Published in final edited form as:

Anal Chem. 2012 October 2; 84(19): 8272–8276. doi:10.1021/ac301764q.

Molecular Beacon Aptamers for Direct and Universal Quantitation of Recombinant Proteins from Cell Lysates

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Abstract

Western blot, ELISA and fluorescent fusion proteins are currently the most common methods for detecting recombinant proteins. However, the former two are cumbersome and time-consuming, and the latter method may interfere with the trafficking and function of the fused recombinant proteins. We report here a rapid, inexpensive and simple approach to detect and quantify recombinant proteins using an anti-His-tag molecular beacon aptamer (HMBA). We demonstrated the technique by detection and quantitation of expressed recombinant proteins directly from *E. coli* cell lysate. The amount of expressed P78-His was determined to be 1.49 μg from the 20 μg cell lysate proteins. To the best of our knowledge, this is the first example directly measuring the concentration and expression yield of recombinant proteins from cell lysate, and the entire procedure required only 5 minutes.

Keywords

molecular beacon aptamer; His tag; cell lysates; quantification

Recombinant proteins are generated from expression of recombinant DNA within host cells. The use of recombinant proteins has expanded greatly in last several decades. A large number of antibodies, antigens, hormones and enzymes used in molecular biology, biochemistry and medicine are obtained from expression systems. For academic and industrial production of recombinant proteins, a simple and rapid detection method as an early operation unit can improve the overall process. However, current methods for detection of recombinant proteins have limitations. Western blot and ELISA are the traditional methods to detect recombinant proteins, but they are cumbersome and time-consuming. For example, because expression proteins have to be separated from other cell lysate proteins before detection, laborious procedures such as gel electrophoresis are employed in Western blot. If recombinant proteins can be directly detected from cell lysate, the detection assay could be largely simplified. Therefore an alternative method was developed by the use of genetically fused fusion partners, such as the fluorescent GFP-fusion protein¹. Because GFP is co-expressed with the target recombinant protein, the expression level of target proteins can be determined through the detection of the presence of GFP. This is a rapid and straightforward approach to detect recombinant proteins.

However, the large size of the fluorescent proteins (around 30 kD) can interfere with the trafficking and function of the proteins to which they are fused, limiting wide application of fluorescent fusion proteins to various recombinant proteins. Smaller tags have less potential to disrupt protein folding or function, and this inspired us to use a small fused tag to detect recombinant proteins directly from cell lysates.

Although many different fusion tag systems, such as chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST) and the poly histidine tag (His-tag) are available, the first choice for most scientists is the His-tag², which is an amino acid motif that usually consists of six histidine (His) residues at the N- or C-terminus of proteins. Because of its small size (~ 1 kD), His-tag rarely interferes with the function, activity, or structure of target proteins, and therefore theoretically it can be used for all recombinant proteins. Furthermore, small His-tag can easily be genetically fused to a target gene by polymerase chain reaction (PCR) techniques³. And as a mature technology, various His-tagged proteins have been successfully expressed from diverse expression systems, including *E. coli*, yeast, mammalian cells, insect cells and plant cells⁴. As a result, because each recombinant protein could be co-expressed with one His-tag, through the monitoring of the His-tag, the detection and quantitation of the target recombinant protein could be realized.

Currently, the most common methods to detect recombinant His-tagged proteins use anti-His-tag antibodies⁵⁻⁷ or probes bearing metal ions, such as Ni²⁺ or Co²⁺, which can bind to the His-tag^{2,8}. However, the use of antibodies lacks accurate quantitation, is costly, and requires extensive time to obtain results. Although they are cheaper than antibodies, methods using metal ion probes still require several hours for detection of His-tagged proteins and lack the ability to provide accurate quantitation. More importantly, neither method is feasible for direct detection of His-tagged proteins from cell lysate. To address all these problems, we developed a novel approach to quickly detect and quantify recombinant proteins directly from cell lysates. This method relying on an anti-His-tag molecular beacon aptamer is universal in scope, inexpensive, and simple enough.

Aptamers are single-stranded nucleic acids generated by SELEX (Systematic Evolution of Ligands by EXponential enrichment)^{9, 10}. Aptamers fold into well-defined three-dimensional structures that enable specific binding to a great variety of targets, such as proteins, with high affinity and specificity comparable to that of antibodies. Anti-His-tag aptamer, 6H5, was first reported in a U.S. patent, in which it was used as an affinity probe to purify His-tagged proteins¹¹. However, since aptamers are much more expensive than metal ion probes, aptamer affinity chromatography is not economically feasible for His-tagged protein purification. Nevertheless, as powerful probes, anti-His-tag aptamers can be used for His-tagged protein detection. For example, anti-His-tag aptamers could be immobilized on a solid surface to act as a DNA microarray for His-tagged protein detection¹². However, the otherwise wide application of this approach has been limited by the need to prelabel target proteins with fluorescent reporters. Our approach eliminates protein labeling by modifying aptamer into a molecular beacon aptamer (MBA).

