Journal of Immunological Methods 473 (2019) 112640

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

An iBody-based lateral flow assay for semi-quantitative determination of His-tagged protein concentration

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ARTICLE INFO

Keywords:
Lateral flow assay
His-tag
Antibody mimetics
Protein quantitation

ABSTRACT

The polyhistidine tag (His-tag) is one of the most commonly used epitope tags in protein engineering. While His-tagged proteins can be detected reliably using immunological methods such as ELISA and Western blot, these methods are costly and time-intensive, necessitating more facile solutions for preliminary qualitative determination and concentration estimation.

To this end, we present a rapid test strip assay based on iBody antibody mimetics that target the His-tag. We compare this strategy to commercial antibody-based assays and discuss the advantages and caveats of lateral flow assay design. Our test strip detected a panel of His-tagged proteins with different tag attachment strategies with a visual detection limit of 1 μM and densitometric detection limit of 0.5 μM. Due to its chemical nature, the presented assay exhibits wide reagent compatibility in comparison to antibody-based assays.

1. Introduction

Since its introduction in 1988 (Hochuli et al., 1988), the polyhistidine tag (His-tag) has become one of the most commonly used epitope tags for production and purification of recombinant proteins. His-tag commonly consists of six to ten consecutive histidine residues, which can be fused to accessible regions of proteins to facilitate their purification and detection. Polyhistidine peptides bind bivalent metal ions, and the most commonly used for protein purification are Ni^{2+} and Co^{2+} immobilized with chelation agents such as nitrilotriacetic acid (NTA, Hochuli et al., 1987) and its multivalent derivatives (Lata et al., 2005). In our previous work, we demonstrated that an iBody polymer conjugated with TrisNTA-targeting ligands can be used to detect His-tagged proteins in complex biological matrices (Sacha et al., 2016).

Due to the common use of this tag in recombinant protein expression, various techniques have been employed for detection and quantitation of His-tagged proteins. In addition to accurate but time- and equipment-intensive methods such as ELISA and Western blot, there is a need for rapid methods for preliminary identification and relative quantitation of His-tagged proteins. To address this need, we present a rapid lateral flow assay for detection of His-tagged proteins based on polymer antibody-mimetics.

2. Materials and methods

2.1. Reagents

Polyvinyl alcohol (PVA, 8148940101) was purchased from Merck Millipore (Burlington, MA, USA). Tween-20 (20605) and PEG-8000 (19966) were purchased from Affymetrix (Santa Clara, CA, USA). The other reagents used in this work were purchased from Sigma (St. Louis, MO, USA). The proteins used for assay validation are listed in Table 1.

Gold nanoparticle stock solution was kindly supplied by Jitka Neburková. Colloidal gold was prepared according to the procedure described by (Frens, 1973). Briefly, 0.01% gold chloride was brought to a boil and trisodium citrate solution was added to 0.05% concentration, followed by a 5-min incubation with rapid stirring. This produced a brilliant red colloidal suspension of nanoparticles with an average diameter of 21 nm verified by dynamic light scattering on a Zetasizer Nano ZSP (Malvern Instruments, Malvern, UK).

2.2. Anti-His-tag iBody

A polymer conjugate (iBody) based on a N-(2-hydroxypropyl)methacrylamide copolymer scaffold targeting His-tag was prepared

Abbreviations: ELISA, enzyme-linked immunosorbent assay; NTA, nitrilotriacetic acid; PVA, polyvinyl alcohol; PEG, polyethylene glycol; MWCO, molecular weight cut-off; IMAC, immobilized metal affinity chromatography; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay (buffer); MES, 2-(N-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethylsulfoxide; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl) phosphine; β-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; FBS, fetal bovine serum; PVC, polyvinyl chloride

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https://doi.org/10.1016/j.jim.2019.112640
Received 27 May 2019; Received in revised form 24 June 2019; Accepted 6 August 2019
Available online 07 August 2019

Table 1
Proteins used in this work and their basic properties. Protein sequences and additional information are available in Supplementary Material.

	M_W [kDa]	Tag
Protein 1	34.4	C-terminal His_6
Protein 2	28.4	N-terminal His_6
Protein 3	46.1	N-terminal His_6
Protein 4	12.1	N-terminal His_6

according to our previously published protocol (Sacha et al., 2016). The iBody, of average molecular weight 73,800 Da (PdI 0.165), carries an average of 3.3 Tris-NTA ligands and 9.8 biotin anchors per polymer molecule.

