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A semi-automated method for purification of milligram quantities of proteins on the QIAcube

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Abstract

A growing number of studies require the purification of multiple proteins simultaneously and the development of simple economical high-throughput purification methods is essential. We have tested the purification of two related proteins in a variety of conditions to benchmark the semi-automated affinity chromatography method for the QIAcube that we have developed. We find that this new QIAcube method can successfully purify milligram quantities of proteins with minimal user involvement and performs as well as methods based on gravity. The method could easily be adapted to other chromatography resins and should prove to be a versatile method for optimizing protein expression or purification conditions for multiple proteins while obtaining sufficient amounts for subsequent biochemical analyses.

Keywords

SH3 domain; protein purification; high-throughput automation; QIAcube; protein expression

INTRODUCTION

In the era of proteomics and high-throughput analyses, it has become increasingly important to develop methods to simultaneously purify recombinant proteins in milligram amounts [1–3]. Currently there are several methodologies available that differ in final yields, the cost of instrumentation/materials required and extent the method is automated. Almost all of these methods are designed for overexpressed recombinant proteins that contain affinity tags (hexahis, GST, MBP etc.) which use affinity resins in their purification [4].

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CONTRIBUTIONS

EJS conceived the idea for this research. JM, PE and SL developed the QIAcube method. VT and OF collected the final data on the SH3 domain proteins. MT, VT and EJS analyzed the data and EJS wrote the paper.

The most economical method to purify protein from multiple cell lysates is to use the appropriate resin in multiple columns or a multi-well plate and manually purify under gravity. The time can be shortened by using the batch method, where the purification takes place in a tube or multi-well plate and is spun in a centrifuge after each step, allowing the resin to separate at the bottom more quickly [5]. A vacuum manifold can also be used to perform the purification on the bench, which can further speed up each step [1, 5]. Although these methods are economical, they are not automated and take 3 hours or more hours of a users time.

For a life science lab wishing to automate the purification of biomolecules such as protein and nucleic acids, there are several relatively inexpensive systems available: Promega's Maxwell 16 (16 samples) and Thermofisher's Kingfisher (24 samples) which both use paramagnetic particle technology for protein and nucleic acid purification [6, 7], the Bio-Rad Profinia (1 sample) that can run 2 sequential columns (i.e. affinity followed by desalting) for protein purification [8] and Qiagen's QIAcube (12 samples) for protein and nucleic acid purification [9].

Our lab chose the QIAcube as it is able to purify both nucleic acids and protein using economical kits cheaper than those based on the magnetic bead technology. The QIAcube is a robotic workstation for the automatic purification of nucleic acid and protein, consisting of a micro-centrifuge, a robotic arm that contains a micro-pipetor and a gripper to transfer spin columns as well as a micro-tube incubator and shaker (Figure 1). It accepts up to 12 micro-tube samples and up to 9 different purification solutions to automatically pipette, mix, incubate, transfer and spin in order to purify nucleic acid or protein. It accepts all of the well established Qiagen spin column kits and runs them automatically after the user includes the samples to be purified, the kit specific buffers, tips and rotor adapters with spin column in appropriate position. Throughout the purification, the spin column containing the chromatographic material is transferred between any of the 3 positions within its plastic rotor adapter unit using the robotic arm (Figure 1).

The QIAcube kit for protein purification uses a Ni-NTA silica membrane to purify his-tagged protein from up to 5 mL of overexpressed bacterial culture and can only provide up to 150 µg of purified protein (Qiagen Ni-NTA spin kit). We sought to develop a method to use the QIAcube to process 10 times the amount of culture and thus purify milligrams of protein using Ni-NTA beads within a Qiagen spin column. This method can easily be adapted to other types of purifications that use other affinity, hydrophobic or ion exchange resins and should prove to be a versatile method for obtaining sufficient protein for subsequent biochemical analyses. In this study we decided to use this method for both native and denaturing Ni-NTA purifications as different proteins have different preferences for this common affinity resin [10].