An MBA, also called an aptamer switch probe¹³ or activatable aptamer probe¹⁴, is a newly developed molecular beacon which can specifically recognize various target molecules, such as ATP, proteins or even cells^{13, 14}. Although inspired by the earlier MBAs, the design of anti-His-tag molecular beacon aptamer (HMBA) was not easy. The simplest way to construct the MBA would be to keep the entire length of 6H5 and add a short cDNA sequence at its 3-prime end with a PEG linker to form a beacon structure. Unfortunately, that MBA cannot bind to His-tag (data not shown). As shown in Figure 1A, 6H5 has a molecular beacon-like hairpin structure with two tails on both ends. We speculated the

hairpin structure could be very important for binding between 6H5 and his-tagged proteins, and the tails might not be that important. Then we decided to cut off the five nucleotide residues (GGCTT), one-by-one, from the 5-prime end of 6H5, and these truncated aptamers were matched respectively with their corresponding short cDNA sequences and a PEG linker to form MBAs. These MBAs were tested, and one of them showed good fluorescence recovery after binding to His-tagged proteins. We named this one as HMBA. As shown in Figure 1C, the HMBA contains a major part of 6H5 with the deletion of 5 nucleotide residues from the 5-prime end, and addition of a partial cDNA sequence containing 7 nucleotide residues to the 3-prime end and PEG36 as a linker. FAM was chosen as the fluorophore, and Dabcyl was used as the quencher.

Thus, in the absence of His-tagged proteins, HMBA forms a stem-loop structure (Figure 1C), and the fluorescence signal of FAM is quenched by Dabcyl. However, in the presence of His-tagged proteins, which bind to HMBA, the stem part will be reorganized to physically separate fluorophore from quencher, allowing a fluorescent signal to be emitted upon excitation (Scheme 1). The entire mechanism for using HMBA to detect recombinant proteins from cell lysates is illustrated in Scheme 1. The recombinant protein is fused with a His-tag. After cell lysis, the HMBA can directly detect and quantify the recombinant protein from cell lysates through the interaction with the His-tag. For this HMBA assay, it should be noted that the recognition of the target protein occurs simultaneously with the optical reporting of that interaction, which is an obvious advantage for any homogeneous high-throughput assay. Neither anti-His-tag antibodies nor metal ion probes have this functionality.

In order to verify the feasibility of using the HMBA to detect recombinant His-tagged proteins, protein Rep78 with a His-tag on its N-terminus (P78-His) was tested as a model study. P78-His was expressed and purified from *E. coli* cells. As shown in Figure 2, the fluorescence of HMBA was largely increased in the presence of P78-His. In addition, HMBA was mixed with its cDNA, and only a slightly stronger fluorescence signal was observed, compared with that produced by P78-His.

To explore the specificity of the HMBA assay for recombinant His-tagged proteins, the kinetic behavior of HMBA with P78-His or P78 was studied by monitoring the fluorescence intensity change as a function of time. As shown in Figure 3, the original fluorescence signal of 25 nM HMBA in buffer was low and stable, but when 300 nM of P78-His was added to the solution, the fluorescence immediately increased to a much higher level and continued to increase slowly as a function of time. In contrast, HMBA showed almost no response to 300 nM P78. These results not only confirm that HMBA is specific to the His-tag, but also illustrate that the binding between His-tagged protein and HMBA is rapid, with nearly 90% response being reached within 5 minutes.

Furthermore, both protein Rep68 with a His-tag on its N-terminus (P68-His) and P68 were tested with HMBA. As shown in Figure 4, HMBA shows strong specificity to the His-tagged proteins. There is a distinguishable difference between the original proteins (P68 and P78) and His-tagged proteins (P68-His and P78-His). In addition, bovine serum albumin (BSA), as another control protein, was tested at two concentrations: 200 nM and 10 μ M. Even when the concentration of BSA was fifty-fold higher than that of His-tagged proteins, it was found that BSA was unable to bind and open the HMBA. Additionally, two other His-tagged recombinant proteins, GSTZ-His and Pdx1-His, were tested in this HMBA assay, and both displayed behavior very similar to that of P78-His (Fig. 4).

