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Competitive, immunometric assay for fusion protein quantification: Protein production prioritization

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ABSTRACT

Effective drug discovery demands the availability of microgram to gram quantities of high-quality protein encoded by novel transcripts. Protein expression vectors designed for large-scale protein production often include one or more specific tags to such transcripts, to simplify the purification of the targeted protein. Optimization of the complex expression and purification process requires the evaluation of multiple expression candidate clones to identify a production-suitable construct in terms of quality and final protein yield. Efficiency of the entire expression screening process is typically assessed by direct visualization of the banding patterns from whole-cell lysates on SDS–PAGE gels, by direct staining and/or immunoblotting, using antibodies against the tag or the protein of interest. These techniques, generally run under denaturing conditions, have proven to be only marginally predictive of the purification yield and authentic folding for native proteins. Small-scale, multiparallel affinity purification followed by SDS–PAGE analysis is more predictive for expression screening; however, this approach is labor intensive and time consuming. Here we describe the development of an alternative expression efficiency assessment technique, designed to evaluate the accessibility of affinity tags expressed with the desired fusion proteins, using acoustic membrane microparticle assay technology on the ViBE protein analysis workstation.

Taking full advantage of high-throughput genomic studies, which have elucidated novel sequences related to disease states or to cellular responses to proposed activity modulators, demands increased throughput in the expression and purification of the proteins encoded by such transcripts. A common approach to fulfilling this need has been the generation of chimeric fusion proteins using specific expression vectors [1–4]. The design of such expression vectors has evolved to increase target protein solubility [5], to promote appropriate target protein folding [6,7], and to simplify the target protein purification processes [2,4]. Unfortunately, the selection of appropriate clones to advance for larger scale production, typically made on the basis of relative band strength on stained SDS–PAGE gels and/or Western immunoblots, has proved to be problematic. Neither gels nor immunoblots are representative of the native state of the desired protein while in solution, nor do they provide quantitative results [8]. Consequently, multiple constructs must be tested in parallel to ensure the generation of the desired protein, generally incurring significant expense and unanticipated delays in downstream discovery research and process development.

One of the most widely used fusion sequences is the polyhistidine affinity tag, which encodes a series of four or more histidine residues within the targeted sequence or on its N- or C-terminus. Upon expression, affinity purification of the polyhistidine structure, typically using chelated metal (Ni^{2+}, Co^{2+}, or Zn^{2+}) affinity chromatography, permits copurification of the attached, targeted protein. However, occlusion, constriction, or other obstruction of the histidine-rich portion of the expressed fusion protein hinders its binding to the affinity matrix, thus compromising the efficiency of the purification [9].

Here we describe the development of a novel technique to provide a rank order of prospective expression clones for purification, using histidine-enriched sequences as an example, on a variety of fusion proteins derived from *Escherichia coli* and insect cell expression systems. This method measures the presence and accessibility of polyhistidine-enriched fusion proteins contained in lysates prepared from such protein production candidate lines, by their ability to competitively inhibit the binding of a tracer

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molecule (fluorescein-labeled monoclonal anti-pentahistidine antibodies) to paramagnetic bead-immobilized protein with an accessible polyhistidine tag (Fig. 1). Acoustic membrane microparticle (AMMP) assay technology combines the specificity of immunological antigen capture techniques with the sensitivity of microelectromechanical systems (MEMS) sensors, using magnetic microparticles to create novel, nonoptical, detection procedures.

For this proof-of-concept study, we used 12 target genes encoding proteins ranging in size from 27 through 235 kDa. Six of these proteins were expressed in *E. coli* and six were expressed in insect (Sf9) cells using a baculovirus expression system. AMMP assays were performed using crude lysates and the resulting data were compared with the actual purification yields following Ni–NTA column purification.

Materials and methods

Principles of the AMMP assay

The AMMP assay has been described previously [10]. In this particular instance, the technique measures the ability of an unknown fusion protein, putatively containing an N-terminal, C-terminal, or internal sequence of 4–10 histidine residues, to competitively inhibit the binding of fluorescein-labeled anti-pentahistidine antibodies (anti-5His) to a similarly expressed fusion protein known to possess an accessible C-terminal hexahistidine sequence, which has been immobilized onto the surface of paramagnetic beads. Signal is generated in the system when the fluorescein-labeled tracer antibody "bridges" the hexahistidine-coupled beads to the anti-fluorescein-coupled sensor surface—the vibrational frequency of the sensor membrane decreases in direct proportion to the number of beads bound to the sensor surface (Fig. 1). Nonspecifically bound materials are washed from the sensor surface using a flow of running buffer (0.02 M phosphate buffer, containing 0.15 M NaCl and 1% (v/v) Tween 20). Between measurements, the sensor surface is regenerated using a similar flow of BioScale regeneration solution (P/N: 75018-0005), followed by running buffer to prepare for analysis of the next series of samples.

