The MagZ™ System: His-Tagged Protein Purification without Hemoglobin Contamination

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Abstract

This novel protein purification system opens up the possibilities for the expression, purification and functional analysis of His-tagged proteins expressed in rabbit reticulocyte-based protein expression systems. The MagZ™ Protein Purification System offers a unique advantage over nickel-based systems by specifically purifying polyhistidine-tagged (His-tagged) proteins from rabbit reticulocyte lysate without co-purification of hemoglobin. Thus, the proteins purified using the MagZ™ System could be used for several downstream applications, which are often limited by the presence of hemoglobin in the eluted samples using other immobilized metal ion affinity chromatography (IMAC) methods.

The MagZ™ Protein Purification System is optimized for efficient recovery of His-tagged proteins without the co-purification of hemoglobin.

Introduction

The use of in vitro-expressed proteins is a rapidly growing area, with applications in basic research, molecular diagnostics and high-throughput screening (1–6). Rabbit reticulocyte lysate is used in eukaryotic protein expression systems, which can have several advantages over bacterial-based in vivo or cell-free (S30) expression systems. Compared to cell-based expression systems, rabbit reticulocyte lysate systems provide a means of rapid functional screening of expressed cDNA libraries. Moreover, this expression method is also amenable to high-throughput applications. Libraries in His-tagged vectors are now available for human, bacteria, yeast and plant proteins. Rabbit reticulocyte lysate expression systems could be a powerful tool for the primary screening of expressed proteins from these cDNA libraries.

Although His-tag is the most commonly used protein fusion tag, currently available nickel purification systems are of limited use for purifying His-tagged proteins expressed in rabbit reticulocyte lysate because of the co-purification of hemoglobin present in rabbit reticulocyte lysate. This often limits the downstream applications of purified proteins in fluorescence-based functional assays or protein:protein interaction studies and also decreases the amount of proteins purified.

We developed a novel protein purification system for the purification of His-tagged proteins expressed in rabbit reticulocyte lysate without hemoglobin contamination. In this article we describe the unique features of the MagZ™ Protein Purification System. We show that the MagZ™ System provides a convenient and flexible method for the purification of His-tagged proteins expressed in rabbit reticulocyte lysate with minimal hemoglobin contamination.

Procedure for Purifying His-Tagged Proteins

The three basic steps involved in His-tagged protein purification from TnT® Reticulocyte Lysate[a–d] using the MagZ™ System are shown in Figure 1. The steps are optimized for efficient recovery of His-tagged proteins without the co-purification of hemoglobin. The magnetic nature of the MagZ™ Binding Particles allows purification to be performed in a single tube, thus simplifying and shortening the purification process.

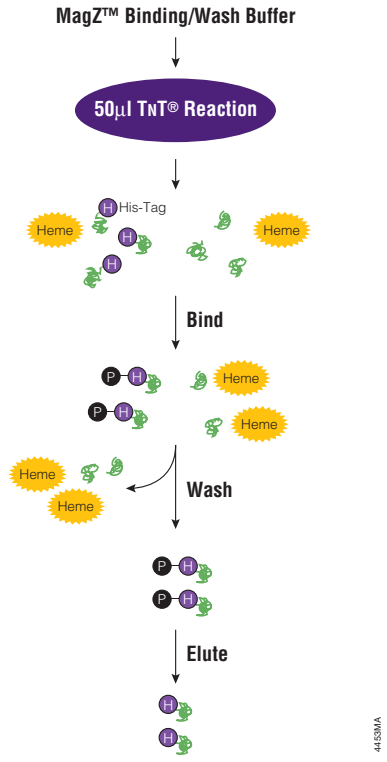

Figure 1. Schematic of the MagZ™ Protein Purification System. A TnT® reaction expressing His-tagged proteins is diluted with MagZ™ Binding/Wash Buffer and added to MagZ™ Binding Particles. The His-tagged proteins bind to the particles during incubation, then are washed to remove unbound and nonspecific bound proteins. The target protein is eluted with MagZ™ Elution Buffer.

