Protocol

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Affinity His-Tag Purification

Utilizing Nickel NTA Magnetic Agarose Beads

Introduction

The purification of recombinant target proteins can be achieved using a powerful polyhistidine tagging system that allows binding of target proteins labeled with a tag consisting of histidine residues to resins/agarose beads containing immobilized metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}). Use of Nickel NTA magnetic agarose beads in this process allows for rapid and easy small scale purification of His-tagged proteins. The protein lysate is first incubated with the magnetic beads, which capture the His-tagged proteins and render them immobile. Then, the beads are washed to eliminate non-specifically bound protein. Finally, His-tagged proteins are eluted from the magnetic beads. Purified proteins can then be analyzed using various methods including SDS-PAGE and Western blotting, or used in protein-protein interaction studies. Here, we present a protocol for purification of His-tagged protein that includes the preparation of the magnetic beads, purification of the protein, and recommendations for purification under native or denaturing conditions.

Materials

- Nickel NTA Agarose Beads (GoldBio Catalog # H-351)
- Binding/Equilibration Buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0)
- Washing Buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0)
- Elution Buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM imidazole, pH 8.0)
- Imidazole (GoldBio Catalog # I-902)
- Magnetic separator
- Urea
- See Table 2 for suitable reagents for specific studies

Method

These resins are adapted for purification performed under native or denaturing conditions. The imidazole concentration used in the binding buffer requires optimization. A low imidazole concentration during equilibration and washing steps will provide a higher binding capacity and a higher concentration will improve purity. The following general procedure is adapted to reach both a high binding capacity and high purity, and may be optimized depending on the specific protein being purified.

However, prior to describing the general procedure, we include various recommendations for purification of His-tagged proteins in native and denaturing conditions.

Native conditions

In native conditions, the presence of a low concentration of imidazole in the lysis and binding buffers (usually up to 20mM) will not affect the target fusion protein. If the tagged protein does not bind to the magnetic beads, then the imidazole concentration should be reduced to 5-10mM.

To prevent nonspecific interactions during binding and washing, the buffers should contain at least 300mM NaCl. Avoid increasing this concentration to 2M.

Denaturing conditions

In denaturing conditions, recombinant proteins often form insoluble inclusion bodies. If this occurs, render these soluble by purifying these tagged proteins under denaturing conditions using urea or guanidine chloride at relevant stages (see Table 2). First, cells are disrupted under native conditions using lysozyme with sonication. After centrifugation, the fused protein is extracted and solubilized by using a denaturing agent, such as urea.

Isolation of inclusion bodies

1. Thaw an E. coli cell pellet on ice (if frozen).

2. Resuspend 1 g of pelleted, wet cells in 5 ml buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0) by pipetting up and down or stirring until resuspension is completed without visible cell aggregates.

3. Add lysozyme to a final concentration of 1mg/ml and stir the solution on ice for 30 minutes.

4. Sonicate the suspension on ice and check the sample appearance after sonication. If the lysate is still viscous, add 5µg/ml DNase I and stir on ice for 15 minutes.

5. Centrifuge the crude lysate at 10, 000 g for 30 minutes at 4°C to collect the inclusion bodies. Discard the supernatant and keep the pellet on ice.

Solubilization of inclusion bodies

1. Resuspend the pellet in 10 ml of 50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0.

2. Centrifuge at 10, 000 g for 30 minutes at 4°C and discard the supernatant.

3. Add 2 ml (per g of wet cells) of 50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, 8M urea, pH 8.0. Homogenization or sonication may be necessary to resuspend the pellet. Dissolve the inclusion bodies by stirring on ice for 60 minutes.

4. Centrifuge at 10,000 g for 30 minutes at 20°C to eliminate any insoluble material and transfer the supernatant to a clean tube. Centrifuge until the supernatant is clear.

Note: This procedure is similar to purification under native conditions, except the sample and buffers contain 8M urea. In some instances, 8M urea is not sufficient to completely solubilize inclusion bodies. If this occurs, then the urea in the Denaturing Lysis buffer can be replaced with 6M guanidine hydrochloride. However, the samples that contain guanidine hydrochloride cannot be applied to SDS-PAGE directly. Dilute the sample or precipitate it with trichloroacetate to remove guanidine hydrochloride.

General Procedure

Preparation of magnetic beads

Determine the quantity of Nickel NTA magnetic agarose beads (5%) needed for your purification. This procedure is described to work with 10µl of magnetic beads.

Note: Binding capacity will vary for each His-tagged protein. The yield of His-tagged protein depends on various parameters such as amino acid composition, 3D structure, molecular weight, etc. Nickel NTA agarose magnetic beads are supplied as a 5% suspension of magnetic beads in 20% ethanol and have a binding capacity of up to 70mg/ml (6 x His-GFP).

Note: Adjust the sample to the composition and pH of the equilibration buffer. Clarification of sample may be needed before applying it to the magnetic beads.

Note: To determine bead suspension volumes suitable for specific protein expression levels, follow table 1). Volumes can be linearly scaled up or down depending on culture volumes.