2.3. Preparation of iBody-gold conjugate

The gold-polymer conjugation protocol was devised according to conventional protein-gold conjugation protocols via physisorption (Hermanson, 1996). Gold colloid was adjusted to pH 8.6 with 100 mM sodium carbonate. A 100 µl aliquot of 500 µg/ml aqueous polymer solution was incubated under gentle stirring with 10 ml of colloidal gold solution for 30 min at room temperature. Afterwards, 1.2 ml of 10% PEG-8000 was added, followed by another 5-min incubation. The conjugate was concentrated by ultrafiltration on 30 kDa MWCO centrifugal filters (Amicon, Merck Millipore, UFC903096) for 10 min at 4000 × g. The filtrate was diluted with 500 µl 40% sucrose, 0.4% sodium azide, 200 µl 10% PEG-8000, and 100 µl 20% Tween-20, and adjusted to a final volume of 2 ml to achieve a final composition of 10% sucrose, 0.1% sodium azide, 1% PEG-8000, and 2% Tween-20 in 100 mM carbonate buffer, pH 8.6.

2.4. Immobilization of capture reagents

Neutravidin (Thermo Scientific, 31,000) and His-tagged Protein 1 were transferred into 100 mM borate buffer, pH 8.5, at concentrations of 10 and 1 mg/ml, respectively. Solutions were dispensed using a Labcyte Echo 550 instrument onto Whatman AE98 Membrane (6 pieces, 25 × 100 mm, GE Healthcare, 15,843,704) in 2 × 2.5 nl dots with a density of 5 dots/mm for the formation of both test and control lines. Lines were formed 11 mm from the edge of the membrane with 3 mm between lines. Once the line dispensing was complete, the membranes were left to dry overnight at ambient temperature and humidity.

2.5. Preparation of conjugate, sample and wicking pad

A glass fiber pad (G041, Merck Millipore, GFCP0008) was cut to 8 × 600 mm, pre-treated by soaking in 0.1% PVA and 0.1% Tween-20, and dried overnight at 54 °C in a forced air convection oven on a non-absorbent support (aluminum foil). The treated pad was soaked in conjugate concentrate and left to dry at room temperature for 30 min and subsequently overnight in a desiccator.

A cellulose fiber sample pad (C083, Merck Millipore, CFSP002000) was cut to 20 × 600 mm and soaked with 1% PEG-8000, 2% Tween-20, and 0.1% sodium azide in 100 mM phosphate buffer, pH 7.4, followed by drying in a forced air convection oven as described above.

Whatman filter paper Grade 470 (GE Healthcare, WHA10539028) was cut to 38 × 600 mm and used as a wicking pad with no further modifications.

2.6. Test assembly

Prepared sheets of material were pasted onto a PVC adhesive backing plate (d-c-fix, OBI, 5200647) as shown in Fig. 1 in the following order: (1) nitrocellulose membrane, (2) conjugate pad with 2 mm overlap with the membrane facing the test line, (3) sample pad with 4 mm overlap with the conjugate pad, and (4) wicking pad with 5 mm overlap with the membrane. The wicking pad was covered with printable adhesive foil of identical width. This master card was then cut into 3 mm strips using a Robotsung YS-100 W automatic cutter. Strips were stored in a desiccator at room temperature before use.

2.7. Test procedure

The test strip sample pad was dipped in 100 µl of analyte for 20 s and laid horizontally for 10 min to allow the sample to fully migrate. Liquid from the saturated sample pad migrates to the conjugate pad where it reconstitutes the anti-His-tag iBody-gold conjugate. Conjugate then joins the sample flow driven by capillary force maintained by the dry wicking pad on the opposite side of the strip. Upon reaching the test zone, the conjugate is captured by the immobilized His-tagged Protein 1, forming a visible test line due to high extinction coefficient of gold nanoparticles. This process is disrupted by the presence of His-tagged protein in sample via competition, resulting in progressive vanishing of the test line with increasing concentration of His-tagged protein as illustrated in Fig. 2. Excess conjugate then continues to flow towards the wicking pad until it is captured by immobilized Neutravidin, forming the control line.

Control and test lines on developed strips were analyzed using a Canon CanoScan 8400F scanner (Ōta, Tokyo, Japan). Monochrome image data was processed using ImageJ (Schneider et al., 2012) to obtain a virtual chromatogram, from which the areas under the control and test line peaks were measured. The ratio of test area to control area was calculated to normalize the values between readings and then divided by the same ratio of negative control (buffer alone, 0.74 for PBS). The resulting percentage value (relative band intensity) expresses the test line density in proportion to the negative control.

