Rapid Detection and Quantitation of His-Tagged Proteins Purified by MagneHis™ Ni-Particles

By Laurie Engel, B.S., Sanchayita Kar, Ph.D., and Tonny Johnson, Ph.D., Promega Corporation

Abstract

In this report we describe methods for on-particle Coomassie® Brilliant Blue (CBB) and BODIPY® fluorescence dye binding assays to rapidly detect and quantitate His-tagged proteins. We used the CBB assay to monitor the expression of an induced His-tagged protein. The binding of CBB dye was proportional to the amount of protein bound to the MagneHis™ Ni-Particles. We were also able to bind BODIPY® fluorescent dye to His-tagged proteins associated with MagneHis™ Ni-Particles. Labeling with fluorescent dyes has the potential to be more sensitive than a CBB assay. Both of these assays should be amenable to high-throughput studies.

Introduction

The polyhistidine tag is one of the most commonly used affinity tags for the expression and purification of recombinant proteins. This method has become an attractive system for the purification of recombinant proteins for structural and functional studies. Various His-tagged proteins have been successfully purified from diverse expression systems including *E. coli*, yeast, mammalian cells, insect cells (reviewed in reference 1) and plants (2) for structure-function studies.

The CBB dye binding assay is an attractive method for the quantitation of His-tagged proteins that overcomes the inherent problems associated with the presence of imidazole in the elution buffer.

One of the primary prerequisites for these types of studies is the availability of rapid detection and quantitation methods for the expressed His-tagged proteins. Antibody-based approaches are commonly used for detection. Polyclonal or monoclonal antibodies directed against His-tags have been used to detect His-tagged proteins by Western blot or ELISA (3–6). Modified enzyme-NTA (nitrilotriacetic acid) conjugates (7) have also been used to detect His-tagged proteins. Protein detection by Western blot is time-consuming and is of limited use for the rapid screening of hundreds of clones at a time.

Quantitation of eluted His-tagged proteins without gel separation is normally limited due to the interference of high concentrations of imidazole present in elution buffers, which inhibit various protein quantitation assays such as Bradford or BCA assays. This problem can be partly overcome by diluting purified His-tagged proteins or by removing imidazole by dialysis. However, these methods are useful mainly for the quantitation of highly expressed proteins and may not be a good choice for high-throughput expression screening or for the optimization of protein expression systems.

Thus, it is necessary to develop simple systems for the quantitation and detection of His-tagged proteins, especially as a tool for primary screening of expressed His-tagged proteins. Such techniques would allow the rapid detection of His-tagged proteins without the need for Western blot or ELISA. These techniques could be used for rapid screening in applications such as optimization of induction conditions, random mutational studies (8), ribosome display (9) or phage display screenings (10). Interestingly, His-tagged proteins have been extensively used for these applications because of the simple purification steps involved.

In this article we describe methods we have developed using Coomassie® Brilliant Blue and fluorescent dye labeling to detect and quantitate His-tagged proteins purified by the MagneHis™ Protein Purification System[a,b] (Figure 1).

Rapid Detection of Expressed His-Tagged Protein by Coomassie® Brilliant Blue (CBB) Dye Binding Assay

We monitored the expression of an induced, His-tagged tRNA methionyl synthetase using an on-particle Coomassie® Blue dye binding assay. We induced bacterial cells expressing a His-tagged methionyl tRNA synthetase and collected cells at various time points. We isolated His-tagged protein from each time point using the MagneHis™ System and labeled the isolated protein with CBB as described in Table 1. Labeled proteins were analyzed by measuring the absorbance of light by CBB at 595nm and by SDS-PAGE analysis (Figure 2, Panels B and C). The CBB binding was proportional to the level of expression and the amount of protein bound to the Ni-Particles as shown by SDS-PAGE analysis. An advantage of this technique is that the level of protein expression can be seen without using a spectrophotometer (Figure 2, Panel A).

