Low Expression His-tagged Protein Purification Kit

Introduction

A polyhistidine tag, also called 6xHis-tag, His-tagged, and His-tag, is a versatile tool for purifying the highly purified recombinant protein from various expression systems, including bacterial, yeast, plant cell, and mammalian cells systems. The tag comprises six or more consecutive histidine amino acid residues positioned at either N or C terminus of a recombinant protein. Due to its small size, His-tag has several distinctive features, including less immunogenicity, hydrophilic nature, and versatility under native and denaturing conditions. Additionally, it is unnecessary to cleave the tag from the recombinant protein since it rarely perturbs the structure and function of its fusion protein. The purification principle of the His-tag depends on immobilized metal ion affinity chromatography (IMAC).

Immobilized metal ion affinity chromatography (IMAC) is a rapid affinity purification chromatography where the his-tagged protein are separated based on their affinity for Ni^{2+} or Co^{2+} that have been immobilized by a chelator to a solid matrix such as beaded agarose or column. At pH 7-8, his tagged protein will bind to Ni^{2+} or Co^{2+}. The binding reaction with the tagged protein is affected by various independent variables such as pH, temperature, salt type, salt concentration, immobilized metal and ligand density, and protein size. The bound protein is eluted by a decreasing pH gradient, increasing imidazole concentration, or adding an EDTA chelator in a buffer. This technique is an ideal tool for capturing and purification of his-tagged recombinant protein in a quick, inexpensive, and straightforward manner.

Nonspecific binding was a potential problem with the purification of his-tagged proteins from various expression systems, especially from the lower expressed recombinant his-tagged proteins or higher expressed proteins interacting with other cellular proteins. Many factors can cause nonspecific binding. Among them, low expressed protein or the tagged protein bound with the endogenous proteins could result in severely nonspecific binding. The binding of His-tagged proteins to the metal ion of the IMAC depends on electric charges. The nonspecific binding occurs when the his-tagged binding sites of resin are only partially bound by the protein of interest due to its low abundance. The rest of the binding sites were non-specifically interacted with other slightly charged proteins such as histidine-rich protein, leading to impurities later on. Multiple lines of evidence prove that reducing reagents and nonionic detergents in the binding and washing buffers could dramatically reduce the highly nonspecific binding and get purer proteins. The most used chelators in IMAC applications are iminodiacetate (IDA) or nitrilotriacetic acid (NTA). These chelators cannot use together with reducing reagents such as DTT (dithiothreitol), β-me (β-Mercaptoethanol), and TCEP (TRIS (2-carboxyethyl) phosphine) since the reducing reagents can strip off the metal from these resins, resulting in the rapid loss of the protein binding capacity.

Moreover, those chelators are mainly immobilized to the traditional affinity chromatography matrices such as agarose resin or column. These solid matrices make the purification process tedious, time-consuming, unable to handle very tiny samples, and challenging to adapt to the automation system. Bioclone introduces a powerful magnetic beads-based IMAC system to address these problems.

BcMag™ Low expression His-tagged protein purification kit uses reducing reagent-compatible magnetic beads to purify his-tagged proteins with nonspecific binding problems such as low expressed protein or the target protein bound with the cellular protein (fig.1). The beads are manufactured using nanometer-scale superparamagnetic iron oxide as core and entirely encapsulated by a high purity silica shell, ensuring no leaching problems with the iron oxide. Our proprietary chelators are immobilized on their surface and bound firmly with Ni^{2+} ions, leading to high resistance to reducing reagents. The pure inert silica makes less nonspecific binding due to fewer side chains of the silica materials than other solid matrices such as agarose. Additionally, the beads combine all the advantages of BcMag™ Low expression His-tagged protein purification kit (low costs, simplicity, high specificity, and capacity) and magnetic properties to perform efficient manual or automatic quick high-throughput purification.

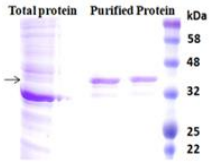

Fig1: Using BcMag™ Low expression His-tagged protein purification kit to purify low expressed protein at high purity.

