N NaOH and equilibration with 10 ml PBS buffer (pH 7.4). Once equilibrated, the columns were loaded with media from the first row of the loading block (A1–A4, Fig. 3). In order to load the 44 ml of hybridoma supernatant, two loading cycles were required

Table 1
General protocol for 4-sample one-step (protein A) purification on MG-SPE system.

One-step protein A purification protocol for MG-SPE
1. Prime loops
2. Sanitize with 0.2 N NaOH 5 ml
3. Equilibrate with PBS buffer 10 ml (pH 7.4)
4. Sample loading 44 ml
5. Column wash with PBS buffer 10 ml (pH 7.4)
6. Elute with sodium acetate buffer 3 ml (pH 3.6)
7. Sanitize with 0.2 N NaOH 22 ml
8. Equilibrate with PBS buffer 22 ml (pH 7.4)

because the maximum working volume of the syringe pumps is 22 ml each due to 3 ml of dead volume. For each cycle, supernatant was drawn into the holding loops with the switching valve in position one (Fig. 2A) keeping the columns out of line. With sample in the holding loop, the switching valve was then moved to position 2 bringing the columns in line and the sample was passed over the columns (Fig. 2B). Flow through was collected in the Flow-through block (rows A1–A4, Fig. 3). Following sample loading, columns were washed with 10 ml of PBS and wash was collected in the Column-wash block (rows A1–A4, Fig. 3). To increase capacity it is possible to divert both flow-through and wash to waste and use the additional deck space for samples. Following wash, mAbs were eluted using 100 mM acetic acid into the 24-well collection block (A1–A4, Fig. 3). For the single-step purification, samples were neutralized with Tris buffer (pH 9.0) previously spotted in the 24-well Collection block. Fig. 4A shows the results from the single-step mAb purification. All samples appear pure and intact by non-reducing SDS-PAGE. Elution volumes were 3 ml and total run time for 4 samples in parallel is ~1.25 h. Analysis by analytical SEC (Superose 6), showed no aggregation or shoulder peaks as expected for the control antibody identical to spectra from the same mAb