Purification of His-Tagged Proteins

The MagZ™ System is flexible enough to be used with different labeling and detection methods. His-tagged proteins expressed in rabbit reticulocyte lysate can be labeled with [^{35}S]met or the FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System[e]. FluoroTect™-labeled His-tagged proteins can be visualized by gel analysis and read on a FluorImager® instrument (Figure 2).

Figure 3 shows that direct analysis of proteins using a fluorometer is also possible, thus eliminating the need to run protein gels. This approach could be used for quantitative expression analysis of expressed protein in cell-free systems. ^{35}S-labeled His-tagged proteins are analyzed by gel analysis and detected using a Storm® Phosphorimager® instrument (Amersham). Our results indicate that labeling with [^{35}S]met or FluoroTect™ tRNA does not interfere with the MagZ™ Particles and hence the purification of His-tagged proteins expressed in rabbit reticulocyte lysate. We also confirmed these results by Western blot analysis using protein-specific as well as anti-polyhistidine tag antibodies (data not shown).

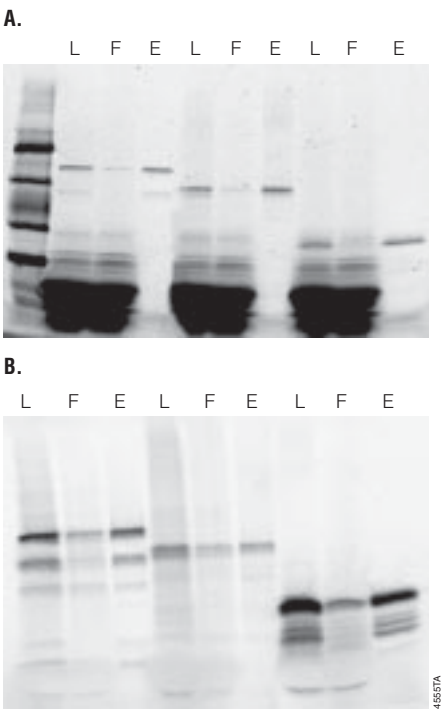

Figure 2. Purification of FluoroTect™- or [^{35}S]met-labeled His-tagged proteins using the MagZ™ Protein Purification System. His-MAPK, His-HGF and His-calmodulin (53, 42 and 25kDa, respectively) were expressed using the TnT® T7 Quick Coupled Transcription/Translation System[a–d,f] (Cat.# L1170) using 1µg of DNA and 2µl of FluoroTect™ Green$_{Lys}$ tRNA (Cat.# L5001) or [^{35}S]met. The reactions were incubated at 30°C for 60 minutes. The His-tagged protein was purified using the MagZ™ System by combining 50µl of lysate and 100µl of MagZ™ Binding/Wash Buffer and adding to 60µl MagZ™ Binding Particles. The particles were incubated for 15 minutes on an orbital rocker. The particles were washed 4 times with 200µl of MagZ™ Bind/Wash Buffer, and the His-tagged proteins were eluted with 100µl of MagZ™ Elution Buffer (1M imidazole) and run on a gel. **Panel A.** FluoroTect™-labeled proteins were visualized using a FluorImager® instrument using a 530nm filter. Lanes L, 2µl lysate; lanes F, 6µl flowthrough; lanes E, 8µl eluate. **Panel B.** [^{35}S]met-labeled proteins were visualized using the Storm® Phosphorimager® (Amersham). Lanes L, 2µl lysate; lanes F, 6µl flowthrough; lanes E, 4µl eluate.