For protein detection, proteins 1–4 were serially diluted in PBS. For determination of reagent compatibility, reagents were dissolved in deionized water (where applicable), which was used as a visual control. The criteria for compatibility were (a) the absence of aggregation artefacts and severe smearing and (b) a ratio of test area to control area > 0.5. Conditions under which (a) and (b) are fulfilled, but in which both bands were not clearly visible by eye compared to the control, were considered incompatible. All tests were performed in duplicate, and averages of test and control areas were used to calculate relative band intensities.

3. Results and discussion

3.1. Assay optimization

The first step in assay design was to determine the optimal pH for iBody-colloid conjugation. The performance of the conjugate was verified in a half-strip format (direct application of conjugate onto a test strip lacking a sample pad). Conjugate performance was consistent across the pH range 6.5–9.1, with a carbonate buffer system at pH 8.6 being only marginally better than other conditions tested. The polymer concentration in the conjugation reaction was also optimized using the half-strip method, as it allowed us to monitor free polymer contamination. High concentrations of free polymer resulted in a decrease in absolute density of both control and test bands. Free polymer also interfered with the sodium chloride aggregation stability test, which is commonly used for protein gold conjugates (Hermanson, 1996).

We also customized the protocol by employing ultrafiltration as a concentration step, rather than pelleting the conjugate by centrifugation. Ultrafiltration allowed us to reduce the scale of conjugate synthesis, due to lower losses of conjugate by aggregation during the concentration step. However, it also opened the possibility of contaminating the conjugate with free polymer, as no washing step is

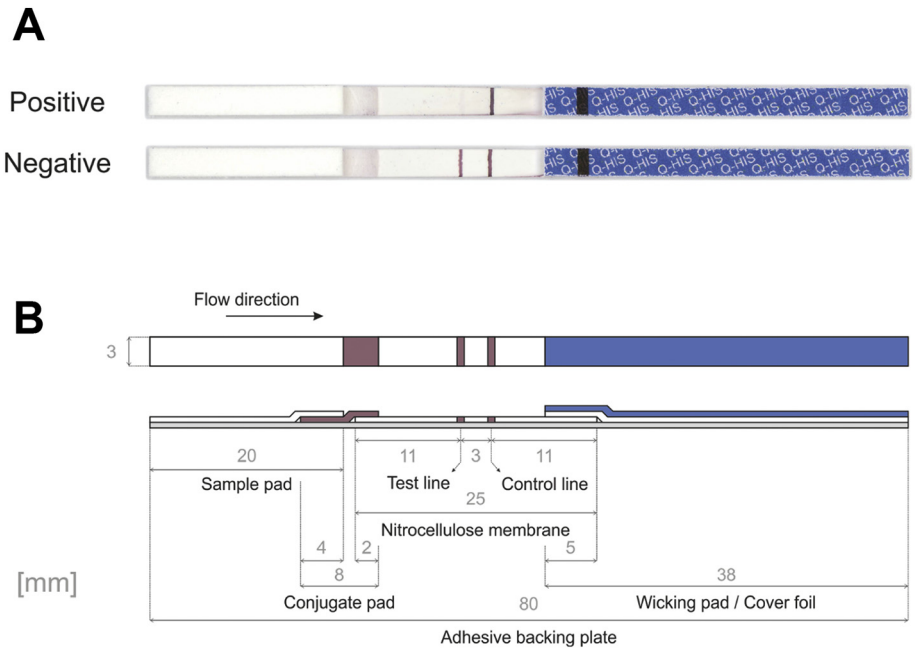

Fig. 1. Graphic overview of test function and composition. (A) Example of qualitatively positive (0.5 mg/ml Protein 4 in 100 mM acetate, pH 4.0) and negative test (100 mM acetate, pH 4.0). (B) Matching diagram of test components, dimensions and relative positions.

included. This was mitigated by optimizing the polymer concentration in the conjugation reaction.

Assembly of the test strips also required optimization. The lengths of the overlaps between sample pad, conjugate pad, and membrane affect assay performance. Longer overlap between conjugate and sample pad increases sensitivity, but also increases time for the test to fully develop and with it also the probability of premature drying and formation of smearing. This effect can be explained by slower conjugate release with increasing overlap, which in turn provides more time for the analyte to react with conjugate before it reaches test band. Considering these counteracting effects, we chose a sample-conjugate pad overlap of 4 mm as a trade-off. These effects were not as pronounced for conjugate pad-membrane overlap, for which we selected an overlap of 2 mm to provide reliable contact for solution transfer. The overlap length of the membrane-wicking pad appeared to have only a marginal effect on assay performance and was set to 5 mm to ensure reliable contact with the membrane.