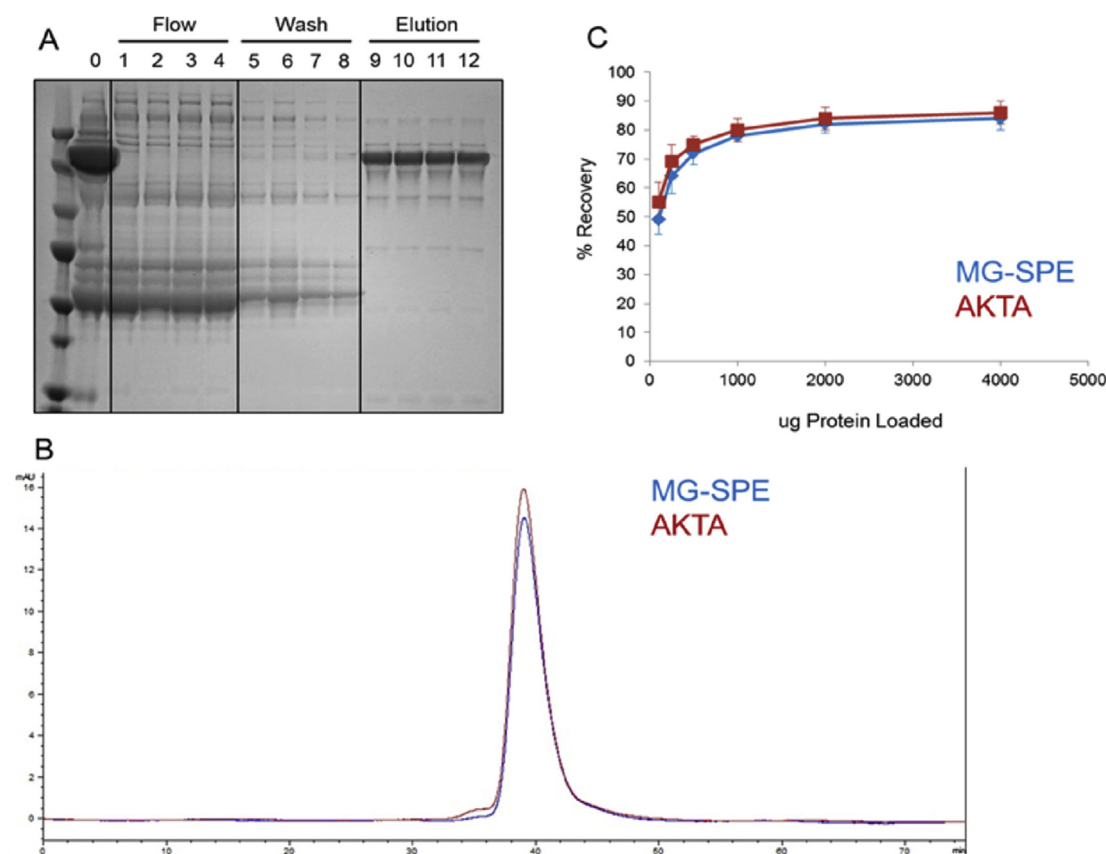


Fig. 4. (A) Non-reducing SDS-PAGE gel for four-sample one-step protein A purification from conditioned media with previously purified mAb added (MW markers represent 250 kDa, 150 kDa, 100 kDa, 75 kDa, 37 kDa, 25 kDa). A: lane 0, mAb in conditioned media; lanes 1–4, flow-through for samples 1–4, respectively; lanes 5–8, column washes for samples 1–4, respectively; lanes 9–12, elution for samples 1–4, respectively. (B) Spectra of analytical SEC for purified protein using Superose 6 column from MG-SPE (Blue) and ÄKTA FPLC (Red). No shoulder peaks or aggregation peaks are shown in either spectra. (C) Recovery for single-step protein A purification on MG-SPE (Blue) and ÄKTA FPLC (Red). Protocol was run at 2 ml/min, slower load speeds did not significantly improve yield (data not shown). Values are representative from 4 individual experiments with identical samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purified from an ÄKTA FPLC (Fig. 4B). To determine yields, a series of experiments were performed by purifying known amounts of previously purified mAb added to conditioned mammalian cell culture media. The yield was greater than 60% when sample loading was lower than 200 μ g and around 80% when sample load was above 1 mg (Fig. 4C). When compared with mAb purification performed using an ÄKTA system, the curve of protein recovery was nearly identical indicating that the MG-SPE can perform at a similar level to the ÄKTA for affinity purification of mAbs.

3.3. One step affinity purification using multiple serial runs

Once we established that samples could be run in parallel with acceptable yield, quality, and reproducibility on the MG-SPE system, experiments were performed to add sequential purification of 12–24 samples in an automated fashion. The main challenge for sequential purification of multiple samples using sample probes and holding loops is to avoid cross contamination by carry-over from previous runs. To account for this, samples were run as described, however following elution of the first four samples (A1–A4) the columns and holding loops were washed with 0.2 N NaOH and reequilibrated with PBS prior to loading the second set of samples (Fig. 3). Then, the purification process was repeated for samples in row B (B1–B4) of the Load block (Fig. 3). The flow-through and eluate were collected in the corresponding places on the tray deck (Flow Through B1–B4 and Elution B1–B4). This was followed by another loop wash and purification of samples in Row C

(Fig. 3). To confirm that we were not getting cross-contamination between samples run serially over each column, cell supernatant containing recombinantly expressed mAb (~150 kDa non-reduced) was used in rows A (samples 1–4) and C (samples 9–12) and cell supernatant containing a recombinantly expressed Fc-fusion protein (~100 kDa non-reduced) was used in row B (samples 5–8) so that molecular weight differences could be seen on SDS-PAGE. The purified eluates were analyzed shown in Fig. 5A. Non-reducing SDS-PAGE shows no detectable cross contamination between samples for serial runs in the same channel confirming that the MG-SPE can process 12–24 samples without intervention.

3.4. Two-step purification including buffer exchange

During single-step mAb purification, the final product can be neutralized to a pH of between 5 and 5.5 for long-term stability [2]. This can be done by manually adding high pH neutralization buffer to the collection plate prior to purification as described above. However, the pH jump can cause aggregation in some antibodies and some downstream processes such as certain cell based assays or protein analytics have specific buffer requirements. To minimize manual manipulation of the samples and make it more convenient for downstream process, we added a buffer-exchange step following affinity chromatography to allow for formulation of the final product in any buffer desired. For buffer-exchange, commercially available PD10 desalting columns (GE Life Science) were set up on top of the multi-well blocks used for collection of the eluate