To test our method, we chose to purify a well-characterized N-terminal his-tagged protein in our lab, the SH3 domain from the yeast protein Actin Binding Protein 1 (Abp1p), which we abbreviate to AbpSH3 [11, 12]. Src Homology 3 (SH3) domains are the most common eukaryotic interaction domains that are frequently involved in cell signaling by binding to proline rich peptide targets [13–16]. AbpSH3 has several known peptide targets including a

17-residue peptide from the yeast protein Ark1p [11, 12, 17, 18]. We recently found that AbpSH3 most likely binds in an intra-molecular fashion to a proline rich sequence upstream of the domain [19] and this prompted us to make an AbpSH3-ArkA hybrid to improve expression and purification of the domain, similar to other studies [20–23].

The N-terminal his-tagged AbpSH3 and AbpSH3-ArkA proteins were purified by methods based on gravity and using the QIAcube under native and denaturing conditions to explore the utility of our newly developed QIAcube method.

MATERIALS

Qiagen spin columns with lid removed (purple or blue) were obtained from any QIAGEN spin kit. A polypropylene frit that can be found in the QIAquick or minelute Qiagen spin columns after the paper disk is removed is fitted at the base of the spin column, allowing 300 μ L of resin to be added to complete the column construction. QIAcube tips, rotor adapters, 2 mL tubes and Ni-NTA superflow resin were also obtained from Qiagen. Ni-NTA resin was also obtained from Clontech as a comparison. 100X bacterial protease arrest inhibitor was obtained from G-biosciences. DNase, benzonase, lysozyme, guanidine, imidazole, ampicillin, polyethylenimine (PEI) and all other chemicals were purchased from Sigma. 20 mL econopac columns were purchased from Bio-Rad. For the AbpSH3 construct, residues 535–592 of the yeast Abp1p protein (uniprot # **P15891**) were expressed from an ampicillin resistant pJexpress414 (DNA 2.0) plasmid to include a N-terminal histag (total size of protein 7800 Da). For the AbpSH3-ArkA construct, residues 535–592 of AbpSH3 were linked to KKTKPPVPPKPSHLKGT based on the yeast protein Ark1p (uniprot # **C7GWN7**) via a 17 amino acid linker GSGSENLVYFQGGSYAMG and were expressed from a pJexpress414 plasmid (DNA 2.0) to include a N-terminal histag (total size 12,400 Da). BL21 (DE3) chemically competent cells were obtained from Lucigen. The extinction coefficients at 280 nm were calculated as 23950 $\text{M}^{-1} \text{cm}^{-1}$ for AbpSH3-ArkA and 20970 $\text{M}^{-1} \text{cm}^{-1}$ for AbpSH3.

METHODS

Protein overexpression

Both AbpSH3 and AbpSH3-ArkA proteins were overexpressed using an autoinduction protocol [24] to aid in the high-throughput expression and purification efforts. BL21 (DE3) cells were transformed and plated on LB-agar plates supplemented with 100 μ g/mL ampicillin. The following morning, several colonies were used to inoculate 4 mL of terrific broth medium (1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4 17 mM KH_2PO_4 0.4% glycerol) with 100 μ g/mL ampicillin, which is left to shake for ~6 hours at 37 °C. This culture was transferred to a 2 L flask containing 500 mL of autoinduction medium (TB media + 0.015% (w/v) Glucose, 0.5% (w/v) lactose, 2 mM MgSO_4) with 100 μ g/mL ampicillin, and left to shake at 25 °C until an O.D₆₀₀ of ~6–8 was reached (within 8–18 hours). The cells were harvested into 50 mL fractions and centrifuged at 4600 rpm for 20 minutes. The pellets were frozen at –20 °C until purification.

Buffer systems for native and denaturing purification methods

The buffer systems used with the 4-part QIAcube method as well as the gravity method can be found in Table 2.