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Workflow (Fig.2)

The purification with magnetic microparticles is straightforward (Fig.2). Mix the microparticles with the sample and incubate them with continuous rotation for a sufficient time. During mixing, the beads remain suspended in the sample solution, allowing the target molecules to interact with the immobilized ligand. After incubation, the beads are collected and separated from the sample using a magnet rack. Then the ultrapure His-tagged recombinant proteins are eluted by imidazole

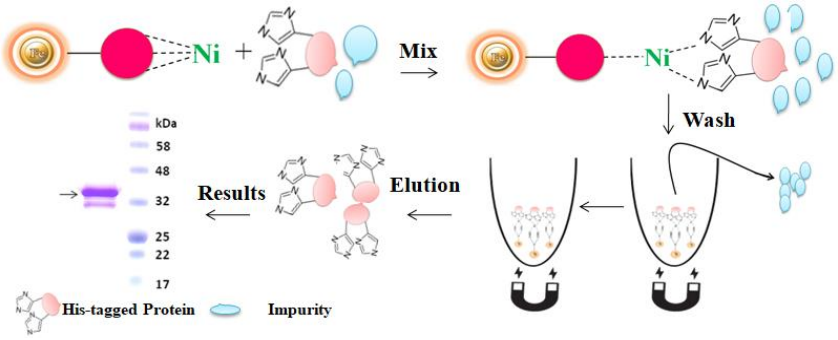

Fig.2

Feature and benefits

- Magnetic beads exhibit less nonspecific binding than porous supports.
- Stable covalent bond with minimal ligand leakage
- The beads resist up to 20 mM EDTA and 20 mM reducing reagents without nickel leaching.
- High protein purity
- Cost-effective: Eliminates columns, filters, repeat pipetting, and organic reagents.
- High-throughput: Compatible with many different automated liquid handling systems.

Applications

- Investigating protein structure and function
- Preparing recombinant proteins for X-ray crystallography
- Ideal for study of protein interactions with protein or DNA
- Immunization to raise antibodies against a protein of interest
- Effective screening protein expression even with crude cell lysatess
- Microscale purification of his-tagged proteins

Specificities	
Composition	Magnetic beads charged with Ni^{2+}
Magnetization	~60 EMU/g
Type of Magnetization	Superparamagnetic
Stability	pH 4-11,100% Ethanol, 100% Methanol,8M Urea, 6M guanidine hydrochloride, 20 mM DTT, 20mM EDTA
Concentration	100 mg/ml (1% $NiSO_4.6H_2O$)
Binding Capacity	>2mg His-tagged GFP /ml of Beads
Storage	Store at 4°C upon receipt

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Protocol

Note

- This protocol is optimized to capture and purify soluble high- or low-expression his-tagged protein from E.coli. For insoluble his-tagged protein, we recommend using BcMag™ IDA--Ni His-tagged protein purification magnetic beads (Cat. No MHN-105). Creating a universal kit for protein purification is difficult because each protein has a different composition and structure. To get the best results, the user should optimize the working conditions for the purification of individual proteins based on the suggestions described in the troubleshooting section. For purification from other expression systems, optimizing the working condition is necessary.
- Recombinant protein expressed in E.coli is classified into two groups: soluble and insoluble. Soluble: The Recombinant proteins remain soluble in the cytoplasm. Insoluble: The recombinant protein is accumulated as insoluble aggregates (called inclusion bodies). The soluble target protein is found in the supernatant while inclusion bodies remain in the pellet. Practically, the solubility of the recombinant protein can be determined by the following method. Harvest the bacterial cells by centrifuge and lyse the cell. Separate the insoluble fraction of the lysed cells from the soluble fraction by centrifugation and perform an SDS-PAGE analysis.
- Before purifying the His-Tagged fusion protein, you should equilibrate all the reagents contained in the kit at room temperature.

