Promega

Automated High-Throughput Protein Purification with MagneHis™ Particles

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Abstract

High-throughput protein production facilitates the study of multiple families of proteins involved in various metabolic pathways. Purified proteins find innumerable applications in biochemical and immunological assays for therapeutic discovery and diagnostics. In order to generate purified proteins for these applications, we developed an automated high-throughput protein purification pipeline. The basic elements of this 96-well format pipeline include generating clones and expressing and purifying proteins. Cell growth and expression are performed in 96-well, deep-well blocks using liquid handling robots where necessary. We have completely automated the protein purification process on the Biomek® FX using a magnetic bead-based affinity purification procedure. In this report, we briefly describe the process of robotic-affinity protein purification and present our purification results with forty-eight polyhistidine-tagged proteins. The pipeline consistently yielded greater than 10µg of protein with ~90% purity from 1ml cultures.

Greater than 10µg of protein was isolated from 1ml of culture using our automated protein purification process.

Introduction

Proteins are an important class of biological macromolecules that maintain the structural and functional integrity of the cell. Several diseases are associated with protein malfunction. Isolation of pure proteins enables researchers to delve into the mechanistic aspects of protein function and design diagnostic and therapeutic agents.

Several protocols have been developed for isolating recombinant proteins in recent years. Briefly, recombinant protein isolation can be described in two stages: 1) expression of the recombinant protein in a heterologous system, and 2) separation of the recombinant protein from other constituents of the cell such as DNA, membrane structures and other host proteins. Conditions are usually optimized for expression and purification of individual proteins. Simultaneous isolation of multiple proteins poses technical challenges, as every protein expresses at a different level in a heterologous expression system and exhibits different folding and biophysical properties.

Affinity tags offer a means to purify multiple proteins on a single platform. A comparison of affinity tags for expression of proteins was previously reported from our laboratory (1). In addition to aiding in purification, the tags also enhance expression of proteins in heterologous systems. GST fusions for native purification conditions (data not shown) and polyhistidine tags for denaturing purification conditions gave us the best results after one-step affinity purification.

Although several hundred proteins can be purified in a 96-well format by manual methods, the tedious and error-prone nature of manually performed high-throughput operations calls for automation of the process. Similar to low-throughput protein purification, high-throughput purification of recombinant proteins involves lysis of the cells and separation from other cellular components to finally yield the protein of interest. Robust protocols are needed for efficient purification of large numbers of proteins with diverse properties. Since the overall goal of high-throughput purification is to achieve the maximum amount of reasonably pure protein from as little as 1ml of culture in a 96-well format, minor improvements in every step of purification can improve yield and purity.

Reagents for cell lysis as well as affinity purification resins coupled to divalent metal ions or other compounds are commercially available from several vendors. Although these reagents are available, very few laboratories have built high-throughput protein purification pipelines for proteome-scale protein purification. We have optimized the protein expression and purification conditions to a certain extent (1–3). In this report, we describe the construction of our automated protein purification methodology using MagneHis™ Ni-Particles[a] (Cat.# V8560 and V8565). In addition, we discuss the purification of forty-eight unique proteins. A schematic representation of the pipeline is shown in Figure 1.

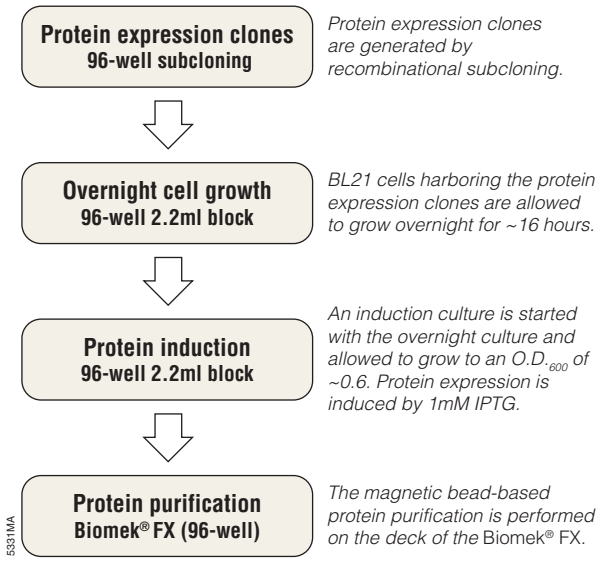

Figure 1. Schematic diagram of the automated protein purification method.

