Metal Affinity Tag for Protein Expression and Purification using the Flexi® Vectors

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Abstract

We have developed a new metal affinity tag, HQ tag, for the purification of recombinant proteins. The HQ tag is incorporated into several Flexi® Vectors for convenient cloning and expression. Protein-coding regions can be easily and efficiently transferred into this affinity-tag vector from other Flexi® Vectors. The HQ Flexi® Vectors come in four formats to provide flexibility in tag location and antibiotic resistance. The HQ tag performs similarly to a polyhistidine tag purified from bacterial cells. However, some HQ-tagged proteins can be eluted from the affinity columns at lower concentrations of imidazole. This property of the HQ tag may be useful for downstream applications such as enzymatic reactions.

As part of several new Flexi® Vectors, we introduce a unique protein expression and purification tag that can be used with nickel-based isolation systems.

Introduction

Many fusion tags are available for the expression and purification of recombinant proteins. The polyhistidine tag is most commonly used for several reasons: the tag is very small, can be used under native or denaturing conditions and does not need to be removed prior to injection into an animal for antibody production. The HQ tag shares similar features with the polyhistidine tag. HQ-tagged proteins can be purified using any nickel-affinity resin in single tubes or a high-throughput format using manual or automated methods. Proteins can be expressed from bacterial, insect and mammalian systems and purified under native and denaturing conditions. In addition, the HQ tag has been incorporated into several Flexi® Vectors[a,b,c] with the versatility of an amino-(N) or carboxy-(C) terminal expression tag and your choice of ampicillin or kanamycin resistance.

The HQ tag consists of 6 amino acids (HQHQHQ; H = histidine, Q = glutamine), which can be appended to any protein-coding region using Flexi® Vectors designed for bacterial or in vitro protein expression. Flexi® Vectors are designed for rapid, high-fidelity transfer of protein-coding regions between vectors containing various expression or peptide tag options. These vectors enable expression of native or fusion proteins to facilitate the study of protein structure and function as well as protein:protein interactions.

High-Efficiency Transfer Between Flexi® Vectors

The Flexi® Vector Systems use two rare-cutting restriction enzymes, *Sgf* I[d] and *Pme* I[e], in a simple yet powerful directional cloning method for protein-coding sequences (Figure 1, Panel A). The *Sgf* I site upstream of the start codon allows the expression of either a native (untagged) protein or a N-terminal-tagged protein by readthrough of the *Sgf* I site (e.g., pFN6A (HQ) Flexi® Vector and pFN6K (HQ) Flexi® Vector). The downstream *Pme* I site contains the stop codon for the protein-coding region and appends a single valine residue to the C-terminus of the protein. Flexi® Vectors are designed for native or N-terminal fusion protein expression and contain *Sgf* I and *Pme* I sites into which protein-coding regions are cloned. Inserts can be easily transferred to other Flexi® Vectors following digestion with *Sgf* I and *Pme* I. This maintains insert orientation and reading frame, eliminating the need to resequence the insert after each transfer (1). The C-terminal fusion Flexi® Vectors (e.g., pFC7A (HQ) Flexi® Vector and pFC7K (HQ) Flexi® Vector), on the other hand, can act as acceptors of a protein-coding region flanked by *Sgf* I and *Pme* I sites, but contain a different blunt-end site, *Eco*ICR I (Figure 1, Panel B). When the blunt-ends of *Pme* I and *Eco*ICR I are joined during the transfer of a protein-coding region, the stop codon is not recreated, allowing readthrough of a C-terminal peptide sequence contained on the vector backbone.

Figure 1. Transferring protein-coding regions in the Flexi® Vector Systems. The features necessary for expression and the options for protein-fusion tags are carried on the Flexi® Vector backbone, and the protein-coding region can be shuttled between vectors using two rare-cutting restriction endonucleases, *Sgf* I and *Pme* I. The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic-resistance marker for selection of colonies containing the Flexi® Vector. **Panel A.** Transfer between Flexi® Vectors designed to express native (untagged) or N-terminal-tagged fusion proteins is reversible. **Panel B.** C-terminal Flexi® Vectors contain *Sgf* I and *Eco*ICR I sites and are designed to allow expression of C-terminal-tagged proteins. Joining *Pme* I and *Eco*ICR I blunt ends removes the stop codon present in the *Pme* I site and allows readthrough to the C-terminal protein-coding sequences in the C-terminal Flexi® Vector. Since both restriction sites are destroyed by joining, transfer into C-terminal Flexi® Vectors is not reversible.

