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An economical and versatile high-throughput protein purification system using a multi-column plate adapter

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TITLE:
An economical and versatile high-throughput protein purification system using a multi-column plate adapter

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KEYWORDS:
High throughput purification, Affinity chromatography, Ion exchange chromatography, MCPA, economical protein purification, SH3 domain

SUMMARY:
A multi-column plate adapter allows chromatography columns to be interfaced with multi-well collection plates for parallel affinity or ion exchange purification providing an economical high throughput protein purification method. It can be used under gravity or vacuum yielding milligram quantities of protein via affordable instrumentation.

ABSTRACT:
Protein purification is imperative to the study of protein structure and function and is usually used in combination with biophysical techniques. It is also a key component in the development of new therapeutics. The evolving era of functional proteomics is fueling the demand for high-throughput protein purification and improved techniques to facilitate this. It was hypothesized that a multi column plate adaptor (MCPA) can interface multiple chromatography columns of different resins with multi-well plates for parallel purification. This method offers an economical and versatile method of protein purification that can be used under gravity or vacuum, rivaling the speed of an automated system. The MCPA can be used to recover milligram yields of protein by an affordable and time efficient method for subsequent characterization and analysis. The MCPA has been used for high-throughput affinity purification of SH3 domains. Ion exchange has also been demonstrated via the MCPA to purify protein post Ni-NTA affinity chromatography, indicating how this system can be adapted to other purification types. Due to its setup with multiple columns, individual customization of parameters can be made in the same purification, unachievable by the current plate-based methods.

INTRODUCTION:

Protein purification techniques to achieve milligram quantities of purified proteins are imperative to their characterization and analysis, especially for biophysical methods such as NMR. Protein purification is also central across other areas of study such as drug discovery processes and protein-protein interaction studies; however, achieving such quantities of pure protein can become a bottleneck for these techniques[1-3]. The principal method for protein purification is chromatography, which includes a variety of methods that rely on the individual characteristics of proteins and their tags. In affinity chromatography, proteins have an additional protein or peptide motif that works as a tag that has an affinity for a certain substrate on the chromatography resin[4]. The most common affinity method is immobilized metal affinity chromatography (IMAC) using His-tagged proteins, whereas another popular method is ion exchange chromatography that separates proteins based on their charge. For highest purity, a combination of affinity chromatography and ion exchange is frequently used together, usually requiring expensive lab equipment for high-throughput.

The evolving era of functional proteomics is fueling the demand for high-throughput techniques for purifying not singular proteins for specific analysis but large numbers of proteins simultaneously for comprehensive analysis and genome wide studies[5]. Immobilized metal affinity chromatography (IMAC) is one of the most widely used methods for high-throughput protein purification[6,7] yet its automated systems are costly and unaffordable for smaller laboratories[8]. The more affordable plate-based alternatives that are currently available employ the use of accessible laboratory-based equipment, such as a vacuum. Although these methods are successful in improving the speed of purification, it can only achieve high-throughput purification on a smaller scale, only yielding protein in the microgram range. These limitations mean that the pre-packed 96 well filter plates (e.g., from GE Healthcare now owned by Cytiva) cannot be used before biophysical techniques[9]. Gravity chromatography is the most cost-efficient method of purification; however, setting up multiple columns is inconvenient and can be prone to error for multiple proteins.

A multi column plate adaptor (MCPA) has been developed and proven to successfully and conveniently run parallel affinity chromatography columns at once to purify His-tagged yeast SH3 domains[10]. The MCPA offers a cost-efficient high-throughput purification method that does not depend on costly instrumentation. Its flexible design can effectively purify milligrams of protein by multiple affinity chromatography columns under gravity or vacuum manifold. Furthermore, resin type, volume, and other parameters can be adjusted for each individual column for faster optimization. This study demonstrates that ion exchange chromatography by the MCPA can be used in conjunction with affinity chromatography by the MCPA to enhance the purification of the Abp1 SH3 domain. Additionally, up to 24 different proteins can be separated in parallel using these methods.

PROTOCOL:

1. Denaturing Ni-NTA chromatography

1.1. Preparing buffers

NOTE: See **Table 1** for details of all buffers.

1.1.1. Make up 500 mL of 0.5 M NaOH, making sure to add the Milli-Q water first and then adding the NaOH slowly whilst the beaker is on a stirrer. Filter using a 0.22 µm filter.

1.1.2. Prepare 100 mL of 0.1 M nickel sulphate and filter using a 0.22 µm filter.

1.1.3. Prepare 50 mL of 2 M imidazole and filter using a 0.22 µm filter.

1.1.4. Prepare and 0.22 µm filter 1.5 L of **denaturing buffer** at pH 8.0 consisting of 5.3 mM Tris-HCl, 4.7 mM Tris-base (Tris (hydroxymethyl)methylamine), 13.7 mM NaH_2PO_4, 86.3 mM Na_2HPO_4 and 6 M guanidine hydrochloride.

1.1.5. Prepare and 0.22 µm filter 500 mL of **lysis buffer** solution of 10 mM imidazole dissolved in denaturing buffer (see 1.1.4) using the 2 M imidazole stock.