Table 1. Volumes required for specific protein expression levels.

Protein Expression Level	µg His-tagged Protein/10 ml culture	Volume Nickel NTA Magnetic Agarose Suspension/10 ml culture	Minimum Elution Volume/10 ml culture
< 0.5 mg/L	< 5 µg	10 µl	25 µl
1 mg/L	10 µg	20 µl	25 µl
5 mg/L	50 µg	100 µl	50 µl
10 mg/L	100 µg	200 µl	100 µl
50 mg/L	500 µg	1 ml	500 µl

1. Gently shake the bottle containing Nickel NTA magnetic agarose beads to achieve a homogenous suspension.

2. Once suspended, pipette 200 µl of the original Nickel NTA magnetic agarose suspension to an appropriate tube.

3. Place the tube in a magnetic separator or use a magnet to remove the preservative from the beads.

Equilibration

1. Remove the tube from the magnetic separator and add 500 µl of equilibration buffer.

2. Mix the bead slurry thoroughly to achieve a homogeneous suspension.

3. Place the tube in the magnetic separator and discard the supernatant.

Note: Equilibration (binding) buffer composition is 50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0. The composition of this buffer depends on the particular properties of the protein. The buffer used most frequently contains 50mM NaH_2PO_4. The pH must generally be neutral (7.0 – 8.0).

Note: To increase the selectivity of the binding of the target protein, it is necessary to add a small concentration of imidazole (10 – 40mM). We recommend 20mM as a general guideline but the concentration must be optimized. If the tagged protein does not bind, then the amount of imidazole should be reduced to 5 -10mM. High purity imidazole must be used to prevent any effects on the absorbance at 280 nm. It is important to avoid the presence of agents like EDTA or citrate at all times.

Purification

Once the resin is equilibrated, the sample containing the tagged protein is applied. In some cases, a slight increase of incubation (contact) time may facilitate binding. Binding capacity may be affected by several factors including sample concentration, binding buffer or contact time.

1. Once equilibration is completed, add the clarified *E. coli* lysate or protein extract.

2. Mix the suspension gently for 30 minutes at room temperature or 1 hour at 4°C.

Note: A slight increase in incubation (contact) time may facilitate binding.

3. Place the tube in a magnetic separator or use a magnet to remove the supernatant. Discard the supernatant.

4. Remove the tube from the magnetic separator.

5. Wash the magnetic beads by adding 500 µl of washing buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0) and mix by vortexing.

6. Place the tube in the magnetic separator again and discard the supernatant.

7. Repeat steps 4 – 6 twice.

8. Remove the tube from the magnetic separator.

9. Add 100 µl of elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM imidazole, pH 8.0.) to the magnetic beads, mix thoroughly, place in the magnetic separator.

10. Collect the supernatant or elution fraction and store on ice.

11. Repeat steps 9 and 10 twice or more and collect each elution fraction in a separate tube to determine the protein concentration of each fraction.

Note: It is possible that a significant amount of His tagged protein may remain bound to the resin. Conditions (volumes, times and temperatures) used for elution may vary among His-tagged proteins.

12. Monitor the eluates using the Bradford protein assay, SDS-PAGE or by measuring the absorbance at 280 nm to determine the yield of the eluted His-tagged protein.

13. For proper storage, remove imidazole by ultrafiltration or dialysis.

Note: For most of the applications it is not necessary to eliminate the His tag. However, elimination of the tag is necessary for certain applications including X-ray crystallography or RMN, where the structure of the protein is determined. To eliminate the tag, use a protease to splice at a protease cleavage site.

Note: For purification under denaturing conditions, the procedure is completed as described. However, the composition of buffers changes as follows:

a) Binding buffer: 100mM NaH_2PO_4, 10mM Tris base, 8M urea, pH 6.3.
b) Washing buffer: 100mM NaH_2PO_4, 10mM Tris base, 8M urea, pH 6.3.
c) Elution buffer: 100mM NaH_2PO_4, 10mM Tris base, 8M urea, pH 4.5.
Due to urea dissociation, adjust the pH immediately before use.

Table 2. Compatibility of reagents with Nickel NTA magnetic beads.

Studies	Reagents	Comments
Buffers	- Sodium phosphate	- Sodium phosphate buffer 50mM pH 8.0 is recommended.
	- Tris, HEPES, MOPS	- Coordinate with metal ions, causing a decrease in binding capacity. Up to 100mM may be used.
	- Sodium Chloride	- Avoids unspecific binding (ionic interactions). - At least 300mM should be used. Up to 2M can be used.
Denaturing Agents	- Urea	- Solubilizes protein. Use 8M for purification under denaturing conditions.
	- Guanidine-HCl	- Solubilizes protein. Up to 6M can be used.
Additives	- Imidazole	- Competes with His-tagged protein. - Reduces nonspecific binding (20mM). - Elute His-tagged protein (100mM).
	- Glycerol	- Prevents hydrophobic interactions between proteins. - Up to 50% can be used.
	- EDTA	- Coordinates with cations, causing a decrease in capacity. - Not recommended, but up to 1mM has been used successfully.
	- Ethanol	- Prevents hydrophobic interactions between proteins but may cause precipitation. - Up to 20% can be used.
Reducing Agents	- Reduced glutathione	- Can reduce Ni^{2+} ions at higher concentrations. - Up to 30mM has been used successfully.
	- B-mercaptoethanol	- Prevents formation of disulfide bonds. Can reduce Ni^{2+} ions at higher concentrations. - Up to 20mm in samples has been used successfully in some cases.
	- DTT, DTE	- Can reduce Ni^{2+} ions at higher concentrations. - Up to 10mM in samples has been used successfully.
	- SDS	- Prevents hydrophobic interactions.