Automated Protein Purification Workstation

The entire protein purification process was performed on the deck of a Beckman Coulter Biomek® FX instrument (Figure 2). The efficiency of the purification process at each step was monitored by observing the purification process and analyzing the proteins. Based on these observations, we adjusted the timing and conditions for purification. The robotic protein purification workbench includes four buffer containers, a 24-pin magnet, a magnet-compatible Greiner plate, a Costar® plate for DNase addition and a Costar® plate for collection of the final eluted protein. Three tip boxes (96 tips each) are used in the purification process. Filter tips are used during resuspension of the cell lysate to prevent the lysate from being aspirated into the 96-well head of the robot.

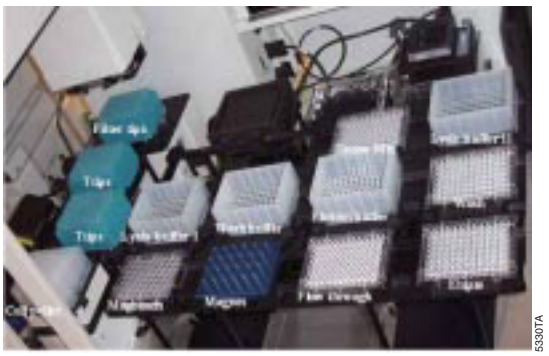

Figure 2. The position of containers on a Biomek® FX deck. The entire purification is performed on the deck of a Biomek® FX instrument. The flowthrough and wash can be collected (optional) or discarded to waste.

Protein Expression

Protein expression was performed according to protocols described elsewhere (1–3). Briefly, *E. coli* BL21 transformants harboring the recombinant plasmids were grown at 37°C as 1ml cultures in a 96-well 2.2ml block (Marsh Biomedical Products) to an $O.D._{600}$ of ~0.6–0.7 and induced with 1mM IPTG. The cultures were harvested after a post-induction growth of 4 hours at 37°C or 6 hours at 25°C. The cell pellets were stored at –80°C for subsequent protein purification.

Protein Purification

After a preliminary investigation of different procedures and high-throughput pipelines, we found that magnetic bead-based affinity purification is most suitable for our applications, which require microgram levels of protein. The purification involves four steps: 1) cell lysis, 2) binding of the recombinant protein to the affinity matrix, 3) washing to remove the other cellular components, and 4) elution to recover the recombinant protein in solution. All the steps for protein purification including cell resuspension, binding, washes and elution were optimized on the Biomek® FX. The buffers used in the purification were prepared in our laboratory and are described elsewhere (1,2). Protease inhibitors are added to prevent proteolysis. Prechilled buffers are used for purification under native conditions to prevent thermal inactivation of the purified proteins.

Cell Lysis: Cell lysis is primarily achieved by freezing the cell pellet at –80°C and thawing at room temperature. The cells are resuspended in lysis buffer and further disrupted by repeated robotic mechanical pipetting followed by vigorous shaking in clockwise and counter-clockwise directions. Lysozyme already present in the cells may aid in lysis of the cell wall. After thorough cell lysis, the lysate is treated with DNase to degrade the chromosomal DNA, forming a nonviscous homogeneous cell lysate. After DNase addition, samples are incubated with shaking for 10 minutes. Improper lysis of cells or shearing of chromosomal DNA results in decreased yield and impure protein preparations. Denaturing conditions for polyhistidine-tagged protein purification are introduced by adding a buffer containing guanidine hydrochloride after the DNase-treatment step.

Binding: The cell lysate is mixed with paramagnetic particles that are coupled to an affinity matrix (MagneHis™ Ni-Particles). The purification uses 30µl of MagneHis™ Particles. To ensure proper binding of the recombinant protein, the lysate and the magnetic particles are resuspended three times by pipetting and allowed to shake vigorously at 900rpm for a total of ten minutes in clockwise and counterclockwise directions.

Washing: Unbound cellular proteins are removed by pipetting and discarded. The beads are washed three times with wash buffer. Thorough washing is ensured by repeated resuspension of the magnetic particles followed by vigorous shaking. Between each wash, the magnetic particles are allowed to sit on the magnet for two minutes. This time is sufficient for separation of most of the particles, allowing the clear solution to be removed by pipetting and discarded. The tips are robotically washed with distilled water between each wash step to prevent contamination.

Elution: The protein is eluted from the magnetic particles by resuspending the particles containing the bound protein in elution buffer containing 500mM imidazole. Fifty microliters of elution buffer is used to elute the proteins of interest. Clumping of the particles in elution buffer is prevented by pipetting and shaking for ten minutes. Greater than 70% of the protein could be eluted in a single step. Although a second elution can recover an additional 10–15% of the protein, it also results in dilution of the protein sample.

Pilot Purification of Proteins

Forty-eight unique proteins with a size range between 9–40kDa were used for polyhistidine purification. Thirty-nine out of the forty-eight proteins were detected on Coomassie® stained gel (Figure 3). The remaining proteins could not be detected, which was probably due to low, or lack of, expression.