Native and denaturing cell lysis

For both methods, 50 mL of bacterial culture yielded a pellet of approximately 1 g wet weight. For the native purification, 1.5 mL of cold native lysis buffer was added to the pellet and resuspended to homogeneity. Then, 1 mg of lysozyme, 7.5 μ L of 100X protease inhibitor, 30 μ L of 10 mg/mL DNase, 30 μ L of Triton X-100 were added and kept rocking for 45 minutes at 4 °C. 30 μ L of cold 10% PEI was added to precipitate the DNA. This was spun in a 2 mL microcentrifuge tube at 14600 rpm for about 20 minutes and the lysate supernatant was separated for subsequent purification. In the simpler denaturing purification, 1.5 mL of cold denaturing lysis buffer was added to the pellet and resuspended to homogeneity. This was kept for rocking for 30 minutes and then spun in a 2 mL microcentrifuge tube at 14600 rpm for about 20 minutes and the lysate supernatant was separated for subsequent purification.

Native and denaturing gravity purifications

For both the native and denaturing purification, the given supernatant was loaded onto the column containing 300 μ L Ni-NTA beads, pre-equilibrated in the relevant lysis buffer. The column was washed with 5 mL before being eluted with 4 mL of the relevant elution buffer collected over two fractions. The elution fractions were dialyzed in 10 mM Tris, 100 mM NaCl, pH 8.

Native and denaturing QIAcube purifications

We divided our QIAcube protocol into 4 parts (A, B, C and D) to allow for the rotor adapters to be emptied, tips to be refilled, samples to be taken or buffers to be changed between the steps. A summary of the purification process can be seen in Table 1. During part A the column with 300 μ L of Ni-NTA beads in 20% ethanol is equilibrated with lysis buffer and the lysate load is added to the column in several steps with incubation periods to maximize binding. During part B, the column is washed and the first 800 μ L elution fraction is collected in elution position 3 of the rotor adapter (Figure 1). During part C, the second 800 μ L elution fraction is collected in the middle position 2 of the rotor adapter and the column is washed. During part D, the column is regenerated and stored for future use. The elution fractions are dialyzed in 10 mM Tris, 100 mM NaCl, pH 8 to remove elution buffer components (guanidine or imidazole) and spun at high speed in a microcentrifuge before the concentration is measured. All steps performed by the QIAcube scripts (Protein Resin Part A, B, C and D) are found in Supplementary File A and can be obtained from applicationslab@qiagen.com to run on any QIAcube. Purification using the Ni-NTA spin kit (Qiagen) was performed according to the manual using 2 mL cell culture for each purification. All elutions were quantitated using absorbance measurements at 280 nm. Any kD Mini-PROTEAN TGX gels from Bio-Rad (Cat. # 456-9036) were stained using Labsafe Gel blue from G-Biosciences (Cat. # 786-35).

RESULTS AND DISCUSSION

We wanted to use the QIAcube and a Qiagen spin column that contains chromatography resin and worked with Qiagen to produce an optimal 4-part method. Part A prepares the column and loads the lysate, Part B washes the column and elutes sample into the elution position 3 of the rotor adapter, Part C elutes more sample into the middle position 2 and washes and stores column in ethanol and Part D regenerates the column. A step-by-step practical protocol is listed below and summarized in Table 1. For reference, every step within the 4 parts that is performed by the QIAcube can be found in Supplemental File A.

1. Decide whether to purify his-tagged protein using denaturing or native method (Table 2) and prepare clarified lysate supernatant (see native and denaturing cell lysis section in methods).
2. Place up to 1800 μ L of supernatant samples (Note 1) in 2 mL tubes (Cat # 990381) into sample rack (Fig 1D) according to the Qiagen scheme, which takes into account the total number of samples.
3. For each rotor adapter (check each is clean), place a column in wash position 1 and a 50 % cropped 1.5 mL tube that has no lid attached into elution position 3 (to ensure the column sits in the adapter correctly). Then load all rotor adapters into the centrifuge according to the Qiagen scheme.
4. Place buffer containers filled to the max line (Table 2) and 1000 μ L tips (light grey, cat # 990352) in their assigned positions in QIAcube (Fig 1B).
5. Run the appropriate version of Part A (Notes 2 and 3).
6. After Part A has finished, for each column, collect or discard flow through (that appears on the outside compartment of the rotor adapter) (Note 4) and add more tips (Note 5).
7. Run Part B (Note 6).
8. After Part B has finished, discard wash.
9. Shift the column position from elution position 3 to middle position 2, recycle tips or refill with new tips and run Part C.
10. After Part C has finished, collect the 800 μ L sample from elution position 3 (which is Elution 1 or E1) and the 800 μ L sample from middle position 2 (which is E2). Empty the rotor adapter reservoir.
11. Replace buffers rack with Part D buffer rack (Table 3), recycle tips or refill with new tips.
12. With the column still in wash position 1, run Part D.
13. Top up columns with 20% ethanol and store at 4 °C for future use (Note 7), take out cropped tubes and store with buffers, rinse out rotor adapters.
14. Collect the NiSO_4 from the rotor adapters using a disposable transfer pipette into an assigned waste container.