3.2. Detection of His-tagged proteins

We pilot-tested our assay with a panel of four His-tagged proteins at various concentrations with PBS as the mobile phase (see Fig. 2). Using color densitometry, we determined that the general reliable detection limit for a relative band intensity drop of at least 10% is 0.5 µM, or 5–20 µg/ml using protein 4 with lowest M_w as lower limit and protein 3 with highest M_w as upper limit. A relative band intensity drop of at least 50% is assumed to be visible to the naked eye, and thus a concentration of a least 1 µM should be visually detectable.

Presented assay sensitivity of 5–20 µg/ml is on par with that of commercially available lateral flow tests for His-tagged proteins, which exhibit sensitivities of 4–20 µg/ml (Thermo Fisher, 2018) and 11–100 µg/ml (Expedeon, 2018) depending on the protein analyzed. It is important to note that manufacturer datasheets for both tests present neither molar concentrations nor M_w of assayed proteins. Due to this, presented sensitivity ranges should be compared with caution.

Fig. 2 also reveals discrepancies in the responses of proteins 1, 2 and 4 and protein 3. These can be explained by differences in protein structure, His-tag linkage and resulting tag availability. These features commonly cause issues with immobilized metal affinity chromatography (IMAC), which works on a similar principle.

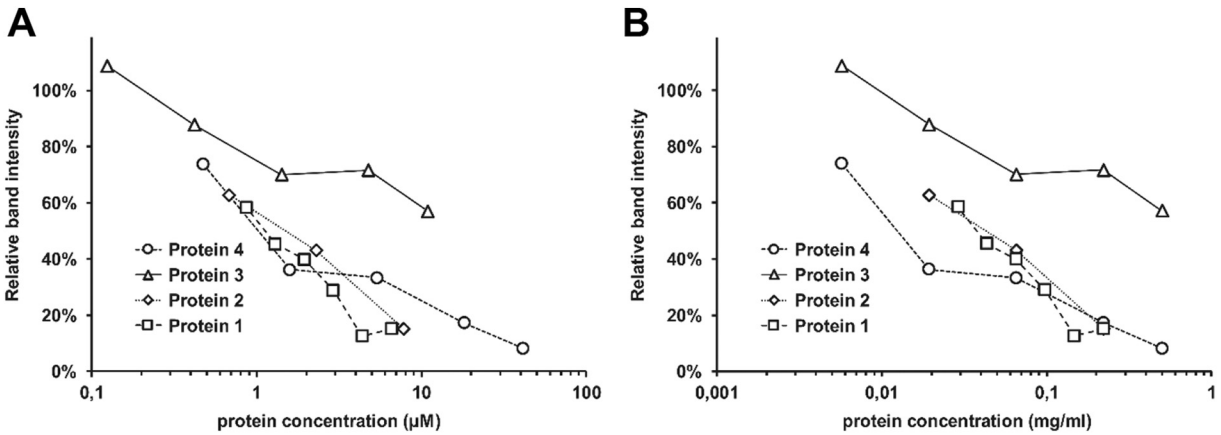

Fig. 2. Relative test band intensity as a percentage of intensity of negative control (PBS) at varying molar (A) and weight (B) concentrations of proteins.

Table 2
Reagent compatibility of our polymer-based lateral flow test compared to published data for commercial antibody-based Test A (Expedeon, 2018), and Test B (Thermo Fisher, 2018). [a]

Buffers						Chelators			
conc.	buffer	pH	This work	Test A	Test B	chelator	This work	Test A	Test B
1×	TBS	7.4	+	+	n.d.	EDTA	< 10 mM	< 100 mM	< 5 mM
	PBS	7.4	+	+	n.d.	imidazole	< 5 mM	< 125 mM	n.d.
	RIPA	7.4	++	+	+				
100 mM	Sodium phosphate	4.5	++	n.d.	n.d.	Solvents			
	Sodium phosphate	6.5	++	n.d.	n.d.	solvent	This work	Test A	Test B
	Tris	8.0	+	n.d.	n.d.	DMSO	< 15%	n.d.	n.d.
	Tris	9.1	−	n.d.	n.d.	acetonitrile	< 30%	n.d.	n.d.
	Sodium carbonate	6.5	−	n.d.	n.d.	isopropanol	< 30%	n.d.	n.d.
	Sodium carbonate	8.6	−	n.d.	n.d.	glycerol	< 10%	n.d.	< 10%
	MES	5.5	+	n.d.	n.d.				
	Sodium acetate	4.0	++	n.d.	n.d.	Reducing agents			
	Sodium acetate	5.0	++	n.d.	n.d.	agent	This work	Test A	Test B
	Ammonium acetate	8.5	++	n.d.	n.d.	DTT	< 50 mM	< 100 mM	n.d.
	Glycine	2.2	++	n.d.	n.d.	TCEP	< 50 mM	n.d.	n.d.
	Glycine	2.7	++	n.d.	n.d.	β-ME	< 500 mM	< 100 mM	n.d.