We measured the transfer frequency of a protein-coding region between various Flexi® Vectors by using LacZ alpha complementation. The insert region encodes a LacZ / -peptide (residues 3–166 of LacZ) and a bacterial promoter to facilitate expression of the LacZ/ -peptide in any *E. coli* host, while the JM109 host encodes the ∅-peptide (residues 15–end of LacZ) on the F´ episome. Any cells transformed with plasmid encoding the LacZ / -peptide express ∈galactosidase activity and are counted as blue colonies on LB plates containing X-gal. The LacZ/ -peptide sequence was transferred from a Flexi® Vector designed to express native (pFN4K) and glutathione-S-transferase (GST) fusion-tagged (pFN2K) protein. The transfer efficiency was calculated as the percent of blue colonies. The transfer was efficient (Table 1), particularly to the pFN6A Vector. Lower transfer frequencies to the pFC7A Vector are due to a higher background of plasmid backbone heterodimers between the pFC7 Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the *Sgf* I and *Pme* I sites, such that plasmid backbone dimers are unstable (2). The pFC7A and pFC7K (HQ) Flexi® Vectors lack these common flanking regions because of the position of the HQ peptide.

Table 1. Transfer Frequencies of LacZ/ -Peptide Between Various Flexi® Vectors.

Donor	Acceptor	%Transfer
pFN2K-LacZ/	pFN6A	98
pF4K-LacZ/	pFN6A	98
pFN2K-LacZ/	pFC7A	77
pF4K-LacZ/	pFC7A	78

Comparison of HQ and Polyhistidine Tags for Protein Expression and Purification

The HQ tag and polyhistidine tag have comparable features with regard to protein expression and purification. The open reading frame for the mouse muscle regulatory protein, Id, was cloned into an N-terminal HQ Flexi® Vector to produce HQ-tagged Id. An insert for polyhistidine-Id was cloned into a Flexi® Vector with the T7 RNA polymerase promoter. Both proteins were expressed and purified using the same conditions. Due to poor solubility of the fusion proteins, the lysates were evaluated for protein expression and purification under denaturing conditions. Similar amounts of Id protein were expressed and purified, regardless of the tag (Figure 2). Furthermore, HQ-tagged proteins can also be purified from insect and mammalian cells (data not shown), and can be expressed in rabbit reticulocyte lysate and purified using the MagZ™ Protein Purification System (Cat.# V8830; data not shown). See references 3 and 4 for experimental procedures used for each system.

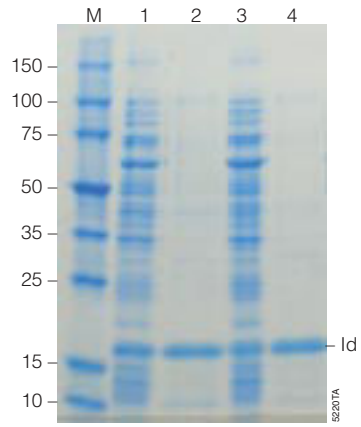

Figure 2. Purification of equal amounts of HQ-Id and polyhistidine-Id. The mouse Id protein coding region was cloned into pFN6K (HQ) Flexi® Vector and polyhistidine-tagged Id was cloned into pF1K Flexi® Vector and separately transformed into a proprietary in-house *E. coli* strain. Following induction, cultures were grown at 37°C overnight. One milliliter of culture was centrifuged and the cells resuspended in a denaturing binding/wash buffer (8M urea, 100mM HEPES, 10mM imidazole, 500mM NaCl). After lysis, the lysate was centrifuged, and 75µl of HisLink™ Resin was added to the supernatant. The resin was washed four times with 250µl of denaturing binding/wash buffer to remove nonspecifically bound proteins. The tagged Id protein was eluted with 100µl of denaturing elution buffer (8M urea, 250mM imidazole) and analyzed by SDS-PAGE. Lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491); Lane 1, 5µl of polyhistidine-Id lysate; Lane 2, 20µl of eluted polyhistidine-Id protein; Lane 3, 5µl of HQ-Id lysate; Lane 4, 20µl of eluted HQ-Id protein.

Elution of HQ-Tagged Protein at Lower Imidazole Concentrations

One unique feature of the HQ tag is that many HQ-tagged proteins elute from metal-affinity resin at a low imidazole concentration (e.g., 50mM imidazole). This can be advantageous for downstream applications. In the following study, HQ-firefly luciferase (HQ-Luc) and polyhistidine-Luc were purified using the MagneHis™ Protein Purification System (Cat.# V8500). Bacterial cultures expressing HQ-Luc and polyhistidine-Luc were lysed with FastBreak™ Lysis Reagent, 10X (Cat.# V8571). The lysate was treated with DNase I to reduce the viscosity of the lysate and decrease the potential for resin aggregation. After lysis, the MagneHis™ Ni-Particles (Cat.# V8560) were added and the protein bound to the resin under native conditions (5). The particles were then washed with MagneHis™ Binding/Wash Buffer (100mM HEPES, 10mM imidazole) to remove any nonspecifically bound protein. Protein was eluted with 50–500mM imidazole (Figure 3). The low imidazole concentration of the MagneHis™ Binding/Wash Buffer (10mM) does not prevent HQ-tagged protein from binding to the Ni-particles (data not shown). In this example, a significant level of HQ-Luc eluted with 100mM imazole.