The HMBA assay was also tested with the His-tag itself. One short His-tag peptide, H-His-His-His-His-NH₂ (H6), opened the HMBA and gave fluorescence recovery. Although

the concentration of H6 was forty-fold higher than that of His-tagged proteins, the efficiency of H6 targeting HMBA was still lower than that of His-tagged proteins (Fig. 4). This could be attributed to the binding between HMBA and the very short peptide H6, which is probably weaker than that between HMBA and His-tagged proteins. It may be because the local environment of protein surface can provide additional support for H6 to bind the small His-tag. In addition, the presence of His-tags in all these His-tagged proteins was verified by Western blot analysis using anti-His-tag antibodies (Fig. 5). The data presented here confirm the specificity of HMBA towards His-tags and evaluate the potential of detection of recombinant proteins through monitoring the fused His-tag.

Different concentrations of P78-His (6.25–400 nM) were tested with HMBA in buffer. As shown in Figure 6, significant fluorescence emission was observed when P78-His reached 100 nM. The inset of Figure 6 shows the fluorescence increase (F/F_0) of HMBA upon addition of different concentrations of P78-His. The linearity of the plot indicates that the HMBA assay can be used to detect and quantify purified His-tagged proteins. In addition, the limit of P78-His detection, based on three times the signal-to-noise level, was estimated to be about 4.2 nM.

To test the potential of this method for direct detection of expressed recombinant proteins from cell lysate, different amounts of P78-His, from 0 to 2 μg , were mixed with an indicated amount of *E. coli* cell lysate so that the total amount of protein in each sample was 20 μg . As shown in Figure 7, the proportional relationship between fluorescence increase ($F/F_0 - 1$) of HMBA and concentration of P78-His in *E. coli* lysate was verified and used as the standard curve. Then, the plasmid containing P78-His gene was transformed into *E. coli*, and IPTG was used to induce the expression of P78-His. After culture, the *E. coli* cells were collected, and cell lysate was prepared. Western blot data confirm the presence of P78-His in the cell lysate from the IPTG treated group, as well as the absence of P78-His in the cell lysate from the group without IPTG treatment (Fig. 5B). Cell lysate (20 μg) from P78-His expressing *E. coli* with IPTG treatment (F) or without IPTG treatment (F_0) was tested respectively by HMBA, and the fluorescence recovery was measured. The experiments were repeated three times and the average value of fluorescence increase ($F/F_0 - 1$) was determined to be 0.441. According to the standard curve in Figure 7, the yield of expressed P78-His in *E. coli* lysate proteins was determined to be 7.45%. Then, we calculated that the amount of expressed P78-His was 1.49 μg from the 20 μg cell lysate proteins. To the best of our knowledge, this is the first example directly measuring the concentration and expression yield of recombinant proteins from cell lysate, and the entire procedure required only 5 minutes.

In summary, this method utilized HMBA to target His-tag to perform detection and quantitation of recombinant proteins. Our design offers several advantages. First, because the HMBA assay can directly detect expressed recombinant proteins from cell lysate, the entire procedure is much more rapid (only about five minutes) than the traditional antibody-based affinity sensing strategies requiring multiple reagents, incubations and washing steps. Second, this is a very simple and low cost method. The HMBA itself is sensitive enough to detect the recombinant proteins from cell lysate. It does not require additional modifications, such as fixing the MBAs on the detection surface¹⁵. Moreover, as a simple method, it is only necessary to add protein samples to a solution containing HMBA and then measure the fluorescence recovery to detect recombinant proteins. This kind of turn-on of fluorescence upon recognition of oligohistidine was also reported by others using chemical probes,¹⁶ and we extended our design for direct detection of recombinant protein in cell lysates. Finally and more importantly, even though a few protein-specific aptamers have been selected and some of them were developed into associated MBAs for protein detection^{15, 17}, HMBA is superior to other MBAs because it can target various different recombinant proteins, as long

as they contain His-tags, while other MBAs can only be used to target one indicated protein. Therefore, this method could become a universal approach for the quantitative detection of expressed recombinant proteins. Consequently, because our method is a rapid, low cost, universal and simple approach, different labs from different research areas such as molecular biology, biochemistry and medicine can easily use this design for the detection of various recombinant proteins. Our method holds great promise to become a routine tool for practical applications in recombinant protein detection and will be very useful in the wide range of research areas of recombinant proteins.

Experimental

1. DNA sequences

6H5 aptamer: 5'-GGCTTCAGGTTGGTCTGGTTGGGTTTGGCTCCTGT GTACG-3'
HMBA: 5'-FAM-CAGGTTGGTCTGGTTGGGTTTGGCTCCTGTGTACG -
(CH₂CH₂O)₃₆-CAACCTG-Dabcyl-3'

The underlined part is a major fragment of 6H5 aptamer, which recognizes His-tag, and the bold parts form the HMBA stem.

2. Fluorescence measurements

All fluorescence measurements were performed using a Fluorolog spectrophotometer (Jobin Yvon Horiba). The HMBA was prepared in PBS 1X without Ca and Mg (Dulbeco's). The fluorescence spectra for all samples were measured at 20° C.