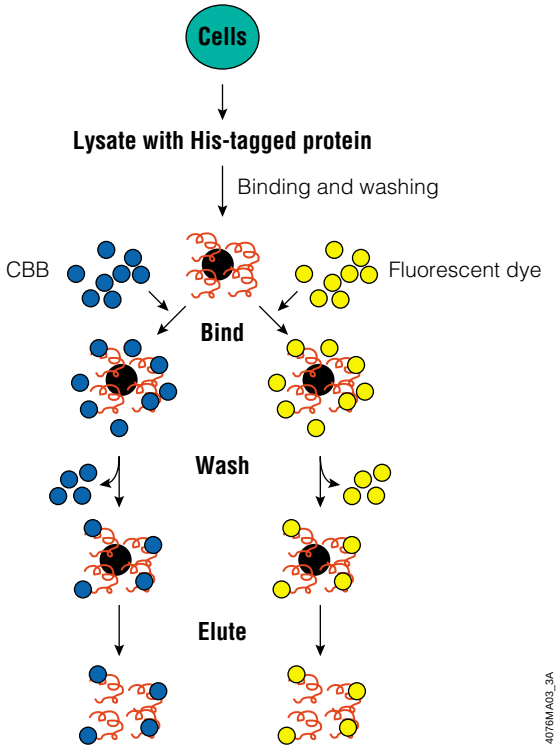

Figure 1. A general method for the Coomassie® Brilliant Blue (CBB) and fluorescence dye binding assays.

Table 1. CBB Dye Binding Assay.

1. Isolate His-tagged protein using the MagneHis™ System as described in Technical Manual #TM060. Do not elute the His-tagged protein from the MagneHis™ Ni-Particles.
2. After washing the MagneHis™ Ni-Particles, resuspend in 200µl of Coomassie® Blue dye solution containing 0.1% Coomassie® Brilliant Blue R dissolved in MagneHis™ Binding/Wash Buffer.
3. Allow the dye to bind to the protein for 3–5 minutes while mixing by pipetting.
4. Capture the MagneHis™ Ni-Particles with a magnetic stand. Remove the dye solution and wash the protein/particles with 200µl of MagneHis™ Binding/Wash Buffer for an additional 5 washes.
5. Elute proteins with 100µl of MagneHis™ Elution Buffer.

The CBB assay could be useful for the initial screening of protein expression, for optimizing the induction or media conditions, and for the selection of protein variants. This method could also be adapted for high-throughput studies in a 96-well format (data not shown). The CBB dye labeling method avoids time-consuming methods like SDS-PAGE, Western blot or ELISA for initial expression screening. Even though this approach would work with other nickel resins or particles (data not shown), it is well suited to MagneHis™ Ni-Particles, which have low levels of nonspecific protein binding.