1.1.6. 1.6 Prepare and 0.22 µm filter 500 mL of **wash buffer** solution of 10 mM imidazole dissolved in denaturing buffer (see 1.1.4) using the 2 M imidazole stock.

1.1.7. Prepare and 0.22 µm filter 500 mL of 100 mM EDTA buffer, adjust pH to 8 using 1 M NaOH.

1.1.8. Prepare and 0.22 µm filter 500 mL of **elution buffer** made up of 7 mL of 99% glacial acetic acid into 6 M guanidine hydrochloride.

NOTE: If using native buffers, adapt according to **Table 1**.

1.2. Lysing cells (denaturing method)

1.2.1. Add 2 mL of lysis buffer to every 1 g of harvested *E. coli* cell pellets that contain the over-expressed His-tagged protein. Vortex for several minutes until cells are re-suspended. Leave the samples on a rocker for 30 minutes at 4 °C.

1.2.2. Centrifuge lysed pellets at 18,000 x g for 30 minutes. Keep 50 µL aside in -20 °C freezer to analyze later on an SDS-PAGE gel.

1.2.3. Carefully pour off the supernatants making sure that the pellet is not disturbed. Lysates should be a clear, light yellow color. If lysates are cloudy, centrifuge sample again; consider sonication if available to degrade nucleic acid.

1.2.4. Filter the supernatants through an empty column (without resin but with filter) and collect in an open collection tray. Then transfer into a tube for use in step 1.3.

NOTE: If using the native buffers, then after step 1.2.1, treat the samples to 3 cycles of freeze/thaw at -80 °C or process by sonication or French Press to lyse cells.

1.3. Preparing vacuum purification setup and equilibrating columns

NOTE: See **Figure 1** for a set up MCPA with 24 columns.

1.3.1. Determine how many columns are needed as well as the desired resin type and volume. As a rule of thumb for 100 mL of autoinduction culture (~2 g pellet) expressing His-tagged SH3 domains using T7 based plasmids, use 0.6 mL of Ni-NTA resin per column to yield about 3 mg of protein from about 6 mL of lysate.

1.3.2. Insert the desired number of 12 mL columns (without resin but with filter) into a pre-assembled MCPA (long-drip plate with punctured sealing mat). The columns should fit snugly in the holes of the sealing mat that have been punctured.

1.3.3. If less than 24 columns are used, close empty holes with an empty closed column.

1.3.4. Take an open collection plate and put this plate into the base of the vacuum manifold and close with the top of the manifold.

1.3.5. Place the assembled MCPA with columns onto top of the vacuum manifold.

1.3.6. Attach tubing from a vacuum pump system to the inlet/nozzle at the bottom of the vacuum manifold.

1.3.7. Ensure that the Ni-NTA beads are in a 50% solution with 20% ethanol. Before doing anything with the beads gently shake/mix the bead/ethanol solution to resuspend evenly. Then, using a 5 mL pipette, pipette 1.2 mL of the 50% solution into the columns. Whilst pipetting make sure the beads remain fully mixed.

1.3.8. After pipetting into all 24 columns, turn on the vacuum pump and run the 20% ethanol through the column and into the collection plate below.

1.3.9. Turn off the pump once all the 20% ethanol has run through all the columns (Ni-NTA resin can tolerate brief drying if the vacuum is still applied after buffer has passed through the column). Dispose of what is in the open collection plate into a waste box.

CAUTION: All waste products from the Ni-NTA beads are hazardous, when handling the beads or disposing of the waste, wear gloves, goggles and other PPE. Furthermore, dispose of all Nickel Sulphate waste in a separate container for individual disposal later.

NOTE: If resin is new or recently regenerated, the rest of these steps can be ignored, move to the next section.

1.3.10. Add **3 resin volumes** (1.8 mL) of **100 mM EDTA buffer** using a 20 mL syringe or a repeater syringe device with a 50 mL syringe. Then switch on the vacuum pump to let the EDTA buffer wash through and switch off the pump once it has washed through.

NOTE: This wash should remove the nickel blue color but if this is not the case then repeat this step until all columns have lost their blue color. Turn off the pump and pour the contents of the open collection plate into the waste box.

1.3.11. Add **3 resin volumes** (1.8 mL) of **0.5 M NaOH** buffer into each column before turning on the pump and running this through each column. Empty collection plate.

1.3.12. Add **4 resin volumes** (2.4 mL) of **100 mM nickel sulphate** into each column. Turn on the vacuum pump and run this through the column once again.

1.3.13. Add **10 resin volumes** (~6000 µL) of **Milli-Q water** and running this through the columns. Turn the pump off and pour off the contents of the collection plate into the waste box.

1.3.14. Wash the columns **twice with 4 resin volumes** (2.4 mL) of **10 mM imidazole in wash buffer** (denaturing or native) each time to remove any excess nickel and equilibrate. Empty collection plate.

NOTE: If any columns are running significantly slower, detach column and check air can be pushed through the MCPA using a large syringe. If this is unblocked, use a different column with a fresh filter.

1.3.15. If multiple different samples/proteins are going to be purified on the MCPA then replace the open collection plate with a 48 x (5 mL/well) collection plate. However, when only running the same protein samples on every column, it is fine to carry on using an open collection plate.

