Development of AMMP® Assay to Determine Presence and Accessibility of His-tag for Candidate Fusion Proteins

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Abstract

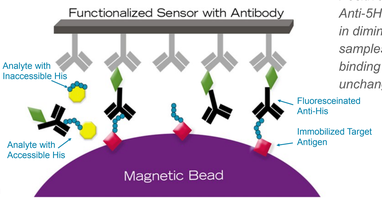
Optimization of conditions for protein expression and purification is a complex process requiring analysis of a large number of expression clones to identify a suitable construct for scale-up. Protein expression laboratories have experienced increased demand for more rapid delivery of characterized proteins. In response to these increased demands, many laboratories have focused upon either *Escherichia coli* or baculovirus-mediated insect cell expression of complex vectors containing not only selective elements, but also chimeric sequences such as hexahistidine or glutathione-S-transferase coding regions, to simplify the purification of the resulting fusion proteins. The *hexahistidine* (HisTag) sequence is particularly popular in most expression laboratories. It can be inserted internally or fused to either the N- or C-terminal of the targeted protein, and is sufficiently small (0.8 kDa) that the sequence rarely requires cleavage.

Here results are presented from a collaboration between a major pharmaceutical company and BioScale to develop and implement a high throughput method for assessing purified yield of target proteins from crude lysate. Results demonstrate that the AMMP assay can reduce dependence on: electrophoretic procedures which are typically unpredictive; Western blots also poorly predictive and extremely labor intensive; and "pilot - purification" which is time-consuming, labor-intensive and costly. These date show that AMMP assays rapidly and efficiently evaluate the status of the affinity tag both qualitatively and quantitatively providing "decision-making" criteria in hours instead of days.

Instrumentation and AMMP Assay

ViBE® Workstation

Competitive Immunometric Assay Format



Positive samples inhibit binding of Anti-5His antibody to bead resulting in diminished signal; Negative samples do not alter Anti-5His binding to bead, leaving signal unchanged.

Materials and Methods

AMMP® Assay Materials

Bead and Standard Preparation

Magnetic Microparticle beads are prepared per BioScale His-tag kit protocol. The LCN-1 protein standard is prepared according to the BioScale His-tag kit protocol.

His-tag Kit Tracer Preparation

The His-tag kit tracer solution is prepared according to BioScale His-tag kit protocol. The final preparation is stored in 0.02 M phosphate buffered saline containing 0.05% bovine serum albumin at 4°C for later use.

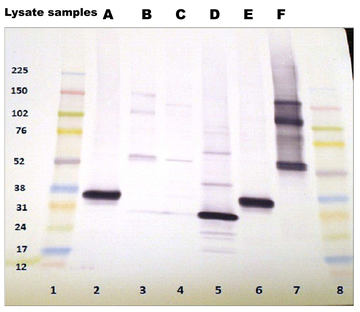
Sample Preparation

Lysates were prepared from six distinct E. coli and Sf9 transfected cell lines, each encoding different protein products with N- or C-terminal hexahistidine residues. 'Null' lysates (not encoding the hexahistidine residues) were provided for both E.coli and Sf9 cells, to control for unwanted specificities. For analysis, 15µg/ml aliquots of each lysate and an aliquot of the LCN-1 protein *standard* spiked into 'null lysate' (500 nM) are serially diluted into PBST (3-fold, through 8 places). Eighty µL samples from each dilution series are dispensed into each of five columns (1-5) in a standard 96-well microplate; column 6 contains 80µl PBST-only. Twenty µL bead suspension (1.5e5 beads/well) is dispensed to columns 2-6 (20 µL *irrelevant* bead suspension added to column 1), and 20µL *tracer* (20ng/mL) is dispensed to each well in columns 3-6, thus making Column 1='Irrelevant Bead Control', Column 2='NSB Control', Columns 2-3='TEST', and Column 4='MAX Signal'. This matrix is repeated for each of the 12 samples. The resulting plates are incubated, shaking, on the ViBE Workstation, and the instrument is started.

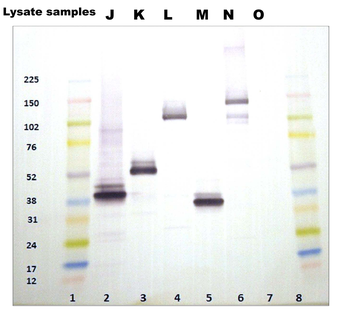
Results

Western Blot Analysis Compared to AMMP Protein Analysis

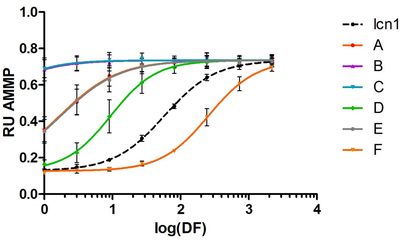
E. Coli Western Blot



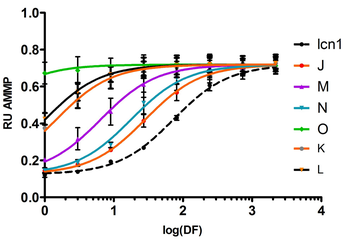
Sf9 Western Blot



E. Coli AMMP Assay



Sf9 AMMP Assay



Western Immunoblots

All samples were fully reduced, then normalized to protein concentrations of about 15 micrograms per lane for SDS-PAGE. After transfer, the resulting blots were stained with murine anti-5HIS antibody conjugated to alkaline phosphatase, then developed with BCIP.