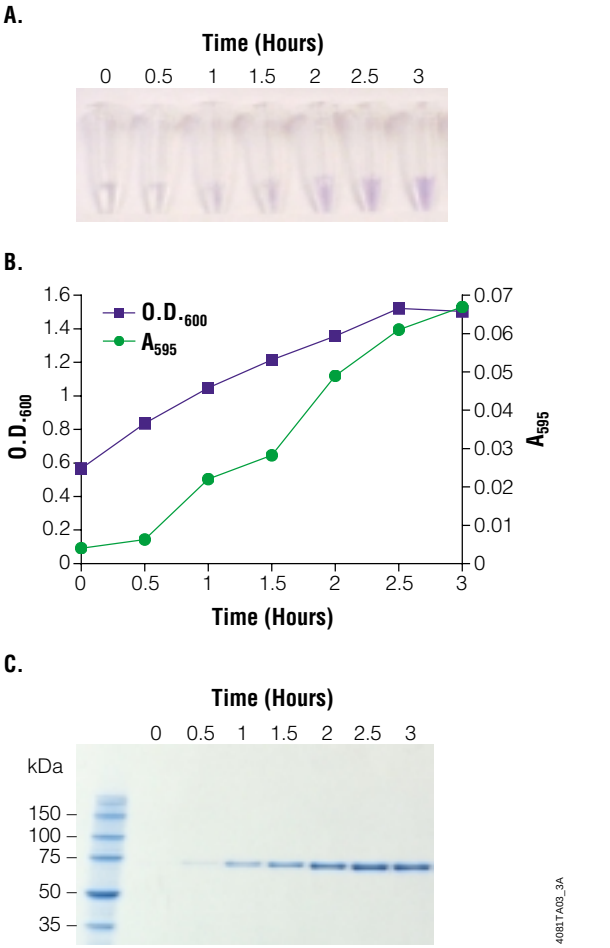

Figure 2. Rapid detection of His-tagged proteins by CBB binding assay. JM109 cells expressing the His-methionyl tRNA synthetase gene were grown to an $O.D._{600}$ of 0.5669 and induced with 1mM IPTG. Samples (1ml) and $O.D._{600}$ values were taken at half hour intervals up to 3 hours. Samples were processed as described in Table 1. **Panel A.** Eluted samples treated with CBB. **Panel B.** Absorbance at 595nm of the eluted CBB-bound proteins and the corresponding $O.D._{600}$ of the culture. **Panel C.** SDS-PAGE analysis of the purified samples.

Linearity and Sensitivity of the CBB Dye Binding Assay

We tested the linearity and sensitivity of the CBB dye binding assay by generating standard curves for two different isolated His-tagged proteins with different molecular weights. We found that the binding of dye to the purified protein is protein-dependent and quantitative. The dye binding and absorbance was proportional to the amount of protein bound to MagneHis™ Ni-Particles in tests using His-tagged ubiquitin (Figure 3A) and firefly luciferase (Figure 3B) with molecular weights of approximately 9kDa and 62kDa, respectively. We confirmed these results by SDS-PAGE analysis (data not shown). The CBB dye binding assay is an attractive method for the quantitation of His-tagged proteins that overcomes the inherent problems associated with the presence of imidazole in the elution buffer. Moreover, the CBB dye binding assay is robust and does not require complicated sample preparation.

The amount of CBB dye that binds to a native protein is dependent on the characteristics of that protein, therefore different proteins will bind different amounts of CBB dye under native conditions. To achieve accurate quantitation of a native protein, a standard curve should be made using the same protein that is being quantitated. Alternatively, one denatured His-tagged protein could be used to develop a standard curve to determine the concentration of several different denatured proteins.

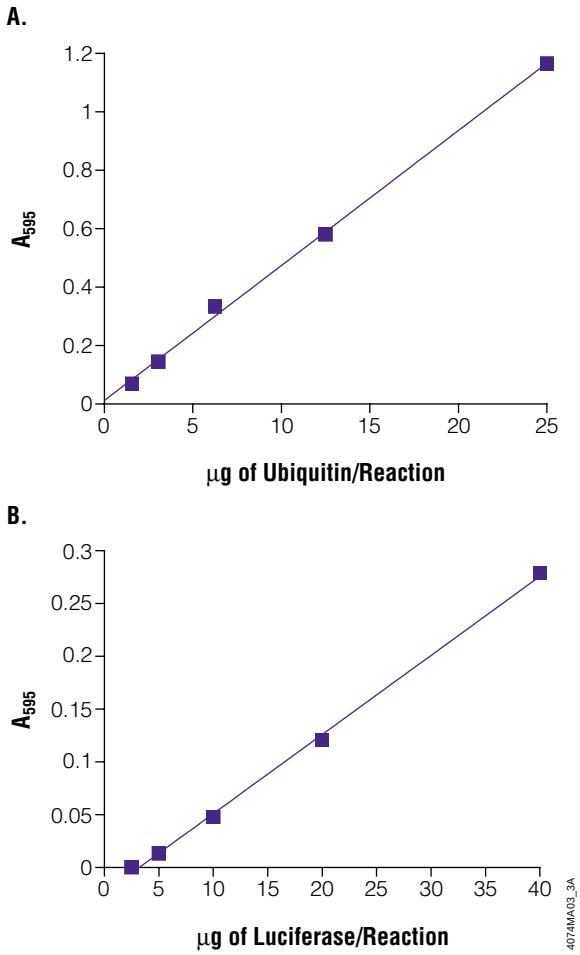

Figure 3. Quantitative CBB dye binding assay using purified His-tagged ubiquitin and firefly luciferase. The indicated amounts of purified His-tagged ubiquitin or luciferase in MagneHis™ Wash/Binding Buffer were combined with 30µl of MagneHis™ Ni-Particles. The protein/particles were treated with CBB as described in Table 1. Absorbance at 595nm of each sample was measured and subtracted for background absorbance. **Panel A.** His-tagged ubiquitin. **Panel B.** His-tagged firefly luciferase.