1.4. Loading, washing and eluting

1.4.1. With vacuum off, load lysates into columns (the amount of lysate will vary, typically from 1 to 12 mL or more and may be required to load in several batches). Use a thin plastic stirrer to gently mix the beads and the lysate in the column to maximize binding.

1.4.2. After gently mixing each column for a few minutes, switch on the vacuum pump and run the lysates through the columns. If multiple protein samples are being run, use a 48 well collection plate and make sure to swap out the collection plate for another 48 well plate after 4 mL has run through as the wells in these plates can only hold 4 mL of solution. Continue running lysate until all the lysates has been run through a column. Freeze collection plates that contain the flowthrough.

NOTE: Columns may become “blocked” causing the lysate to flow through the column much slower than it should. This is easily spotted as the neighboring columns will be flowing at a normal rate. If this does occur, it is recommended that the lysate/resin mixture be transferred to a column containing a fresh filter.

1.4.3. Replace the collection plate with an empty open collection plate and then add 9 resin volumes (5.4 mL) of 10 mM imidazole wash buffer to each column and turn on the vacuum and run the wash through. Repeat this 4-5 times. To avoid overflow, periodically empty waste plate.

1.4.3.1. Then replace the collection plate with a clean appropriate plate (open plate for just one protein and 96 well if there are multiple proteins, ensuring that A1 is in the top left corner).

1.4.4. Using a repeater syringe with a 12.5 mL syringe, pipette ~1 resin volume (0.75 mL) of denaturing elution buffer into each column.

1.4.5. To avoid protein foaming, turn on the vacuum pump at the lowest setting. Gently lift the MCPA to check nothing is left to drip into the collection plate.

NOTE: An alternative to eluting with the vacuum or gravity is pushing a 10 mL syringe plunger into the top of the column to gently push the elution buffer through the column. Do this for every column, being careful when removing the syringe plunger not to disturb the beads at the bottom of the column. Once the elution buffer has been pushed through, gently remove the syringe plunger from the last column it was used in and briefly turn on the vacuum pump to remove the last drops from the columns.

1.4.5.1. If a 96-well collection plate has been used, then check the collection plate to ensure that what is flowing from each column through the long drip plate is only flowing into one well and nothing is eluting into neighboring wells on the collection plate.

1.4.6. For the next elution repeat the above 2 steps and collect in a fresh multi-well (different samples) or open collection plate (same samples).

1.4.7. Optional step, take a 50 µL aliquot of each elution for purity and concentration analysis.

1.4.8. Add 2 mL of 20% ethanol to each column and run this through using the vacuum pump. Then add another 2 mL of 20% ethanol to the columns and use a fresh thin plastic stirrer to mix up the 20% ethanol and the beads before transferring to a 50 mL tube for storage at 4 °C. Clean the columns and check that water flows freely through each column and then clean everything and put away for later use.

NOTE: Some columns will eventually have their filters blocked and will run too slowly, these columns should be replaced, or filters cleaned. As such, it is recommended to periodically test filters by removing resin and filling the column with water to see if it flows through quickly. Filters can be cleaned by leaving a closed column filled with denaturing elution buffer and shaking overnight.

2. Ion Exchange - Single protein purification

2.1. Preparing the buffers

NOTE: See **Table 1** for details of all buffers

2.1.1. Prepare a 2 L low salt buffer: 10 mM Tris pH 8.1 (5 mM Tris Acid, 5 mM Tris Base). Filter solution using a 0.22 µm filter.

2.1.2. Prepare a 0.5 L high salt buffer: 10 mM Tris pH 8.1, 4 M NaCl. Measure 116.9 g of NaCl and make up to 0.5 L with the 10 mM Tris pH 8.1 from above. Filter solution using a 0.22 µm filter.

2.2. Making salt concentration series: 0-1 M NaCl.

NOTE: The range of salt concentrations can be adjusted for every protein.

2.2.1. Setup 24 x 50 mL labeled centrifuge tubes in a rack

2.2.2. Using the buffers made above, prepare 24 x 14 mL NaCl dilutions ranging from 0-1000 µM. Start by adding the required volumes of 4 M NaCl 10 mM Tris. This could be done by increments of 25 mM or 50 mM depending on protein.

2.2.3. Then add the required volumes of 10 mM Tris to each tube. Invert each tube several times to mix.

2.3. Preparing samples

2.3.1. Obtain the samples to be purified by Ion exchange. Optionally, take a 50 µL aliquot for analysis later.

NOTE: Samples should always be kept cold; these may be lysates, or maybe post Ni-NTA. Samples from Ni-NTA in denaturing buffers require dialyzing or buffer exchange in 10 mM Tris buffer. Samples from Ni-NTA in native buffers could be diluted with 10 mM Tris buffer to reduce the salt concentration so that the protein can bind to IEX resin.

2.3.2. Spin samples at 18,000 x g for 10 minutes.

2.3.3. Carefully pour off the supernatant into a weigh boat. Avoid disturbing the pellet.

2.3.4. Take up the supernatant carefully into a 20 mL syringe and attach a 0.22 µm filter.

2.3.5. Slowly eject the supernatant out through the filter into a fresh 50 mL tube.

2.3.6. If dilution is required, dilute supernatant with 10 mM Tris buffer made above (In this experiment the supernatant was diluted 1 in 2). Store at 4 °C in the meantime.