3. His-tag protein expression and purification

The human *Pdx1* gene was cloned into pET28b vector. The full-length human GSTZ cDNA was cloned into pQE30 vector. The Rep68 or Rep78 of AAV2 was subcloned into pET-15b. All proteins contained N-terminal His-tags. Soluble protein expression was obtained in *E. coli* strain BL21 (DE3) by growth at 37°C overnight, then 30°C for 3 h, followed by induction of IPTG with final concentration of 1 mM at 18°C for 24 h. Harvested cells were treated by three freeze/thaw cycles in 20 mM Tris, pH7.5, 0.5 M NaCl, 15 % glycerol and 10 mM imidazole buffer, followed by Benzonase Nuclease (Novagen) treatment. Soluble proteins were directly loaded onto a prepacked HisTrap column (GE) and eluted from the column using a gradient of imidazole from 10 mM to 400 mM. Fractions containing His-tagged proteins were combined and dialyzed against 20 mM Tris (pH7.5), 1 mM EDTA, 0.5M NaCl and 10% glycerol. Protein concentrations were measured by the Bradford method. Proteins of Rep 68 and Rep78 without His-tag were kindly provided by Dr. Nick Muzyczka, University of Florida.

4. Cell lysate preparation

Harvested *E. coli* cells were treated by repeated freeze/thaw cycles in 20 mM Tris, pH7.5, 0.5 M NaCl and 15 % glycerol. Samples were centrifuged at 14000g for 20 min to remove the cell debris. The cell lysate was then incubated with Benzonase Nuclease for 30 minutes at RT. Five mM EDTA was added to cell lysate, and, finally, the total protein concentration was measured by the Bradford method.

5. Western blot

The purified His-tagged proteins or cell lysate was loaded into an SDS-PAGE system. After electrophoresis and membrane transfer, the membrane was blocked, washed and incubated with the anti-His-tag antibodies (1:2000 dilution). Secondary antibodies (1:2000 dilution) were used to visualize targeted proteins.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (20975034) and the National Key Scientific Program of China (2011CB911001, 2011CB911003). This work was also supported by grants awarded by the National Institutes of Health (GM066137, GM079359 and CA133086).

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Figure 1.
The secondary structures of (A) 6H5, (B) truncated 6H5 and (C) HMBA.

Figure 2. Fluorescence emission spectra under different conditions. From bottom to top: 800 nM P68-His only (green line); 25 nM HMBA (black line); 25 nM HMBA incubated with 800 nM P78-His (blue line); 25 nM HMBA incubated with its cDNA at a concentration of 125 nM (red line). Excitation: 480 nm. Emission: 518 nm. The incubation time was 30 minutes.

Figure 3. Fluorescence restoration of HMBA by P78-His or P78 as a function of time. HMBA (25nM) was incubated in buffer for six minutes, and then proteins were added. Excitation: 480 nm. Emission: 518 nm.

Figure 4.

Fluorescence intensity changes ($F/F_0 - 1$) of the HMBA (25nM) toward different proteins. F_0 and F are fluorescence intensities at 520 nm in the absence or presence of detected proteins, respectively. Excitation: 480 nm, emission: 518 nm. Concentrations of all proteins were 200 nM, except BSA*, whose concentration was 10 μ M. The concentration of peptide H6 was 8 μ M.

Figure 5. Western blot analysis by anti-His-tag antibodies A) Analysis of purified His-tagged proteins; B) Analysis of *E. coli* lysate: 1) lysate from cells transformed by Rep78-His and induced by IPTG; 2) lysate from cells transformed by Rep78-His without treatment with IPTG.

Figure 6.

Fluorescence emission spectra of HMBA (25 nM) A) with addition of different concentrations of P78-His. From bottom to top: 0, 6.25, 13, 25, 50, 100, 200, and 400 nM. B) linear relationship between F/F_0 and P78-His concentration. Data were obtained from the average of three parallel experiments.

Figure 7.

Direct quantification of recombinant protein from cell lysate. HMBA (25nM) was incubated with 20 μ g protein mixtures (purified P78-His plus *E. coli* lysate), in which the amount of P78-His varied from 1.25% to 10%. The proportional relationship between fluorescence increase of HMBA and concentration of P78-His in *E. coli* lysate was used as the standard curve. After that, fluorescence emission spectra of HMBA were measured with addition of 20 μ g cell lysate of *E. coli* expressing P78-His (F) or 20 μ g cell lysate of *E. coli* which did not express P78-His (F_0). This F/F_0-1 value (red spot) caused by P78-His expression was measured to be 0.441, corresponding to 7.45% of expression yield. Data were obtained from the average of three parallel experiments.

Scheme 1.

HMBA used to detect and quantitate expressed recombinant proteins directly in cell lysate.