Materials Required

Equipment

- Centrifuge
- Ultrasonic processor
- Ice bucket filled with ice
- Magnetic rack (for manual operation)
- Based on sample volume, the user can choose one of the following magnetic Racks: BcMag rack-2 for holding two individual 1.5 ml centrifuge tubes (Cat. # MS-01); BcMag rack-6 for holding six individual 1.5 ml centrifuge tubes (Cat. # MS-02); BcMag rack-24 for holding twenty-four individual 1.5-2.0 ml centrifuge tubes (Cat. # MS-03); BcMag rack-50 for holding one 50 ml centrifuge tube, one 15 ml centrifuge tube, and four individual 1.5 ml centrifuge tubes (Cat. # MS-04); BcMag™ rack-96 for holding a 96 ELISA plate or PCR plate (Cat. # MS-05). For larger scale purification, Ceramic magnets Block for large scale purification (6 in x 4 in x 1 in block ferrite magnet, Applied Magnets, Cat# CERAMIC-B8)
- Corning 430825 cell culture flask for large scale purification (Cole-Parmer, Cat#EW-01936-22)
- Mini BlotBoy 3D Rocker, fixed speed, small 10" x 7.5" platform w/ flat mat (Benchmark Scientific, Inc. Cat# B3D1008) or compatible

Buffer

- **Soluble protein purification**
- BcMag™ IMAC-Ni reducing reagents compatible magnetic beads
- 1x Equilibration buffer: 50 mM Tris-HCl pH 8.0, 10% glycerol, 0.2 M NaCl, 5 mM imidazole, 0.5% Triton-x 100, 0.5% Tween20, 12 mM DTT (Add fresh)
- 1x Lysis buffer: 100 mM Sodium phosphate pH 8.0, 10% glycerol, 0.5M NaCl, 0.5% 10 mM imidazole, 0.5% Triton-x 100, 0.5% Tween20, 12mM DTT (Add fresh),
- Option: Additives/1x lysis buffer per 50 ml: 2 tablets cOmplete™, Mini Protease Inhibitor Cocktail (sigma Cat# 11836153001). Add 30 µl BcMag™ Benzonase Nuclease (Bioclone Cat#BE-103).
- 1x Wash buffer: 50 mM Tris-HCl pH 7.5, 10% glycerol, 0.5 M NaCl, 20 mM Imidazole,12mM DTT (Add fresh).
- 1x Elution buffers: 50 mM Tris-HCl pH 7.5, 10% glycerol*, 0.3 M NaCl, 200-1000***** mM imidazole, 2mM DTT(Add fresh)
- PBS buffer

Procedure

A. Cell Extract Preparation

1. Harvest cells by centrifugation at 6000 rpm for 6 minutes, remove the supernatant and wash the pellet with 1 x PBS buffer.
2. Remove the supernatant entirely and freeze the cell pellet at -80ºC for 6 hours or overnight.
3. Thaw the cell pellets at room temperature for 15-30 minutes until they detach from the wall.

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4. Suspend the cell pellets with an appropriate volume of 1x Lysis buffer (30 ul lysis buffer per 1ml bacteria culture) and incubate at room temperature for 30-60 minutes with gentle stirring.
5. Lyse cell by French press or sonication at 4°C.
   - Lyse cell by French press: Pass through French press 2-3x to ensure efficient lysis. French press should be chilled and operated at 4°C.
   - Lyse cell by Sonication: Sonicate the sample on ice using three 10-second bursts at high intensity and let the mixture cool down for 30 seconds on ice until the sample is not viscous.
6. Centrifuge at 16000 rpm for 15 minutes at 4°C.
7. Remove the supernatant containing soluble recombinant protein and transfer it to a new tube.
8. Save the cell pellet, which contains insoluble protein.

- Aspirate 10 µl of the supernatant from soluble and insoluble fraction, add an equal volume of 2x SDS sample loading buffer, boil for 5 minutes and run SDS-PAGE to determine the expression level and solubility of His-tagged protein. If protein is insoluble, follow the insoluble purification protocol described in BcMag™ IDA--Ni His-tagged protein purification magnetic beads (Cat. No MHN-105).