2.4. Preparing the vacuum purification setup and equilibration setup

NOTE: In this protocol, only one column is used for IEX purification of one sample. For multiple purifications, see next section.

2.4.1. Determine the desired resin type and volume. As a rule of thumb for up to 20 mg of partially pure His-tagged SH3 domains, use 0.4 mL of Q-sepharose fast flow resin per column (see step 2.4.8).

2.4.2. Insert 24 open empty columns (empty without filter at bottom) into a pre-assembled MCPA. The columns should fit snugly in the holes of the sealing mat that have been punctured.

2.4.3. Place a 10 mL syringe plunger into every empty column except the first position, where the purification will start.

2.4.4. Push the bottom of an open column (with filter) through a 5 mL syringe plunger rubber gasket (that has been pierced) to form a rubber ring at the end of the column. This rubber ring will ensure a good seal when this column is inserted into each empty column above. For now, insert this column into the first empty column on the MCPA.

2.4.5. Take an open collection plate and put this plate into the base of the vacuum manifold and close with the top of the manifold.

2.4.6. Place the assembled MCPA (with columns) onto top of the vacuum manifold.

2.4.7. Attach tubing from a vacuum pump system to the inlet/nozzle at the bottom of the vacuum.

2.4.8. Cut a blue pipette tip ~ 2 cm from the bottom and use to take up 800 µL of Q-sepharose beads (or any other ion exchange resin), ensuring that the 50/50 bead-ethanol solution is mixed to avoid layering. Pipette 800 µL into the one open column (with filter) in the first position and once the beads settle to the bottom of the columns switch on the vacuum enough to run through the 20% ethanol.

2.4.9. Wash the column containing sepharose beads with 2 x 2 mL of 10 mM Tris. Turn the vacuum off just before the Tris buffer runs through to prevent the resin drying out. Run off can be discarded.

NOTE: If resin has been used previously, regenerate by washing in 5 resin volumes of 4 M NaCl and then 5 resin volumes of 10 mM Tris before step 2.4.9.

2.5. Loading, washing and eluting

2.5.1. Transfer all sample to be purified (see section 3) to the Q sepharose column in the first position, use a small plastic loop to stir sample and beads for around ~2 minutes before switching on the vacuum pump.

NOTE: In cases of excess supernatant (>12 mL), beware of overfilling the column. Let the sample run through then add more, mixing before letting run through again.

2.5.2. Store/Freeze the flowthrough for analysis later.

2.5.3. Place another open collection plate into the vacuum manifold and then wash each sepharose column with 2 x 2 mL of 10 mM Tris and store.

2.5.4. Insert a 96 well collection plate, making sure A1 is in the top left corner.

2.5.5. To collect the first elution fraction, remove the syringe plunger from the next column along, move the Q sepharose column into this position and put the syringe plunger in the previous position. Then pipette 1 mL of the first salt concentration into the Q sepharose column. Turn on the pump and collect the elution. Turn off the pump just as the liquid lines near the beads to avoids drying out the beads.

2.5.6. To collect the next elution fraction, remove the syringe plunger from the next column along, move the Q sepharose column into this position and put the syringe plunger in the previous position.

2.5.7. Add 1 mL of the next salt concentration to the column and repeat the process until all concentrations have been used and 24 elutions have been collected in 24 separate wells. Check no liquid has entered any neighboring wells.

2.5.8. Wash the column with 2 mL of 4 M NaCl 10 mM Tris. Let this run through.

2.5.9. Wash with 2 mL of 20% ethanol. Let ethanol run until it is just above the beads and seal column for storage.

2.5.10. Store 96 well collection plate and analyze by UV spectroscopy and SDS PAGE.

3. **Ion Exchange - Simultaneous purifications of 24 different proteins**

NOTE: See steps 2.1-2.3 for details on making buffers, a series of salt concentrations and preparing samples

3.1. Preparing the purification setup

NOTE: How the purification system is set up is very much dependent upon how many proteins being purified and how many salt conditions will be used. To purify 12 samples up to the maximum of 24 samples simultaneously, a collection plate for every salt concentration used to elute is required. For less than 12 samples, it is possible to move the chromatography columns a few positions within the same MCPA before changing collection plates. In this case, there is no need to place the columns in empty columns and they can be directly attached to the MCPA sealing mat. The protocol below is for 24 simultaneous ion exchange purifications.

3.1.1. Place the assembled MCPA directly attached to 24 empty columns with filters onto top of the vacuum manifold that contains an open collection plate and prepare and wash 24 ion exchange columns as in single purification method above. For this purification, it is convenient to use an Eppendorf repeater or syringe to rapidly pipette into columns.

3.2. Loading, washing and eluting

3.2.1. Place a 48-well collection plate into the base of the vacuum manifold before fitting the MCPA (with the washed purification columns attached). Ensure A1 is in the top left corner.