Bivalents					Detergents			
conc.	Reagent	This work	Test A	Test B	detergent	This work	Test A	Test B
100 mM	Cobalt (II) chloride	−	n.d.	n.d.	Tween-20	< 4%	< 5%	n.d.
	Nickel (II) chloride	−	n.d.	n.d.	Triton X-100	< 1%	< 5%	< 1%
	Calcium (II) chloride	−	n.d.	n.d.	SDS	< 2%	< 1%	< 0.2%

Media					Miscellaneous			
conc.	Medium	This work	Test A	Test B	reagent	This work	Test A	Test B
1×	LB-Miller	++	n.d.	n.d.	urea	< 4 M	< 125 mM	< 400 mM
	Gibco advanced RPMI 1640	+	n.d.	n.d.	guanidine HCl	< 1 M	< 100 mM	n.d.
	Gibco Opti-MEM	−	n.d.	n.d.	sodium chloride	< 500 mM	n.d.	< 500 mM
	Gibco freestyle 293	−	n.d.	n.d.				
5%	FBS in PBS	++	n.d.	n.d.				

[a] Legend: ++, Conditions with clear, sharp bands; +, Minor inconsistencies in background; −, Incompatible reagent. For commercial tests: +, Compatible, − Incompatible.

3.3. Reagent compatibility

Recently, two commercial His-tag immunochromatographic tests became available - His-Tag Check&Go! kit (Expedeon, 2018) and Pro-Detect™ Rapid His Competitive Assay Kit (Fisher, 2018) these are discussed as Test A and Test B, respectively. The reagent compatibilities disclosed in the product data sheet can be used to illustrate the differences between antibody- and iBody-based assays. For a side-by-side comparison of reagent compatibilities, see Table 2. Overall, the commercial antibody-based assay is more resistant to common contaminants, such as chelators (in case of Test A), but less resistant to the chaotropes urea and guanidium hydrochloride. Other reagents exhibited similar compatibilities. This may make antibody-based Test A more suitable for preliminary testing of IMAC elutions, in which high concentrations of imidazole are expected. On the other hand, the iBody-based assay might be more effective for monitoring protein solubilization and refolding due its higher tolerance for chaotropes.

Besides direct competition (e.g., divalent ions) or sequestration of metal ions (chelators), incompatibilities are chiefly driven by increasing ionic strength, which induces gold nanoparticle aggregation, and increasing viscosity, which disturbs membrane capillary flow. Based on the simplest example of uni-univalent sodium chloride, we estimate that the limit for ionic strength is 500 mM. Based on the viscosities of aqueous glycerol(Segur and Oberstar, 1951) and DMSO(LeBel and Goring, 1962) mixtures at room temperature, we estimate that the dynamic viscosity limit lies between 1.17 and 1.18 mPa. However, these assumptions might not hold true for all electrolytes and solvent systems, which might have additional modes of test disruption.

4. Conclusion

In the present study, a lateral flow assay based on iBody antibody mimetics was developed, optimized, and shown to have sensitivity suitable for preliminary protein concentration estimation and qualitative testing. The test is able to detect multiple His-tagged proteins with different tag attachment strategies with a visual detection limit of 1 μM and densitometric detection limit of 0.5 μM.

Our test was compared with two commercially available antibody-based assays in terms of reagent compatibility and sensitivity. The antibody-based tests can exhibit higher tolerance to chelating agents, but generally exhibit lower tolerance to chaotropic agents. The sensitivity of all three assays is comparable.

Acknowledgment

The authors would like to thank Jitka Neburková for preparation of stock gold nanoparticle solution, Jakub Staníček for supplying recombinant His-tagged proteins for testing, as well as Vladimír Šubr and Libor Kostka for preparation of anti-His-tag polymer conjugate. The study was supported by the Czech Science Foundation (No. 19-10280S), by Ministry of Industry and Trade of the Czech Republic (No. FV10490) and by the European Regional Development Fund; OP RDE (No. CZ.02.1.01/0.0/0.0/16_019/0000729).

Declaration of Competing Interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.112640.

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