The optimal imidazole concentration for elution of specific protein fusions may differ. We recommend washing the resin with increasing imidazole concentrations to determine the best concentration.

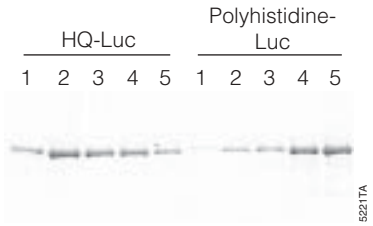

Figure 3. Titration of imidazole required to elute tagged luciferase. No salt was added to focus on the effect of imadazole on the elution characteristics of HQ-Luc and polyhistidine-Luc. The HQ-tagged and polyhistidine-tagged firefly luciferase were purified using the MagneHis™ Protein Purification System following the protocol described in Technical Manual #TM060. The MagneHis™ Elution Buffer (contains 500mM imidazole) was diluted with MagneHis™ Binding/Wash Buffer to a final concentration of 50mM, 100mM and 200mM imidazole. The MagneHis™ Ni-particles were washed sequentially with an increasing concentration of imidazole. Gel lanes were loaded with 15µl eluate. Lane 1, 50mM imidazole; Lane 2, 100mM imidazole; Lane 3, 100mM imidazole; Lane 4, 200mM imidazole; Lane 5, 500mM imidazole.

Purification of HQ-Tagged Proteins in Multiple Formats

The HQ-tagged proteins can be purified using multiple formats such as: 1) small-scale purification using the MagneHis™ Ni-Particles; 2) small-scale vacuum-based format using the HisLink™ 96 Protein Purification System (Cat.# V3680); and 3) large-scale purification of milligram quantities of protein using the HisLink™ Protein Purification Resin (Cat.# V8821).

We examined the effect of NaCl in the binding/wash buffer in combination with HQ-tagged protein purification with HisLink™ Resin and MagneHis™ Ni-Particles (Figure 4). For the HisLink™ Resin, the addition of NaCl in the binding reaction did not increase the amount of HQ-tagged protein purified. With MagneHis™ Ni-Particles, however, the addition of NaCl during the wash and binding steps increased the amount of HQ-tagged protein purified for most proteins. Adding NaCl (<500mM) may reduce nonspecific binding of proteins.

HisLink™ Resin can be adapted to many protein purification techniques including column chromatography, batch chromatography and automated methods such as FPLC. Figure 5 shows a large-scale purification of HQ-GST under native conditions. Thus, the HisLink™ Resin can be used for the purification of milligram quantities of HQ-tagged proteins. The binding capacity of HisLink™ Resin for HQ-tagged proteins is similar to that for polyhistidine-tagged proteins (e.g., 15mg/ml HisLink™ Resin).

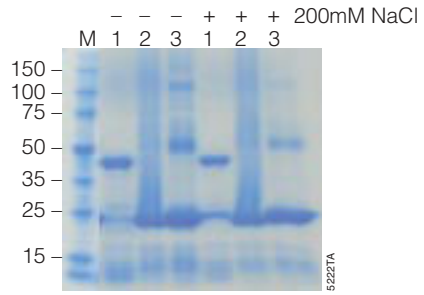

Figure 4. HQ-tagged proteins purified under native conditions using the HisLink™ 96 Protein Purification System in a manual format. Lane 1, maltose binding protein; Lane 2, chloramphenicol aceyltransferase (CAT); and Lane 3, GST coding regions were cloned into pFN6A (HQ) Flexi® Vector and transformed into BL21(DE3) cells. Cultures were induced with 1mM IPTG and grown at 25°C overnight. To 1ml of culture, 110µl of 10X FastBreak™ Cell Lysis Reagent, 1µl of DNase and 75µl of HisLink™ Resin were added. NaCl to a final concentration of 200mM was added to half the samples. The mixtures were incubated for 30 minutes on a rocking platform and transferred to a HisLink™ 96 Filtration Plate. The resin/protein complex was washed with MagneHis™ Binding/Wash Buffer, and the protein was eluted with MagneHis™ Elution Buffer. For SDS-PAGE analysis, 13µl of the eluted protein was evaluated. Lane M = Broad Range Protein Molecular Weight Markers (Cat.# V8491).