Bead preparation

Solutions containing 10, 20, and 40 μg (0.5, 1.0, and 2.0 nmol, respectively) of a 20-kDa recombinant protein including a C-terminal hexahistidine sequence (His target) are coupled to 1-mg aliquots (33 μl) of paramagnetic microbeads according to the manufacturer's recommendations. The resulting mixtures are incubated with continual end-over-end mixing, for 18 h at 25 °C, then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, and resuspended to a final volume of 33 μl in PBS containing 1% bovine serum albumin, for storage at 4 °C prior to use. Similar microbead preparations are made using 20 μg bovine serum albumin (BAH65; Equitech-Bio) per milligram of beads for use as the "irrelevant bead" control. These reagents are available from BioScale (P/N: 75081-0003).

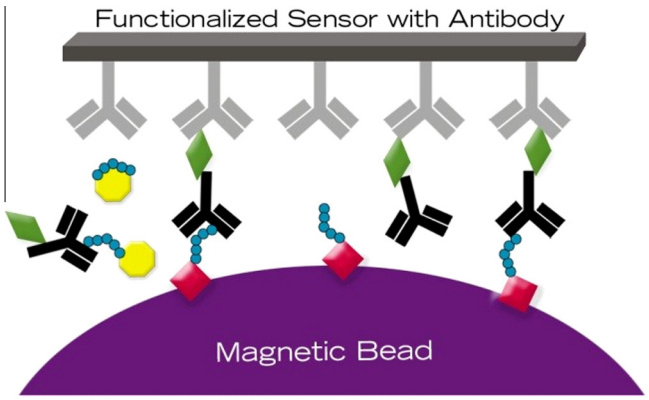

Fig.1. Schematic representation of AMMP competitive immunometric assay. Red squares represent His-tagged standard "target" protein; blue circles represent multi-histidine tag; yellow octagons represent unknown, His-tagged protein; green diamonds represent fluorescein label on (black) anti-histidine "tracer" antibody. Immobilized (gray) antibody on sensor surface represents anti-fluorescein.

Tracer preparation

An anti-pentahistidine antibody (34440; Qiagen) was conjugated with fluorescein according to the manufacturer's recommendations (50541; BioScale). The resulting tracer antibody preparation was then stored in 0.02 M phosphate-buffered saline containing 0.05% bovine serum albumin at 4 °C for later use. "Irrelevant antibody" control samples were prepared as above, using rat anti-human IL-6 monoclonal antibody (75062-0003; BioScale).

Standard preparation

Standards (500 nM) were prepared by the addition of 100 μg of the His target fusion protein (200 μl × 500 μg/ml) to 9.8 ml *E. coli* or Sf9 "null" lysate preparations. Resulting standard preparations were stored at −80 °C as 1-ml aliquots for later use.

AMMP assay protocol

For analysis, all samples and standards were serially diluted (threefold, eight places) into sample dilution buffer (20 mM sodium phosphate buffer containing 450 mM NaCl and 0.05% Tween 20). Three columns of a standard 96-well microplate were used for each dilution series—80 μl sample was dispensed in duplicate to columns 2 and 3 while column 1 contained only diluent. All three columns received His target protein-coated beads (1.5×10^5 beads/well). The ViBE workstation was programmed to deliver additional reagents at time-controlled intervals to ensure appropriate incubation periods. After 30 min of shaking preincubation, the ViBE workstation was programmed to deliver tracer antibody to each column in the sample set at regular intervals, thus permitting 60 min incubation for each well. Following the incubation, aliquots from each sample column (8 wells/column) were injected for analysis. Additionally, irrelevant bead (20 μl immobilized bovine serum albumin (BSA)–beads, 1.5×10^5/well) and irrelevant tracer (20 μl fluorescein-labeled rat anti-IL-6 monoclonal antibody, 200 ng/ml) controls were included periodically to ensure assay performance and assess freedom from host-cell-attributable nonspecific assay interference.

Cell culture and protein expression

A total of 12 cultures of *E. coli* or Sf9 cells, each encoded with a different human hexahistidine affinity tag fusion protein, were used in this study (Table 1). Six *E. coli* proteins (P1–P6) were cultured in 150 ml cultured TB medium at 37 °C and induced at 18 °C with 0.25 mM isopropyl-β-D-1-thiogalactopyranoside for 16 h. Exponentially growing Sf9 cells were cultured in 150 ml culture vessels, at a density of 1.2×10^8 cells/ml. After 24 h incubation at 27 °C, the cells were infected (1:50, v/v) with six different viral constructs. Infected cells were incubated for 72 h at 27 °C, shaking at 100 rpm. Cell viabilities and diameters were measured with a Vi-CELL XR analyzer (trypan blue exclusion method; Beckman