3.2.2. Pipette each protein sample (up to 5 mL at a time) into their corresponding column. Stir each column using a thin plastic loop (one loop for every column to avoid contamination) for approximately 2 minutes. Then switch on the vacuum pump and let the supernatant flow through.

NOTE: In cases of excess supernatant, beware of overfilling the columns. Let the sample run through its column then add more, mixing before letting run through again.

3.2.3. Store/Freeze the 48-well collection plate for later analysis as this contains the flowthrough for every protein.

3.2.4. Place another 48-well collection plate into the vacuum manifold and then wash each sepharose column with 2 x 2 mL of 10 mM Tris.

3.2.5. Insert the first 96-well collection plate into the manifold, ensuring that A1 is in the top left corner and then pipette 1 mL of the first salt concentration into each column and then turn on the vacuum pump to run this through the columns. Remove the collection plate, cover with parafilm and store in at 4 °C for analysis.

3.2.6. Repeat step for each successive salt concentration used to elute. Make sure that a new collection plate is used for every elution and that each collection plate is labelled and stored.

3.2.7. Wash each column with 2 mL of 10 mM Tris, 4 M NaCl.

3.2.8. Wash each column with 2 mL of 20% ethanol. Let ethanol run until it is just above the beads and seal columns for storage.

3.2.9. Store 96 well collection plates and analyze by UV spectroscopy and SDS-PAGE.

REPRESENTATIVE RESULTS:
As an example, the MCPA has successfully purified 14 AbpSH3 mutants in denaturing conditions via Ni-NTA (**Figure 2A**). A small contaminant ~ 25 kDa can be seen, however the protein is still largely pure. This contaminant is believed to be YodA, a common co-purified protein found in *E. coli* [11]. **Figure 2B** shows the purification of 11 different SH3 domains under native conditions. The small contaminant seen in denaturing conditions is removed in native conditions. This shows that the MCPA can be used for comparison of purifications composed of native or denaturing buffers as listed in Table 1.

Representative data for the purification of a lysate via IEX MCPA are shown in **Figure 3.** This suggests that AbpSH3 can be separated from the majority of the contaminants as it elutes later between 425 mM to 700 mM. The concentration of salt needed to elute the protein from the column relates to the strength of electrostatic interaction between the protein and the resin. The majority of bacterial proteins have low pIs; however the protein of interest is very negative and appears to have a lower pI. Good yields of considerably pure AbpSH3 protein were recovered with some various higher molecular weight contaminants AbpSH3 seen as bands at ~ 5 kDa. IEX via MCPA can therefore be used as the first step in a purification protocol as it can isolate sufficient quantities of protein from the present contaminants in a lysate.

Further purification by IEX using the MCPA has been successfully demonstrated on fractions eluted from an Ni-NTA MCPA run (**Figure 4**). Noticeably, two main peaks have been resolved with maxima at 400 and 700 mM salt, which may correspond to separating an N-terminal truncated version of this protein. Through further DSF data analysis it was made apparent that peak 1 was slightly less stable and had a slightly lower T_m relative to peak 2. In comparison to the IEX run of the lysate, the fractions overall are much cleaner and show the benefit of running a Ni-NTA step before IEX. Although there is slight contamination with proteins of a higher molecular weight, the fractions are still largely pure and have yielded good biophysical data using NMR and thermal/chemical denaturation assays.

FIGURE AND TABLE LEGENDS:
Figure 1: **A front view of the MCPA instrument.** (**A**) Front view of the MCPA instrument with 24 columns attached. Columns are spaced out evenly within the 96 well sealing mat to guide the elutions into a 96, 48 or open collection plate. (**B**) Top view of the sealing mat. (**C**) Bottom view of the same sealing mat. (**D**) Front view of the MCPA with columns and syringe plungers attached. This figure has been modified from Dominguez et al.[10].

Figure 2: **An example of a 1 x 24 column configuration, the purification of various SH3 mutants**

under denaturing and native conditions. (**A**) Denaturing purification of various AbpSH3 mutants. A slight contamination can be spotted of ~ 25 kDa across each lane. (**B**) Native purification of 11 different yeast SH3 domains. The contaminants have been removed from each lane and no longer visible on gel. This figure has been modified Dominguez et al.[10].

Figure 3: **Purification of lysate using IEX via MCPA.** (**A**) Absorbance readings of all elutions from the ion-exchange (IEX) were measured by an LVis plate (BMG). The readings are plotted against the corresponding NaCl concentration of the elution. The peak with the highest protein concentration is seen at 700 mM NaCl. (**B**) SDS-PAGE analysis of the IEX salt elutions presented in (**A**). The molecular weight marker is shown on the left of the gel.

Figure 4: **Purification of VJM2 Pool (post Ni-NTA) using IEX via MCPA system**. (**A**) Absorbance readings of the collected elutions from the ion-exchange (IEX) were measured using a NanoDrop. The readings are plotted against the corresponding NaCl concentration of the elution. (**B**) SDS-PAGE analysis of the IEX salt elutions presented in (**A**). The molecular weight marker is shown on the left of the gel.

Table 1: **Composition of buffers, Ni-NTA denaturing and native purifications and Ion exchange low and high salt buffers.**

DISCUSSION:

The method is robust and simple to use for relatively inexperienced protein biochemists, however there are a few considerations to bear in mind.