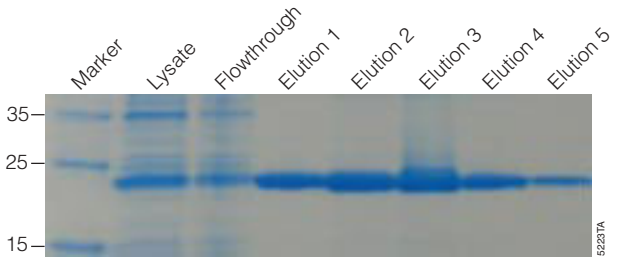

Figure 5. Batch purification of HQ-CAT Using the HisLink™ Protein Purification Resin. The CAT gene was cloned into the pFN6K (HQ) Flexi® Vector and transformed into BL21(DE3) cells. The culture was induced with IPTG and the tagged protein expressed overnight at 25°C. The 40ml culture was pelleted and frozen at –20°C. The cell pellet was resuspended in 12ml of 1X FastBreak™ Cell Lysis Reagent plus 40µl of DNase I, and lysed for 15 minutes. HisLink™ Resin (0.5ml) was added to the lysate (0.25ml bed volume) and mixed for 30 minutes. The resin was then washed with 5ml MagneHis™ Binding/Wash Buffer three times and HQ-CAT eluted with 250µl of MagneHis™ Elution Buffer 5 times. The samples (5µl) were analyzed by SDS-PAGE. Marker = Broad Range Molucular Weight Markers (Cat.# V8491).

HQ-tagged and polyhistidine-tagged proteins purify with similar yields using an immobilized metal-affinity column (IMAC) such as HisLink™ Protein Purification Resin. We compared direct lysis of bacterial cell cultures using 10X FastBreak™ Cell Lysis Reagent and resuspension of a cell pellet in 1X FastBreak™ Cell Lysis Reagent for HQ- and polyhistidine-tagged proteins. We observed that the amount of tagged protein purified increased for both tagged proteins when starting with a bacterial cell pellet (data not shown). We also compared competitor lysis reagents to FastBreak™ Cell Lysis Reagent for both bacterial cell cultures and cell pellets, and similar results were observed for both fusion tags (data not shown).

Conclusion

The new affinity purification tag in the HQ Flexi® Vectors is present as a N- or C-terminal tag, and these vectors provide the ability to transfer protein-coding regions using the Flexi® Vector System. The advantages of the HQ tag are: 1) small-size; 2) expression and purification properties similar to polyhistidine-tagged proteins; 3) isolation using immobilized metal-affinity columns and 4) elution of HQ-tagged proteins at lower imidazole concentrations.

HQ-tagged proteins are not only expressed and purified similarly to polyhistidine-tagged proteins, but can also be purified from bacterial cells under native and denaturing conditions using multiple formats. In addition, some HQ-tagged proteins can elute at lower imidazole concentrations, which may minimize inhibition of downstream applications.

References

1. Slater, M. *et al.* (2005) *Promega Notes* **89**, 11–5.
2. Yoshimura, H. *et al.* (1986) *J. Gen. Appl. Microbiol.* **32**, 393–404.
3. Betz, N. (2004) *Promega Notes* **87**, 29–32.
4. Godat, B. *et al.* (2004) *Promega Notes* **88**, 9–12.
5. *MagneHis™ Protein Purification System Technical Manual* #TM060, Promega Corporation.

Protocol

- *Flexi® Vector Systems Technical Manual* #TM254, Promega Corporation.
  www.promega.com/tbs/tm254/tm254.html
- *MagneHis™ Protein Purification System Technical Manual* #TM060, Promega Corporation.
  www.promega.com/tbs/tm060/tm060.html
- *HisLink™ Protein Purification System Technical Bulletin* #TB327, Promega Corporation.
  www.promega.com/tbs/tb327/tb327.html
- *HisLink™ 96 Protein Purification System Technical Bulletin* #TB342, Promega Corporation.
  www.promega.com/tbs/tb342/tb342.html

Ordering Information

Product	Size	Cat.#
Flexi® System, Entry/Transfer	1 system	C8640
Flexi® System, Transfer	1 system	C8820
Carboxy Flexi® System, Transfer	1 system	C9320
pFN6A (HQ) Flexi® Vector	20µg	C8511
pFN6K (HQ) Flexi® Vector	20µg	C8521
pFC7A (HQ) Flexi® Vector	20µg	C8531
pFC7K (HQ) Flexi® Vector	20µg	C8541

Related Products

Product	Size	Cat.#
10X Flexi® Enzyme Blend (*Sgf* I and *Pme* I)	25µl	R1851
	100µl	R1852
MagneHis™ Protein Purification System	65 reactions	V8500
	325 reactions	V8550
MagneHis™ Ni-Particles	2ml	V8560
	10ml	V8565
HisLink™ Protein Purification Resin	50ml	V8821
HisLink™ 96 Protein Purification System	1 × 96	V3680
	5 × 96	V3681
MagZ™ Protein Purification System	30 purifications	V8830
FastBreak™ Cell Lysis Reagent, 10X	15ml	V8571
	60ml	V8572
	100ml	V8573

[a] Patent Pending.

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