Notes

1. Lysis buffer is used to make all of the samples the same volume as the largest volume sample and that they are all close to one of the following starting volumes; 400, 800, 1400 or 1800 μL .
2. There are 4 versions of Part A, PartA_400, PartA_800, PartA_1400 and PartA_1800, which refer to the volume (in μL) of lysate that will be taken from the sample tube.
3. There are optional steps in Part A according to your choice (see Supplementary File A). The default settings include an optional water wash and lysis buffer equilibration before loading the lysate. Regardless of these options, all lysate loading steps include a 1 minute incubation to increase binding time and all elution steps include a 30 second incubation time to maximize elution from the beads.
4. For quickly discarding flow throughs or washes we use an aspiration system (Vacusafe unit from Integra).
5. For 12 samples, 36 tips are used in Part A_1800, 6 tips are used in Part B, 8 tips are used in Part C and 8 tips are used in Part D. If recycling tips, then the tips from Part B are re-racked into the top left position and used in Part C, saving 6 tips. Likewise, the tips from Part C are recycled in Part D, saving a further 8 tips.
6. There are optional steps in Part B according to your choice (see Supplementary File A). The default settings include an optional additional 400 μL wash with buffer A2 and an optional additional 400 μL elution with buffer F, making the E1 fraction 800 μL . It might be desirable omit this step and reduce the E1 to 400 μL as frequently the first part of the elution contains weaker bound contaminants, thus allowing greater pure protein in the E2 fraction.
7. All centrifuge steps are at 1000 $\times g$ for 1 minute to maintain the integrity of the resin. In general, we found that we could reuse the same column about 10 times before we removed the resin from the column and removed any particulates that may have built up over time. This also allows for the filters to be cleaned as well. The columns can then be remade from the same resin. Columns could be constructed with a frit placed on the top to increase longevity, although this has not been tested.

For use with these methods we separately overexpressed N-terminal his-tagged AbpSH3 and AbpSH3-ArkA proteins using autoinduction [24]. Next, we prepared bacterial cell lysates using both native and denaturing buffers (which took about 1.5 hours to prepare) and ran the 4-part method with either the native or denaturing buffers. For 12 samples, it took ~2 hours to run through protocol A, B and C, which includes a few minutes manual intervention that was required between each part where we collected flow through, wash or elution samples for analysis (Table 1). Protocol D took 30 minutes more to regenerate the columns. With a total run time of 2–2.5 hours for 12 lysate samples, the machine can be run several times through the day to purify 36 or more different proteins.

Thus, the 4-part method was used with 2 different proteins with 2 different buffer systems, which along with the gravity column system totaled 16 purifications (each from 50 mL of cell culture) as seen in the factorial design in Table 3, which also shows the amount of purified protein obtained as determined by absorbance values at 280 nm.