Caution about overfilling collection plates

The 48-well collection plate itself only holds 5 mL per well while each 96-well only holds 2 mL. This needs to be kept in mind when adding buffer and running sample through the column as there is the risk of overfilling the wells. In particular, care needs to be taken when transferring the larger samples to the chromatography column for purification. In cases where there is excess supernatant, divide in parts, sufficient to fill the column, which should be allowed to run through before adding the next part to prevent any overfill and loss of sample. After each addition of supernatant, column should be mixed with a small plastic loop, to increase the likelihood of protein binding to the beads, before turning on the pump. To keep track of the plate's orientation and therefore content of the wells, ensure that the labelled corner 'A1' is always at the top left corner of the plate before starting the purification.

Eluting Protein

When eluting in the IEX and affinity step, the vacuum is used on the lowest setting to pull the elution buffer through the column. This speeds up the flow rate compared to gravity although if the protein concentration is high, it can lead to the protein solution to froth and potentially denature. If this is the case, an alternative is to use a syringe plunger on top of the open column to push the buffer through the column. In this case, the syringe plunger should be gently (not forcefully) pushed down into the column to push the liquid through and into the collection plate beneath. Care should be taken when removing the collection plate to ensure any elution drops remaining on the MCPA do not spill into neighboring wells, causing contamination.

Maintenance of resin and columns

A critical step in this protocol is the regeneration of the Ni resins and columns. Columns should be regenerated at either the start or end of the purification protocol. If regeneration occurs at the end, the resin should be stored in 20% ethanol at 4 °C. The chromatography column filters may become "blocked" causing the sample to flow through the column at a much slower rate than it should. If this is the case, columns need to be replaced or filters removed and cleaned by soaking in denaturing buffer overnight and rinsing with water.

As demonstrated under step 3, the diversity of the MCPA instrument can be exploited for the purification of multiple proteins as effectively as a single sample. The ion exchange protocol can be tailored to suit the needs of the experiment, adapting to the number of different samples and number of salt concentrations being used. For example, if less than 12 samples are simultaneously purified, it would involve any combination of moving the columns after each elution within the same plate and/or swapping collection plates for each elution. If for example, only 4 proteins were being purified in parallel, the columns can be moved across one collection plate to collect elutions of up to 4 salt concentrations before changing plates. MCPA is capable of purification using various resins - affinity and ion exchange have already been discussed but hydrophobic interaction chromatography is also possible and there is further potential for immunoaffinity chromatography[12]. Although this method has focused on multiple small-scale purifications, the MCPA can be used for just a single large chromatography column for purification from several liters of bacterial culture, without the need for a sample pump or an expensive FPLC.

Using the MCPA for high-throughput ion exchange as discussed would require multiple, up to 24, separate collection plates. This could be impractical and require a lot of space on a standard laboratory bench top and increased risk of human error. Furthermore, measuring protein absorbance of elutions from 24 different samples may be challenging. In this situation a multi-channel pipette would be beneficial and would make the transfer of multiple samples quicker and easier. For small volumes, consider using an LVis plate (BMG) containing 16 microdrops as it enables measurement of the concentrations directly, without the need to use any other reagents such the Bradford assay reagent.

While the use of a vacuum pump allows for a 3x quicker purification speed than what is achieved using just gravity[10], without compromising the integrity of the resin, it does create some other issues. Maintaining the strength of the vacuum during the purification, for example, requires all the columns to be blocked with a 10 mL syringe plunger which needs to be taken out before inserting the column packed with resin. Taking the plungers out one by one is also a timely process and the resistance of the vacuum can make them difficult to remove from the column.

Details of a multiple protein IEX purification using the MCPA are given in the protocol. This higher-throughput method is time efficient and controllable, all parameters for each purification can be manipulated by the user. However, in most protein biochemistry labs, including industry, fast protein liquid chromatography is the preferred method of protein purification, which is superior in terms of throughput, reproducibility and method transfer and robustness. These systems such as the Protein Maker by Protein Biosolutions and the AKTA FPLC biomolecule purification system can alleviate the purification bottleneck problem with great success. Despite these systems obtaining superior results, the separation we see using the MCPA system is still good enough to obtain high purity protein. Interestingly, our lab also uses the AKTA start FPLC to perform ion exchange chromatography and although the resolution may be higher with a linear gradient capable with this machine, it is notably more time consuming to run multiple samples and it is much more challenging to train inexperienced students on this system.

Other significantly cheaper plate-based purification alternatives exist. For example, GE Healthcare life sciences (now Cytiva) and Sigma Aldrich sell pre-packaged 96 well filter plates and cartridges with specific purification resins. These filter plates offer small scale high-throughput purification but only purifying in the microgram yield range. Furthermore, the QIAvac 24 Plus from QIAGEN uses spin columns under vacuum however, it is not practical for collecting flow through or washes.