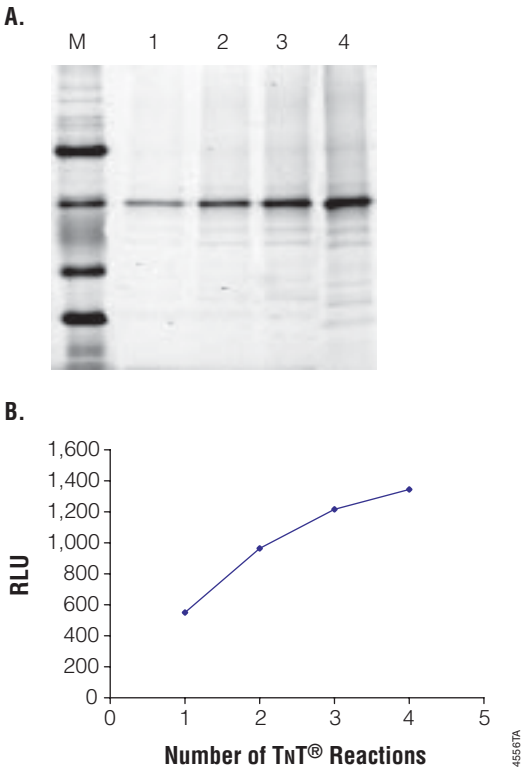

Figure 3. Scale-up purification of His-tagged proteins. The MagZ™ Protein Purification System was used following the procedure described in Figure 2 with the following exception: a master mix of 1ml His-HGF expressed in TnT® lysate and 2ml of MagZ™ Bind/Wash Buffer was used for all samples. One hundred and fifty microliters of the master mix was added to 60µl of MagZ™ Particles and incubated as in Figure 2. To concentrate the His-protein, additional master mix was added to the same particles three more times. The particles were washed and the sample eluted as described in Figure 2. **Panel A.** Following fluorometric analysis, the eluted proteins were analyzed by SDS-PAGE and visualized using a FluorImager® instrument and a 530nm filter. Lane 1, marker; lane 2, one TnT® reaction; Lane 3, two TnT® reactions; lane 4, three TnT® reactions. **Panel B.** The eluted proteins were transferred directly to a 96-well white Labsystems plate and read on a Victor™ Multilabel reader at 485/535nm.

Proteins expressed in rabbit reticulocyte lysate and purified using the MagZ™ Protein Purification System have less than 0.1% hemoglobin contamination. To demonstrate this, we compared the MagZ™ System to two other His-tag protein purification systems. We used each system to purify protein from TnT® Coupled Reticulocyte Lysate and compared the eluted samples to a titration of the TnT® Lysate (0–5%) by Western blot analysis. The MagZ™ Protein Purification System shows minimal hemoglobin contamination (Figure 4). It is evident from the Western blot that the percent of hemoglobin that is being co-purified with the His-tagged protein is less than 0.15%. In fact, the presence of co-purified hemoglobin in the eluted protein can be visualized without running a protein gel (Figure 4). This is also an indication of the