As can be seen in Fig. 2 and Table 3, the method is successful at purifying both proteins, however, AbpSH3-ArkA protein purifies overall much better than AbpSH3 which may be due to the fact that the peptide binds and protects the domain from unfolding and subsequent aggregation or degradation, consistent with studies from another SH3 domain-peptide hybrid [23]. It should be noted that the band at the same position as AbpSH3-ArkA in the native purification flow through is lysozyme, which is used to lyse the cells in this method but does not have a his-tag and bind to the Ni-NTA columns (data not shown). Other general observations of the data reveal that the denaturing method is more effective at purifying AbpSH3-ArkA while the native method appears to be better for AbpSH3. Some of these observations are consistent with other studies that show different proteins have different preferences towards the chosen buffer system [10]. Most striking however is that our new QIAcube method is able to purify equivalent amounts of protein to the corresponding manual based gravity methods and that this method is able to purify orders of magnitude more protein compared to the previous QIAcube spin column method.

This study highlights that our QIAcube method can successfully purify 12 proteins simultaneously with minimal manual intervention. For AbpSH3, the QIAcube was able to purify up to 1.4 mg of protein from 50 mLs of bacterial culture (using our method) compared to 0.031 mg of protein from 2 mLs using the Ni-NTA spin column from the Qiagen kit. Likewise, for AbpSH3-ArkA, the QIAcube was able to purify up to 5.5 mg of protein from 50 mLs of bacterial culture compared to 0.04 mg of protein from 2 mLs using the Ni-NTA spin column. The significant increase in purified protein will allow QIAcube users more flexibility in their protein purification method choices. For example, when compared to our newly developed method, the current Ni-NTA spin column for the QIAcube requires less user involvement, automating at an earlier stage (after the initial bacterial pellet has been produced) and is useful for those needing to quickly produce a small amount of purified proteins for assays such as western blots or expression trials. However, our method is considerably cheaper, both in terms of producing the columns and regenerating them after use and provides considerably more protein.

Since developing this method we have successfully purified over 50 different proteins using both the denaturing and native method on the QIAcube with yields up to 10 mg of protein. We have found it a convenient, time saving tool for analyzing protein expression optimization experiments, especially with students that are inexperienced in protein purification methods. Furthermore, with milligram amounts of purified protein using our method, it is possible to perform further purification steps as well as thermal/chemical denaturation, enzyme activity, nuclear magnetic resonance or isothermal titration calorimetry assays, which would be much more challenging with the previous Ni-NTA spin column method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SH3	Src Homology 3
Abp1	Actin Binding Protein 1
AbpSH3	The SH3 domain from Abp1
AbpSH3-Ark1	The chimeric protein containing AbpSH3, a 17 residue linker followed by a 17 residue binding peptide from Ark1

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Highlights

A new method to purify milligram quantities of proteins was developed on the QIAcube.

It allowed optimal purification conditions to be found.

It gave comparable yields to equivalent manual gravity based protocols.

It allows much more protein to be purified than the current method.

Figure 1.

Overview of the QIAcube system. A. Robotic arm with gripper to transfer spin columns and a micro-pipettor to dispense liquids. B. Tips and small volume buffer holder section. C. Micro-centrifuge with 12 buckets to accept rotor adapters (2 are currently shown with spin-column in wash position 1). D. Sample holder/incubator/shaker (2 samples are present). E. Buffer holders (5 are present). The lower panel shows an image and schematic of the rotor adapters.

Figure 2.

SDS-PAGE analysis of the flow through (F.T) and first elution (E1) for the purifications outlined in Table 2 (left gel uses gravity column and right gel uses QIAcube, where C and Q refer to Ni-NTA from Clontech and Qiagen respectively). The AbpSH3-ArkA protein band appears slightly higher than AbpSH3 due to its higher molecular weight. The band in the flow through of the Native purifications that is approximately the same position as AbpSh3-ArkA is lysozyme, which is used for cell lysis and does not bind to Ni-NTA.

Table 1
Table summarizing steps and times of our QIAcube method designed to purify milligram quantities of proteins.