The entire 96-well-format protein purification pipeline represents a high-throughput scenario for production of thousands of proteins. As expected, variable yield was observed for different protein samples. The yield of proteins ranged from 1–30µg depending on the expression level of the protein. We observed ~90% purity for polyhistidine purification for most proteins (Figure 3). This is suitable for most of our high-throughput protein assays.

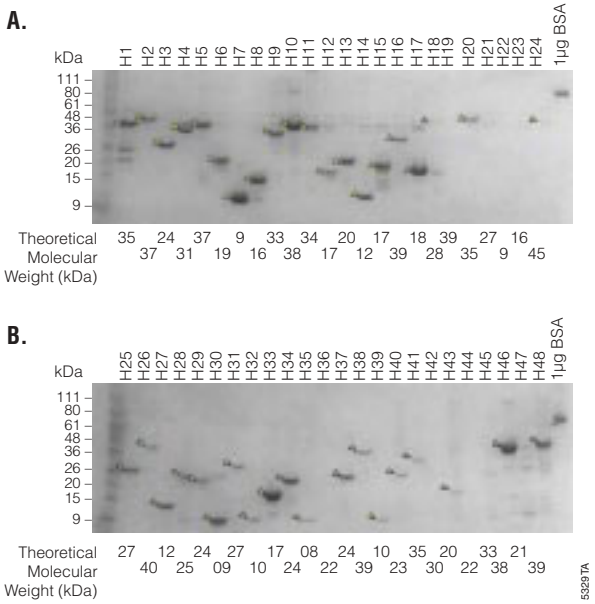

Figure 3. Coomassie® gel analysis of the proteins purified by the automated 96-well protein purification pipeline. Purification of forty-eight polyhistidine fusion proteins under denaturing conditions. Nearly 17% of the final eluate is analyzed on a 4–20% gradient Coomassie® gel. Thirty-nine of the forty-eight proteins are detected on the Coomassie® gel. The purified recombinant protein is indicated with a triangle on the left side of the protein band.

A volume of 30µl of MagneHis™ magnetic particles was found to be optimal for purification. Use of higher amounts of magnetic beads improved yield in cases where expression levels are known but also increased the background proteins (data not shown). Further purification of proteins was achieved by additional washes, but underexpressed proteins were not detected. However, if higher purity is required, proteins that show high levels of expression can be separated from those with low expression and purified separately by incorporating an additional washing step.

Since reproducibility is critical to the success of high-throughput methods, we also investigated the reproducibility of protein purification using the pipeline. For this purpose, we repeated the 96-well purification three times using eight different proteins. Analysis of the eight independent proteins, which exhibit different molecular weights and show varied expression levels, is shown in Figure 4. The purification pattern indicated that the pipeline is reproducible in terms of expression and purification. Automated, 96-well capillary-based protein analysis to determine size, purity and quantity is underway.

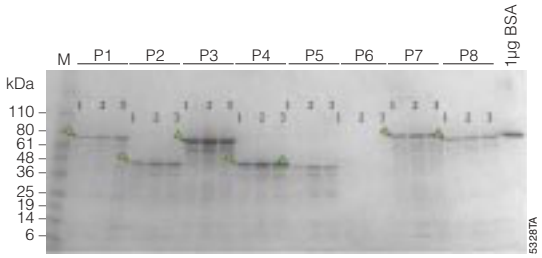

Figure 4. Reproducibility of the protein purification pipeline. The 96-well subcloning, protein expression and purification were repeated three times. Eight independent proteins (P1 to P8) of different molecular weights that also show varied expression levels were chosen for analysis on SDS-PAGE. Lanes 1 and 2 correspond to the protein expressed and purified in different blocks on the same day, and lane 3 corresponds to the protein expressed and purified on a different day. Less than 10% variation was observed between the purified samples.

Conclusion

A 96-well format protein purification pipeline was established using the MagneHis™ Ni-Particles for protein purification. From 1ml cultures, greater than ten micrograms of protein can be obtained with ~90% purity. The proteins are currently being used in high-throughput biochemical and immunological assays.

Acknowledgments

The work presented here was performed at the Harvard Institute of Proteomics. We thank Dr. Pascal Braun for the protocols on protein expression and Dr. Joshua LaBaer for his support.

References

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3. LaBaer, J. *et al.* (2004) *Genome Res.* **14**, 2190–200.

Ordering Information

Product	Size	Cat.#
MagneHis™ Protein Purification System	65 reactions	V8500
	325 reactions	V8550
MagneHis™ Ni-Particles	2ml	V8560
	10ml	V8565

These products require the use of a magnetic stand.

[a] Patent Pending.