Rapid Detection of His-Tagged Proteins by Fluorescence Dye Labeling Assay

We have fluorescently labeled proteins attached to MagneHis™ Ni-Particles to develop an on-particle assay. We used BODIPY® dye, which is an amine-reactive dye that binds covalently to the N-terminus of a protein and the ε-amine of lysine residues. Figure 4 shows SDS-PAGE analysis of various His-tagged proteins that were labeled with BODIPY® dye while bound to MagneHis™ Ni-Particles. This method allows visualization of the protein bands without staining and destaining steps. These labeled proteins could be used for further downstream applications. For example, fluorescently labeled protein purified with the MagneHis™ System can be used as bait to study protein-protein interactions because the purified protein would be free of contaminating free fluorescent dye. Different colored dyes could be used in place of or in addition to BODIPY® dye for labeling multiple His-tagged proteins for multiplex analysis.

Figure 4. Fluorescence labeling of His-tagged proteins attached to MagneHis™ Ni-Particles. Purified His-tagged proteins were bound to 30µl of MagneHis™ Ni-Particles. The protein/particles were treated with BODIPY® dye as described in Table 2 and analyzed by SDS-PAGE followed by fluorescent imager scanning. Lane 1, His-firefly luciferase (62kDa); lane 2, His-*Renilla* luciferase (36kDa); lane 3, His-RNasin® Inhibitor[c,d] (45kDa); lane 4, His-methionyl tRNA synthetase (76kDa).

Table 2. BODIPY® Fluorescent Dye Binding Assay.

1. Isolate His-tagged protein using the MagneHis™ System as described in Technical Manual #TM060. Do not elute the His-tagged protein from the MagneHis™ Ni-Particles.
2. After washing the MagneHis™ Ni-Particles, resuspend in 100µl of BODIPY® dye solution containing 1mg/ml BODIPY® FL SE dye (Molecular Probes, Cat.# D-2184) dissolved in 50% acetonitrile with 100mM HEPES (pH 7.5).
3. Incubate for 5 minutes at room temperature.
4. Remove unbound dye by capturing the MagneHis™ Ni-Particles with a magnetic stand followed by three washes (0.5ml each) with MagneHis™ Binding/Wash Buffer.
5. Elute proteins with 100µl of MagneHis™ Elution Buffer.

Since BODIPY® dye binds specifically to unprotonated, aliphatic amine groups, it would be of limited use to create a quantitative assay. Commercially available noncovalent fluorescent dyes that bind to proteins in the same manner as CBB could be used to develop a fluorescence-based protein quantitation assay and would not require organic solvents. Since fluorescence-based methods have the potential to be more sensitive than the CBB dye binding assay, low-expressing His-tagged proteins could be detected.

Conclusions

In this article, we describe two novel methods for the rapid detection and quantitation of His-tagged proteins by CBB and BODIPY® fluorescent dye binding. Applications for these methods include: 1) primary screening for selecting high-expressing His-tagged proteins; 2) optimization of media, temperature, additives, induction parameters and promoters for cells expressing a particular His-tagged protein; 3) high-throughput screening of His-tagged proteins in mutational studies; 4) quantitation of His-tagged proteins; and 5) preparation of fluorescently labeled His-tagged proteins for downstream applications.

References

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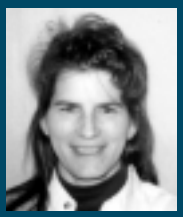
Protocols

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



Laurie Engel, B.S.
Research Scientist

Not Pictured
Sanchayita Kar, Ph.D.
Research Scientist

Not Pictured
Tonny Johnson, Ph.D.
Research Scientist

Ordering Information

Product	Size	Cat.#
MagneHis™ Protein Purification System[a,b]	2ml	V8500
	10ml	V8550
MagneHis™ Ni-Particles	2ml	V8560
	10ml	V8565

[a] Patent Pending.

[b] 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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[d] 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.