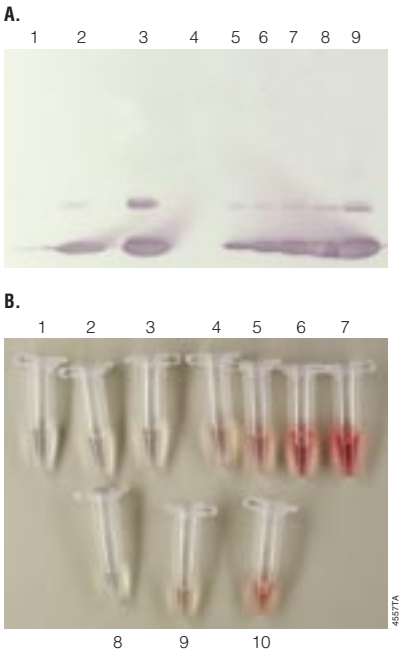

Figure 4. Comparison of hemoglobin contamination in samples purified with different protein purification systems. Fifty microliters of lysate was purified using either the MagZ™ Protein Purification System, the MagneHis™ Protein Purification System[g,h] or the Qiagen Ni-NTA magnetic resin. The MagZ™ and MagneHis™ Systems were performed using the appropriate technical literature, #TB336 and #TM060, respectively. The Qiagen Ni-NTA magnetic resin was used following the manufacturer's protocol. **Panel A.** Western blot analysis detecting hemoglobin in eluates. Sheep anti-human hemoglobin-AP was used at a 1:1,000 dilution and detected using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841). Lane 1, MagZ™ Protein Purification System. Lane 2, MagneHis™ Protein Purification System. Lane 3, Qiagen Ni-NTA magnetic resin. Lanes 4–9 are dilutions of lysate from 0–5% (0, 0.15, 0.6, 1.25, 2.5, 5). **Panel B.** Tubes 1–7 contain 0, 0.15, 0.31, 0.62, 1.25, 2.5, 5% TnT® lysate, respectively. Tube 8, eluate from the MagZ™ System; tube 9, eluate from the MagneHis™ Protein Purification System; tube 10, eluate from Qiagen resin.

inherent limitations of nickel-based systems for the purification of proteins for fluorescent-based assays. Hemoglobin intself generates background fluorescence that can be seen in our FluoroTect™ experiments (Figure 2).

Optimizing Imidazole Concentration for Improved Background

The concentration of imidazole used to elute His-tagged proteins from the MagZ™ Binding Particles should be optimized. The elution of His-tagged proteins from the MagZ™ Binding Particles is protein dependent; different proteins elute from the particles at varying concentrations of imidazole. For example, His-calmodulin can be eluted with 100mM imidazole, whereas His-actin requires >1M imidazole for the elution (Figure 5). Many purchased His-tagged proteins eluted at 0.5M imidazole.

Although elution with 1M imidazole produces the highest yield of His-tagged proteins from the MagZ™ Particles, these eluates will have more background proteins than those eluted using lower concentrations of imidazole. Our experiments with different proteins have shown that not all proteins require 1M imidazole for elution. There are some proteins such as His-calmodulin that can be eluted with 100mM imidazole (Figure 5). In the two experiments where we used ≤500mM imidazole, little to no background proteins were vissible (Figure 5, Panel A). Thus, we strongly recommend optimizing the imidazole concentration for each protein of interest.

To optimize the elution conditions, the concentration of the MagZ™ Elution Buffer (1M imidazole [pH 7.5]) can be titrated by diluting the buffer with Nuclease-Free Water. For example, the MagZ™ Elution Buffer can be diluted to 50, 100, 250 and 500mM imidazole. The His-tagged proteins were eluted off the MagZ™ Binding Particles with the diluted imidazole, increasing