B. Protein purification

a. Magnetic beads preparation

1. Vigorously shake the bottle until the magnetic beads become homogeneous and transfer an appropriate volume of the magnetic beads (20 ul beads per 1ml bacteria culture) from the bottle to a new tube or flask.

   Note:

   - ***Optimize the number of beads used for each application. Too many beads will cause higher background. Insufficient beads will lead to lower yields.***
   - ***Do not allow the beads to sit for more than 3 minutes before dispensing. Resuspend the magnetic beads every 3 minutes.***

2. Place the tube on the magnetic rack for 1-3 minutes. Remove the supernatant while the tube remains on the rack. Add ten bead-bed volumes of H_2O_2 and shake it to mix the beads. Again, place the tube on the magnetic rack for 1-3 minutes and remove the supernatant while the tube remains on the rack.
3. Repeat step (2) one more time.
4. Equilibrate the beads by adding ten bead-bed volumes of 1x Equilibration buffer and shake it to mix the beads. Incubate at room temperature with continuous rotation for 2 minutes. Place the tube on the magnetic rack for 1-3 minutes. Remove the supernatant while the tube remains on the rack. The beads are ready for purification.

b. Purification

1. Add the equilibrated beads to the cell lysates (Step A (4) and incubate on Mini BlotBoy 3D Rocker with continuous rotation for 5-10 minutes.
2. Place the tube on the magnetic rack for 1-3 minutes. Remove the supernatant while the tube remains on the rack. Add ten bead-bed volumes of Wash Buffer and shake it ten times to wash the beads. Again, place the tube on the magnetic rack for 1-3 minutes and remove the supernatant while the tube remains on the rack.
3. Repeat step (2) six times.

   Note: It may be necessary to wash the beads more than six times for some proteins to reduce the nonspecific binding.

4. Elute protein with an appropriate volume of elution buffer (10 ul elution buffer per 1ml bacteria culture) by vigorously shaking by magnetic rack.

   Note: Optimize elution condition by adjusting the imidazole concentration for each protein, and it may be necessary to elute the protein 2-3 times.

5. Transfer the supernatant to a new tube.

C. Troubleshooting

Problem	Probable cause	Suggestion

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The yield of the purified protein is too low or undetectable in eluted protein solution by SDS-PAGE.	The open reading frame is wrong.	• Confirm sequence to ensure that the reading frame is correct. • Reclone and move the tag to the other end of the protein. • Purify the fusion protein under denaturing conditions.
	His tag is not accessible due to protein conformation	• Purify the fusion protein under denaturing conditions. • Reclone and move the tag to the other end of the protein.
	The protein does not bind to the Magnetic Beads.	• Check the pH of all the buffers and solutions.
	The protein is unstable.	• Add protease inhibitor to Binding/Washing Buffer.
	The protein is not efficiently eluted from beads.	• Increase imidazole concentration to 0.5-1.0 M in the Elution Buffer • Use 0.5-1.0 M sodium citrate (pH 4.0) to elute protein. After elution, immediately adjust pH to 7.0 using 1.0 M Tris-HCl (pH 9.0).
	The target protein appears in the washing buffer.	Reduce the concentration of imidazole. Increase pH slightly in Binding/Washing Buffer.
	The beads used are insufficient for purification	• Increase the number of magnetic beads.
Observe multiple bands in the eluted protein.	Degradation of the fusion protein	• Add appropriate protease inhibitor. • Use protease-deficient expression host.
	The washing condition is not optimized.	• Increase the concentration of imidazole. • Increase washing time and washing volume
Eluted proteins lose activity	Some proteins will lose or decrease activity when fused toHis-tag.	• Try to fuse His-tag at the other end of the protein. • Switch to other fusion tags, such as GST

Related Products	
IDA Magnetic Beads	Secreted His-tagged protein purification Kit
IDA-His-tagged protein purification Kit	Low Expression His-tagged Protein Purification Kit
GST-tagged Protein Purification Kit	

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