Step	Action	Time
1	Grow 50 mL of cells using autoinduction method.	16 hrs
2	Harvest cells (~1 g) and fully resuspend into 1.5 mL cold lysis buffer and leave gently rocking for 30 minutes at 4 °C.	60 min.
3	Harvest lysed cells and carefully separate the supernatant from pellet.	30 min.
4	Ensure columns are in position 1 and tips, buffers and samples are in place	2 min.
5	Run Part A to equilibrate and load	60 min
6	Collect flow through or discard from reservoir.	2 min.
7	Run Part B to wash and make 800 µL Elution 1 (position 3).	30 min.
8	Move column to position 2 and discard wash from reservoir.	2 min.
9	Run Part C to make 800 µL Elution 2 (position 2) and then wash column in position 1.	30 min.
10	Collect Elution 1 from position 3 and Elution 2 from position 2.	2 min.
11	Change to regeneration buffer set.	2 min.
12	Run Part D to regenerate column.	30 min.
13	Top up columns with 20% ethanol and store at 4 °C. Rinse out rotor adapters.	5 min.

Table 2

Buffer positions for protein purifications

Composition of the buffers used in both native and denaturing purifications.

Position	Reagent for part A	Reagent for part B	Reagent for part C	Reagent for part D
1	Water	Water	Water	6 M Guanidine, 0.2 M Acetic Acid.
2	A1: Lysis Buffer (10 mM Imidazole)	A1: Lysis Buffer (10 mM Imidazole)	A1: Lysis Buffer (10 mM Imidazole)	100 mM EDTA
3	A2: Wash Buffer (20 mM Imidazole)	A2: Wash Buffer (20 mM Imidazole)	A2: Wash Buffer (20 mM Imidazole)	100 mM Nickel Sulfate
4	F: Elution Buffer (250 mM Imidazole or low pH)	F: Elution Buffer (250 mM Imidazole or low pH)	F: Elution Buffer (250 mM Imidazole or low pH)	Water
5	20% ethanol	20% ethanol	20% ethanol	20% ethanol
6	-	-	-	-

Native lysis and wash buffers contain 20 mM Tris, 300 mM NaCl, pH 8.0 and 10 or 20 mM Imidazole respectively. Denaturing lysis and wash buffers contain 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, 6 M GuHCl, pH 8.0 and 10 or 20 mM Imidazole respectively. Native elution buffer contains 20 mM Tris, 300 mM NaCl, 250 mM Imidazole. Denaturing elution buffer contains 6 M Guanidine, 0.2 M Acetic Acid, pH 3.0.

Table 3

Two 2-level factorial designs have been created to explore the performance of two Ni-NTA resins (Factor A), two types of buffer (Factor B) and two purification systems (Factor C). The total amount of purified protein as calculated by 280 nm absorbance values is indicated. Furthermore the amount of purified protein as calculated by 280 nm absorbance values from 2 mL cell culture using the Qiagen Ni-NTA spin column in the QIAcube is also indicated in the last column.

Protein	Factor A (Resin)	Factor B (Buffer)	Factor C (system)	Total (mg)	Spin col total (mg)
AbpSH3-ArkA	Clontech	Native	Gravity	1.6	
AbpSH3-ArkA	QIAGEN	Native	Gravity	1.7	
AbpSH3-ArkA	Clontech	Denaturing	Gravity	4.3	
AbpSH3-ArkA	QIAGEN	Denaturing	Gravity	3.1	
AbpSH3-ArkA	Clontech	Native	QIAcube	0.5	
AbpSH3-ArkA	QIAGEN	Native	QIAcube	0.7	0.012
AbpSH3-ArkA	Clontech	Denaturing	QIAcube	5.5	
AbpSH3-ArkA	QIAGEN	Denaturing	QIAcube	1.3	0.040
AbpSH3	Clontech	Native	Gravity	0.8	
AbpSH3	QIAGEN	Native	Gravity	0.8	
AbpSH3	Clontech	Denaturing	Gravity	1.0	
AbpSH3	QIAGEN	Denaturing	Gravity	0.7	
AbpSH3	Clontech	Native	QIAcube	1.0	
AbpSH3	QIAGEN	Native	QIAcube	1.4	0.031
AbpSH3	Clontech	Denaturing	QIAcube	0.2	
AbpSH3	QIAGEN	Denaturing	QIAcube	0.2	0.017