The flexible design of the MCPA allows for parallel protein purification, although manually moving columns and plates using the MCPA method potentially increases human errors compared to standard FPLC systems. However, manually loading the samples onto columns is more reliable for inexperienced users than loading samples onto columns using standard FPLCs, where mistakes can be more easily made as it involves switching valves and pumps that requires more extensive training. It is clear that fully automated systems for protein purification are better suited than the MCPA for purification groups in industry and academic labs which routinely work on purification. However, for small laboratories which cannot afford the expensive equipment and upkeep and want to avoid extensive training or only occasionally work on protein purification, the MCPA offers an effective alternative system which still obtains good separation and is cheap and easy to set up.

The MCPA consists of simple and inexpensive instrumentation which permits multiple columns to be interfaced for simultaneous parallel purification to produce milligram quantities of proteins. Furthermore, this technique allows modularity of the individual columns increasing the throughput. This is unique to this method and cannot be achieved using current plate-based purification kits.

Protein purification will remain essential in the study and characterization of proteins and the development of therapeutics. Biophysical techniques such as NMR and protein crystallography rely on milligram quantities of pure protein, therefore the current expression and purification systems need further development to improve the cost and time of achieving this[2,13,14]. As discussed, automated purification systems have many advantages over un-automated methods however they remain too costly for smaller scale laboratories requiring expensive instrumentation and training. The MCPA is considerably cheaper with a starting cost of $45[10]. Additionally, this MCPA does not need extensive training or continuous maintenance and should any problems arise these can be easily solved. Corrosive buffers such as the denaturing buffers used for the Ni-NTA can corrode purification systems if they are not cleaned properly. However, the flexible design of the MCPA allows for quick cleaning, repairing and changing of compartments if necessary. In conclusion, the MCPA will facilitate effective, higher-throughput protein purification for smaller laboratories until more affordable automated systems are established[10].

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

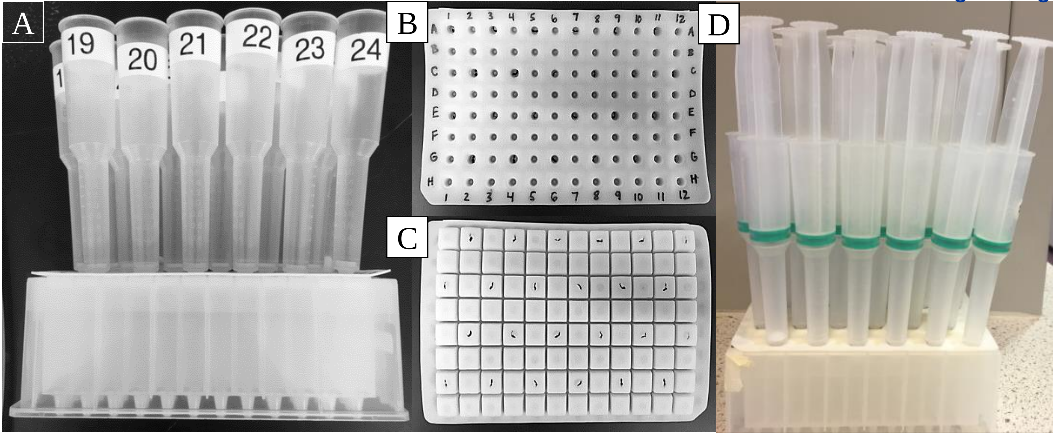

Figure 2

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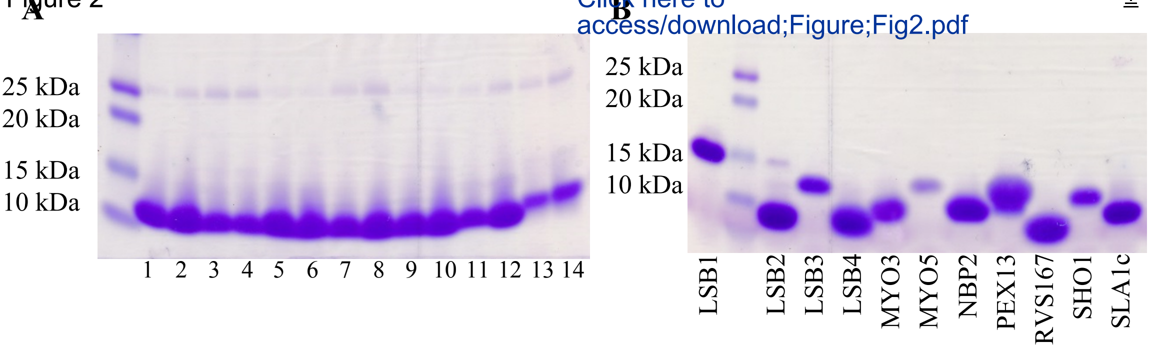

Figure 3

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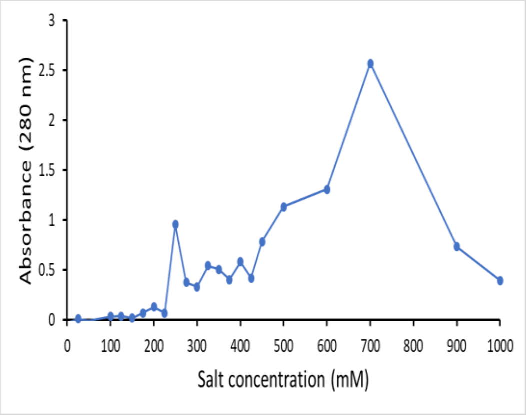
A