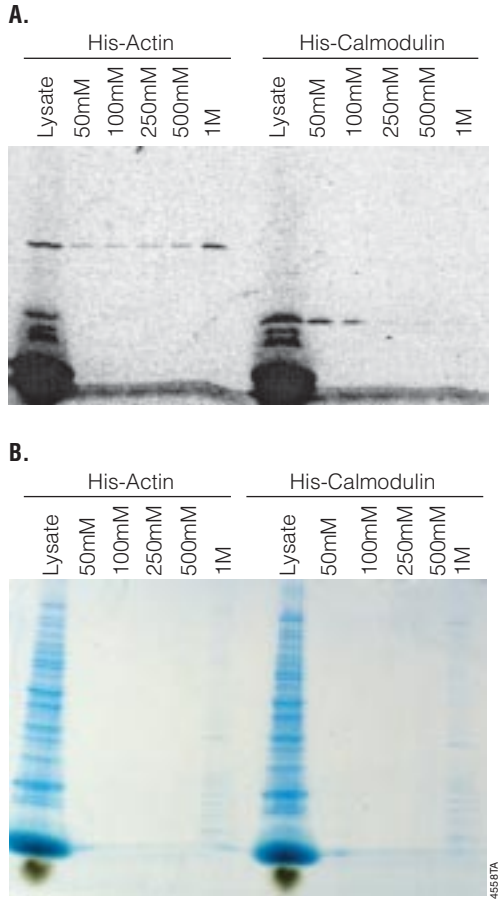

Figure 5. Optimization of elution conditions for different His-tagged proteins. TnT® T7 Quick Coupled Transcription/Translation System was used to express two His-tagged proteins labeled with FluoroTect™ tRNA: His-calmodulin and His-Actin. The His-tagged proteins were purified using the MagZ™ Protein Purification System. The optimal elution conditions were determined by diluting the MagZ™ Elution Buffer (1M imidazole [pH7.5]) to 50, 100, 250, 500mM imidazole. MagZ™ Binding Particles were washed with 100µl of each dilution of the MagZ™ Elution Buffer, increasing sequentially from the lowest to highest concentration. **Panel A.** Eluted samples were collected and analyzed by SDS-PAGE and visualized on a FluorImager® instrument using a 530nm filter. **Panel B.** SimplyBlue™ staining.

sequentially from the lowest to highest concentration. The flowthrough is collected for analysis. Figure 5 shows the FluoroTect™-labeled His-tagged proteins that eluted with 50mM to 1M imidazole. Titrating the MagZ™ Elution Buffer allows the best yield of His-tagged protein with minimal background using the lowest concentration of imidazole.

Scale-Up Purification

The MagZ™ Purification System can be used to purify expressed His-tagged proteins from multiple in vitro translation reactions. There are two ways to perform scale-up purifications: 1) pool more than one 50µl in vitro translation reaction; 2) sequentially add 50µl in vitro translation reactions to the same MagZ™ Particles. In the first case, the amount of MagZ™ Particles and Binding Buffer must be increased in proportion to the number of in vitro translation reactions used. For example, for purification from a 200µl in vitro translation reaction, 240µl of MagZ™ Particles and 400µl of MagZ™ Binding Buffer should be used. For sequential binding, multiple 50µl in vitro translation reactions can be added to the original MagZ™ Particles. Results of these studies are shown in Figure 3. These results show that we successfully bound the protein from four 50µl in vitro translation reactions to 60µl of MagZ™ Particles. This could be an efficient way to purify the same His-tagged protein from multiple in vitro translation reactions.

Conclusions

The data presented here show that the MagZ™ Protein Purification System is a simple and fast method to purify His-tagged proteins from rabbit reticulocyte lysate. The unique feature of the MagZ™ System is its ability to purify His-tagged protein expressed in rabbit reticulocyte lysate while eliminating 99.9% of hemoglobin contamination. The elimination of hemoglobin is very beneficial for downstream applications such as in vitro expression cloning (7), His-tag pull-down experiments (8), ribosome display (9), in vitro mutagenesis (10) and directed-evolution studies (11). The system's flexibility allows for detection of His-tagged protein by labeling with the FluoroTect™ System or [^{35}S]met, and purified proteins can be visualized by gel analysis, FluorImager® instrument or Western blot.

Acknowledgments

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Protocols

- *MagZ™ Protein Purification System Technical Bulletin* #TB336, Promega Corporation. (www.promega.com/tbs/tb336/tb336.html)
- *MagneHis™ Protein Purification System Technical Manual* #TM060, Promega Corporation. (www.promega.com/tbs/tm060/tm060.html)

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Ordering Information

Product	Size	Cat.#
MagZ™ Protein Purification System	30 purifications	V8830

(a) U.S. Pat. Nos. 5,324,637 and 5,492,817, Australian Pat. No. 660329 and other patents.

(b) The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

(c) U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

(d) Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and other patents.

(e) FluoroTect™ Green$_{Lys}$ incorporates dye conjugates made with the BODIPY®-FL fluorescent reactive dyes, which are licensed from Molecular Probes, Inc., under U.S. Pat. Nos. 4,774,339, 5,274,113 and 5,433,896 for IVE analysis for research use only including GPR and ASR applications and Fluorotag® technology and under U.S. Pat. No. 6,306,628. BODIPY is a registered trademark of Molecular Probes, Inc., and Fluorotag is a registered trademark of AmberGen, Inc.

(f) U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

(g) Certain applications of this product are covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used.

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