		- Coordinates with cations, causing a decrease in capacity. Not recommended but up to 0.3% in samples has been used.
Detergents	- Nonionic detergents (Tween, Triton, etc.)	- Removes background proteins. Up to 2% can be used.

Troubleshooting

We delineate the potential problems at each step in the protocol that might explain poor performance and possible solutions.

Table 3. Troubleshooting guide.

Observation	Possible Causes	Recommendation
High viscosity sample	- Presence of DNA in the sample/lysate.	- Lysate may remain viscous. Add 5µg/ml DNase I and incubate on ice for 10 minutes.
	- Presence of insoluble material in the sample/lysate.	- Use centrifugation or filtration (0.45 µm membrane) to avoid clogging of the column.
Highly diluted or concentrated sample	- Highly diluted sample	- It is preferable to concentrate the sample before its purification in the column.
	- Highly concentrated sample	- It is preferable to make a previous dilution of the sample before its purification in the column.
Target protein not bound to the column	- His-tag is not present or has been degraded.	- Check it. If the tag has been degraded, perform the purification at lower temperatures (4°C) reducing degradation. Try not to reduce the purification time.
	- His tag is not exposed (inaccessible).	- Purify in denaturing conditions or add the tag on a different site (N-terminus, C-terminus, or in both positions).
	- Inadequate binding conditions.	- Check the buffer and binding pH. - If the binding has been done in the presence of imidazole, reduce its concentration or eliminate it in this step. - Verify if some of the reagents used in the adsorption step interferes with the binding reaction.
High amount of co-eluted proteins (contaminants)	- Insufficient washing stage.	- Increase volume of washing buffer. - Increase the concentration of imidazole in the buffer during washing and equilibrating steps.

	- Inadequate adsorption conditions.	- Add or increase saline concentration in the binding buffer to prevent nonspecific ionic interactions. - Low concentrations of nonionic detergents can also be added. - Add small quantities of glycerol in the binding buffer to avoid nonspecific hydrophobic interactions. - Increase imidazole concentration in the binding buffer. Note: In general, higher imidazole concentrations can compete with the binding of the target protein. This concentration can be modified with the type of protein to be purified.
Target protein binds only partially to the resin	- Resin capacity is exceeded.	- Apply less fused protein to the resin.
	- The resin has been previously used during several purification cycles without regeneration. This causes a diminution of the binding capacity. This diminution varies in each case and increases with the number of purification cycles of the resin.	- Use fresh resin.
	- Loss of chelating metal in the resin.	- Apply a regeneration step in the resin. Avoid use of reducing chelating agents.
	- Histidine tail is not very exposed.	- Increase the contact time between resin and fused protein. Note: A greater exhibition would be obtained working in denaturing conditions.
	- Poor protein expression.	- Optimize bacterial expression conditions.
	- The fused protein forms inclusion bodies.	- Modify bacterial growth conditions. - Work in denaturing conditions.
High amount of co-eluted proteins (contaminants)	- The resin used in the purification shows low selectivity to bind the fused protein.	- Employ Single Step Elution procedures to separate the target protein from the rest of retained proteins.

Target protein elutes poorly	- Too smooth elution conditions. Sometimes protein binding with chelating metals is too strong. Note: Also the position of the histidine tail can influence the strength of the binding of the target protein.	- Increase imidazole concentration or reduce pH in the elution step. - Try, if possible, an elution at a higher temperature. - Make an elution with a chelating agent such as EDTA. - Increase imidazole concentration up to 1M in the elution buffer. - Elute in denaturing conditions. - Add solubilizing agents (see compatibilities). - Incubate the resin with the elution buffer for 8-10 hours and elute with the elution buffer.
Elution profile is not reproducible in different cycles of purification	- Samples nature could have been modified. The histidine tail could have been lost due to protease action.	- It is necessary to prepare a fresh sample. Run the protocol at 2-8°C. Add protease inhibitors (see Table 2).
	- Proteins or lipids could have precipitated.	- Use fresh resin.
	- pH or ionic forces could have been modified.	- Prepare new buffers.
	- Samples do not contain same tagged protein.	- Keep all the parameters and same conditions.

Associated Products

- Nickel Agarose Beads (GoldBio Catalog # H-351)
- Imidazole (GoldBio Catalog # I-902)

References

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