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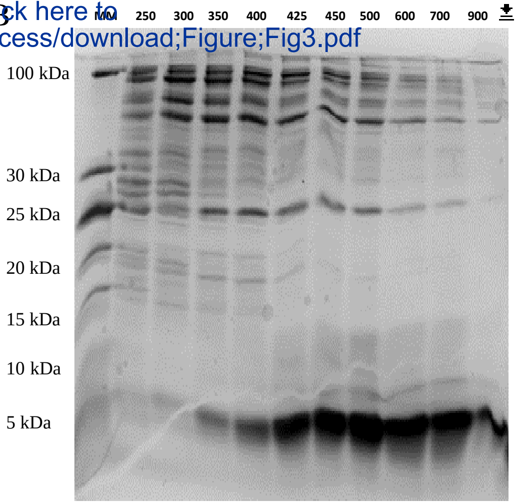
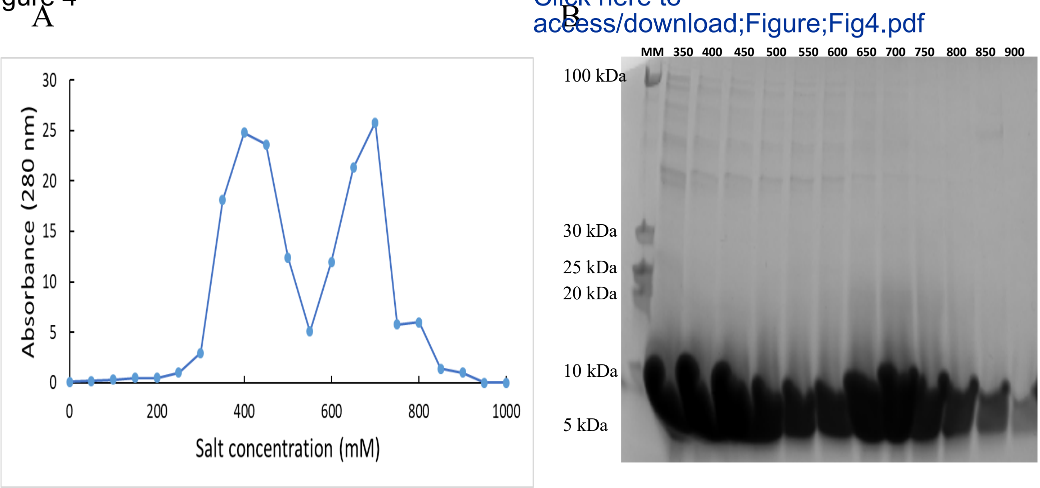


Figure 4


A
B
30
25
20
15
10
5
0
Absorbance (280 nm)
0
200
400
600
800
1000
Salt concentration (mM)
MM 350 400 450 500 550 600 650 700 750 800 850 900
100 kDa
30 kDa
25 kDa
20 kDa
10 kDa
5 kDa

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2 mL/ well collection plate	Agilent technologies	201240-100	
5 mL/ well collection plate	Agilent technologies	201238-100	
12 mL chromatography columns	Bio-Rad	7311550	
96 well long drip plate	Agilent technologies	200919-100	Come with 0.25 um filters which are to be removed.
96 well plate seal/mat	Agilent technologies	201158-100	Should be peirceable
His60 Ni Superflow Resin	Takara Bio	635660	
HiTrap Q HP anion exchange colur	GE Healthcare (Cytiva)	17115301	
Lvis plate reader	BMG LABTECH		Compatible with FLUOstar Omega plate reader
Male leur plugs	Cole-Parmer	EW-45503-70	
PlatePrep 96 well Vacuum Manifold Starter kit	Sigma-Aldrich	575650-U	
Reservoir collection plate	Agilent technologies	201244-100	
The Repeater Plus	Eppendorf	2226020	With 5 mL and 50 mL syringes
VACUSAFE vacuum	INTEGRA	158 320	The vacusafe vacuum has a vacuum range from 300 mBar to 600 mBar and a 4 L waste collection bottle

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January 31, 2021

Dear Professor Nguyen

Thank you for your recent email concerning our manuscript "*An economical and versatile high-throughput protein purification system using a multi-column plate adapter*" by Kineavy *et al*.

I have made the following changes in response to the editors and reviewers comments:

- Proof read the manuscript, figures and tables and made a variety of changes to include removing embedded figures/tables, personal pronouns. Added more detail to protocol steps where required. Reordered 4 figures and made consistent throughout.
- Removed the comment about Mass Spec requiring mg quantities of proteins
- Updated the section "Significance of the method with respect to existing/alternative methods" to include discussion of other methods and acknowledge that automated systems are superior and that the MCPA method is useful for small laboratories which cannot afford the expensive equipment and upkeep, want to avoid extensive training or only occasionally work on protein purification.

I hope these changes are satisfactory. Thank you for your time and consideration in reviewing this paper for publication in JOVE.

Sincerely,

Elliott Stollar

Elliott Stollar, Ph.D.

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