Visual inspection reveals the presence of his-tagged proteins as strong banding patterns in E. coli lysate samples A [lane 2], D [lane 5], E [lane 6], and F [lane 7], with no appreciable signal observed for samples B [lane 3] or C [lane 4]. Similar patterns were observed for the Sf9 lysate samples: J [lane2], K [lane 3], L [lane 4], M [lane5], and N [lane6] showing strong banding, while no such banding is observed in sample O [lane 7], thus demonstrating the difficulty in assigning rank order of his-tag expression to samples solely on the basis of Western blot band strength. The same was true for SDS PAGE electropheragrams (data not shown).

AMMP Results

In these studies, the presence of accessible his-tagged proteins is visualized as inhibition of the signal normally generated by the binding of anti-5His antibody to bead-immobilized, his-tagged LCN1 protein. Soluble (non-immobilized) his-tagged LCN1 is an effective inhibitor of this reaction (Ki ≈ 7.7 nM), and is used a 'standard' to quantify the resulting inhibitory effects of the lysates in the assay. The dilutional ratio obtained between sample and standard ID_{50} values (ID_{50}Sample/ID_{50}Standard) is a valid measurement of sample potency relative to that of the standard.

E. coli

The ID_{50} ratios for these samples are reflected in the table below, and shows samples A and E with ratios of 0.031 and 0.032 respectively contain his-tagged proteins at concentrations of 15-16 nM (both ranked number 3), and sample E with a ratio of 0.032 equates to a total his-tagged protein concentration of 16 nM. Samples D (ratio = 0.172) and F (ratio 4.47) contain concentrations of about 86nM and greater than 2200nM, respectively, making these cultures viable candidates for large scale protein production. In contrast, samples B and C, with ID_{50} ratios below 0.002, contain concentrations lower than 1 nM.

Insect

Here, samples J, N, and M are clearly more productive in terms of accessible HisTag than the remaining samples, with ID_{50} ratios of 0.395, 0.253, and 0.096, indicating accessible fusion protein concentrations of 197, 126, and 48 nM, respectively. Samples K (ratio= 0.020) and L (ratio=0.014) are less desirable in terms of accessible HisTag with apparent concentrations of about 10 and 7 nM. Finally, sample O, with an ID_{50} ratio of about 0.002, suggests an accessibly tagged protein concentration less than 1 nM.

AMMP Assay Summary Analysis and Sample Ranking

The summary of the results shows the differences in molecular weights of the proteins which vary from 31kD to 235kD does not impact the assay. Also shown is the position of the His-tag which does not appear to impact the accessibility in the assay nor does the total protein concentration of each preparation which ranges for 8 -26 mg/mL for both E.coli and Sf9 lysates. The AMMP assay ranking is consistent with visual inspection for both models and is also consistent with measurements made with samples normalized for total protein content. The collaborating pharmaceutical company purified the same samples by Ni-NTA affinity chromatography. Actual rank and is shown in row 10. All actual assessments agree with the AMMP assay results.

	E. coli Lysates						Sf9 Lysates					
ID	**A**	**B**	**C**	**D**	**E**	**F**	**J**	**K**	**L**	**M**	**N**	**O**
Molecular Weight	35,600	27,400	50,100	31,700	35,600	59,300	37,100	48,600	96,800	35,600	121,100	235,000
His Orientation	C'	N'	N'	C'	N'	N'	N'	C'	C'	N'	C'	N'
Wet Weight (g) 10^6 **cells**	0.9	0.9	0.8	0.4	1.0	0.9	191	124	97	105	122	99
Protein (mg/mL)	10.06	8.14	11.96	9.42	10.06	25.76	12.30	14.60	11.20	18.30	10.50	10.60
ID_{50} Mean	1.695	0.104	0.094	9.306	1.725	241.767	27.497	1.409	0.969	6.679	17.583	0.109
ID_{50} Ratio	0.031	0.002	0.002	0.172	0.032	4.472	0.395	0.020	0/014	0.096	0.253	0.002
nM His	15.673	0.962	0.865	86.069	15.950	2235.958	197.732	10.132	6.965	48.030	126.444	0.784
AMMP Rank	3	5	6	2	3	1	1	4	5	3	2	6
Purified Rank	3	5	6	2	3	1	1	4	5	3	2	6

Summary and Conclusions

This work established and verified a method determining the presence and accessibility of small, common peptide fusion sequence on a variety of *candidate* fusion proteins using a single, commercially available antibody. The study done to detect, measure and rank His-tagged proteins from E.*coli* and insect (Sf9) cell lysates utilizing an AMMP Competition Assay configuration demonstrated that AMMP is a robust assay platform for screening constructs for expression and for their purified protein yields. In addition, the data was obtained rapidly (96 data points in <2 hours) as compared to > 1 day using currently employed assessment technology. These results provide critical "decision-making" criteria in hours instead of weeks for continued investigation and/or bulk purification by Ni-NTA affinity chromatographic procedures for the *candidate* fusion proteins

Key summary points

- The results were independent of protein molecular weight, the His orientation (N' or C') or the total protein concentration.
- Rank Order determined by AMMP assay correlated with the rank order by purification.
- AMMP assay is suitable for comparing proteins of various sizes. The molecular weight of the protein does not contribute to the signal.
- Minimal manual intervention is required once the lysates are made.
- Further assay development is not required for different proteins if the same tag is detected.
- Rapid results provide critical decision making criteria in hours
- Incorporation of a standard His-tagged protein into the assay permits the return of quantitative rather than qualitative results.
- AMMP assays are repeatable, reproducible, require minimal amounts of sample lysate and are significantly less time-consuming and labor-intensive than other alternative assays.

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