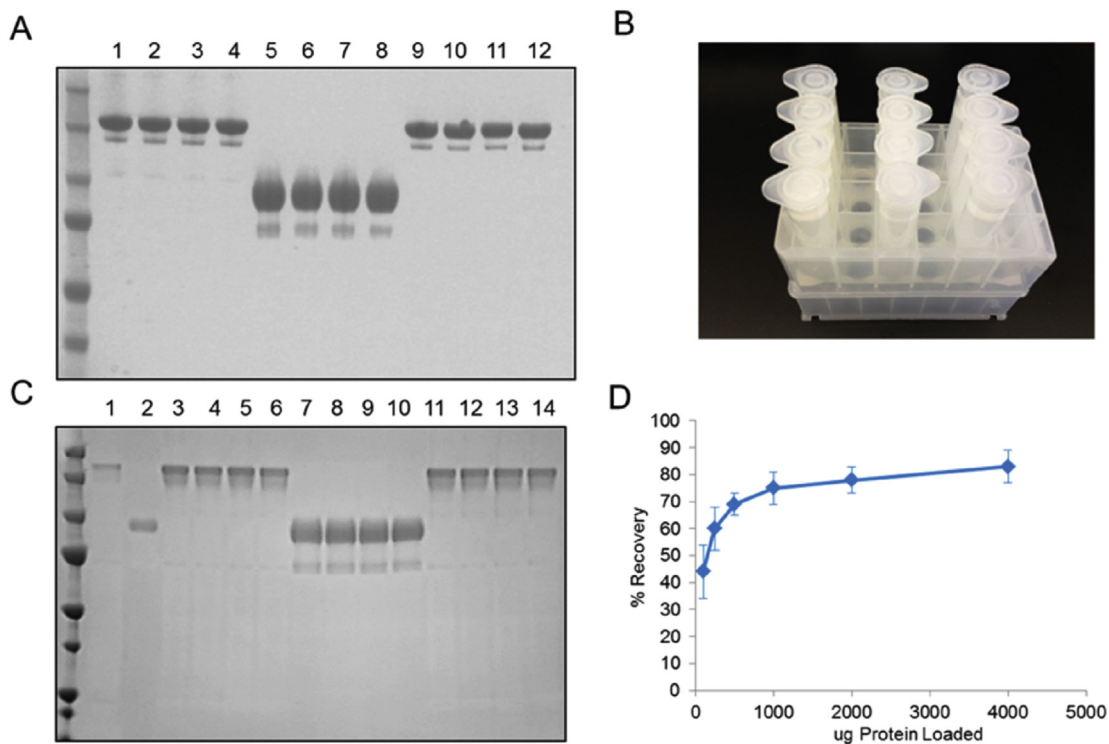


Fig. 5. (A) Non-reducing SDS-PAGE for cross-contamination study. Lanes 1–12 correspond with elution for samples 1–12 (MW markers represent 250 kDa, 150 kDa, 100 kDa, 75 kDa, 37 kDa, 25 kDa). (B) Customized column holding block with PD-10 columns (top part). Commercially available 24-well collection block (bottom part). This was placed in the “Elution Block” spot on the plate deck as indicated in Fig. 3. (C) Non-reducing SDS-PAGE for twelve-sample two-step purification on MG-SPE. Lane 1: mAb before purification; lane 2: Fc fusion protein before purification; lanes 3–14: elution for samples 1–12, respectively. (D) Recovery for two-step Protein A following desalting purification on MG-SPE. Values are the average from 4 individual experiments with identical samples.

in the single-step purification. Desalting columns were chosen because the load volume is much larger than normal SEC. In order to do this, a rack was manufactured using a 3D printer (Fig. 5B). In the two-step protocol, the 3 ml eluate from the protein A columns (run identically to the one-step protocol) was directly loaded onto the desalting columns equilibrated with the desired buffer. Buffer was exchanged by gravity flow followed by dispensing an additional 4 ml of buffer on the column. Each row of samples was desalted immediately after Protein A elution prior to loading of the next row of samples to minimize time in low pH buffer. The results from a 12-sample two-step purification are shown in Fig. 5C. The recovery for two-step purification was only slightly lower than the one-step purification, which indicated there was negligible sample loss during desalting (Fig. 5D). The only drawback to adding the second step is that the recovery volume is increased to 7 ml so low-titer antibodies or other proteins will be recovered at a fairly low concentration and need to be concentrated. The total run time for purifying and desalting 12 samples was ~6 h so it would be possible to purify 24 samples overnight.

4. Conclusion

We determined that a parallel mid-scale protein purification system would increase efficiency and help alleviate a bottleneck in the protein generation process for both large molecule drug discovery programs. To meet this need, we developed a purification system based on a modified Gilson SPE system that can run 4 affinity purifications in parallel using commercially available affinity columns. Using mAbs as a model system for affinity purification, a one-step Protein A purification method was developed that allows the purification of 24 samples of 44 ml each overnight. Protein

yields and quality were comparable with industry standard ÄKTA systems, however throughput was significantly increased. Additionally, we developed a two-step purification method that allowed for buffer exchange following the affinity step. Yields were similar to the one-step purification and the sample could be exchanged to any desired buffer.

The major advantage of the MG-SPE is that it is fully automated and capable of walk-away purification of up to 24 samples with no manual input between each run allowing 24 mid-size samples to be processed overnight. The system is mounted on a cart so it is mobile (for transfer between cold room and main lab space) and it uses exclusively micro-titer based format blocks so that sample mix up is all but eliminated and barcoding of sample blocks can be used if desired. Additionally this format aligns well with other high-throughput formats for upstream processing such as HT-cloning and expression [11] and hybridoma supernatant generation. For downstream processing the standard 24-well sample collection block is ideal to interface with assays and analytics that use multi-well plate formats so manual transfer of samples is minimized. Although the system lacks a UV detector so that eluates must be collected based on elution volumes rather than peak fractionation and gradient chromatography cannot be performed, the system fills a void in the protein generation process. The MG-SPE can handle the mid-scale affinity purifications from hybridoma campaigns and initial recombinant expressions while the ÄKTA FPLC's are used mid-to large-scale multi-step purification.

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Detailed plans for the GX-274 modification are available from Gilson Inc., 3000 Parmenter Street, Middleton WI 53562, ph. 608-836-1551.

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