PureCube Ni-INDIGO MagBead-based protein purification

Fast purification of His-tagged membrane proteins in reaction tubes

1. Description

PureCube Ni-INDIGO MagBeads are ferrimagnetic spheres covered with 6% agarose and coupled with nickel ions allowing efficient immobilization and purification of recombinant His-tagged proteins. It selectively binds His-tagged proteins through coordination between immobilized nickel ions and the imidazole side chains of histidine residues. Magnetic particles are utilized to easily separate the beads from the surrounding liquid with the help of a magnet, making PureCube Ni-INDIGO MagBeads perfectly suitable for batch purifications. They exhibit a binding capacity of approximately 80 mg/mL, determined using a 27 kDa 6×His-tagged GFP protein, and provide higher protein purity compared to conventional Ni-NTA resins. A key advantage of the INDIGO ligand is its tolerance to various buffer components, including EDTA, DTT, phenanthroline, guanidinium hydrochloride, Triton, urea, and acetonitrile. Furthermore, the Ni-INDIGO resin can be regenerated using an established regeneration protocol, allowing repeated use while maintaining consistent binding capacity.

This protocol describes the purification of His-tagged proteins under native (see 3.1.) or denaturing (see 3.2.) conditions.

2. Required material and recommendations

All necessary buffers for protein purification and subsequent regeneration of the resin are listed in the following tables and should be prepared freshly.

Table 1: Buffers/solutions for purification under native conditions (3.1.)

Buffers/solutions	
Binding Buffer	50 mM phosphate buffer pH 8, 300 mM NaCl, 10 mM imidazole (*Optional*: Add protease inhibitor cocktail)
Wash Buffer	50 mM phosphate buffer pH 8, 300 mM NaCl, 20 mM imidazole
Elution Buffer	50 mM phosphate buffer pH 8, 300 mM NaCl, 250 mM imidazole

Table 2: Buffer/solutions for purification under denaturing conditions (3.2.)

Buffers/solutions	
Denaturing Binding Buffer	100 mM phosphate buffer pH 8, 10 mM Tris base, 8 M urea
Denaturing Wash Buffer	100 mM phosphate buffer pH 6.3, 10 mM Tris base, 8 M urea
Denaturing Elution Buffer	100 mM phosphate buffer pH 4.5, 10 mM Tris base, 8 M urea

The composition of all purification buffers can be modified to suit the properties of the target protein (to solubilize inclusion bodies, 8 M urea contained in the Denaturing Binding Buffer can be replaced with 6 M guanidine hydrochloride (GuHCl)). Ni-INDIGO tolerates up to 20 mM EDTA and 20 mM DTT. A full list with compatible reagents is available at https://cube-biotech.com/products/protein-purification-products/his-tag-affinity-resins-magbeads/ni-indigo/purecube-100-ni-indigo-agarose/75103#datasheet-tab-content. Please note that the resin tolerates pH 2-14, while protein binding is stable at pH 5-8. A pH below 5 can lead to a reduced binding capacity.

Table 3: Buffers/solution for regeneration (3.3.)

Buffers/solutions	
Regeneration Buffer	500 mM NaOH
Neutralization Buffer	200 mM phosphate buffer pH 7.0, 150 mM NaCl

For fast and simple separation of magnetic beads from buffer or sample, we recommend the application of our Cube MagBead Separator (Cat. No. 16941) if the protein purification occurs in reaction tubes (1.5-2 ml). If a larger number of samples should be purified, we recommend the use of round-bottom 96-well plates and an appropriate magnetic device. Alternatively, we also offer ready-to-use 96 well plates pre-filled with magnetic beads (PureHT™ Ni-INDIGO Plate, Cat. No. 90030 (KingFisher) or Cat. No. 90010 (Nunc)).

You can adjust the volume of magnetic beads according to your target protein concentration. For proteins >90 kDa, optimal binding might not be reached under the conditions described. More information on how to improve your protein yield can be found in section 4.

3. Protocol

3.1. Protein purification under native conditions

- 20 µL of the 25 % suspension contain 5 µL magnetic beads.
- Thorough mixing of the sample is essential to optimize the yield.
  For low sample and magnetic bead volumes, best results can be achieved by incubating the samples on a thermoshaker at 1000-1300 rpm.

3.1.1. Resuspend the magnetic beads by by vortexing. For **250 µL cleared lysate** (prepared with Binding Buffer), pipette **20 µL of the 25 % suspension** (5 µL beads) into a reaction tube, place it on the magnetic separator to separate the beads and remove the supernatant.

3.1.2. Equilibrate beads in **500 µL Binding Buffer**. Separate the beads in the magnetic separator and remove the supernatant. Repeat this step twice.

3.1.3. Resuspend the magnetic beads with the **250 µL cleared lysate** containing the target protein. Incubate for **15 min - 1h at 4°C**. Keep the beads in suspension by inverting the tube occasionally, or by placing the tube on a roller.

Depending on protein type and concentration, incubation time of 15 min can be sufficient. To maximize yield, incubate for 1h.

3.1.4. Place the reaction tube in the magnetic separator and carefully remove the supernatant.

3.1.5. Remove tube from the magnet and add **500 µL Wash Buffer**. Resuspend carefully and place the reaction tube in the magnetic separator to collect the beads. Remove the supernatant and repeat this step twice.

3.1.6. Elute the His-tagged protein using **100 µL Elution Buffer**. Repeat elution once and collect each elution fraction in separate tubes to determine the protein concentration of each fraction.

3.2. Protein purification under denaturing conditions

- 20 µL of the 25 % suspension contain 5 µL magnetic beads.

3.2.1. Resuspend the magnetic beads by by vortexing. For **250 µL cleared lysate** (prepared with Denaturing Binding Buffer), pipette **20 µL of the 25 % suspension** (5 µL beads) into a reaction tube, place it on the magnetic separator to separate the beads and remove the supernatant.

3.2.2. Equilibrate beads in **500 µL Denaturing Binding Buffer**. Separate the beads in the magnetic separator and remove the supernatant. Repeat this step twice.

3.2.3. Resuspend the magnetic beads with the **250 µL cleared lysate** containing the target protein. Incubate for **1h at 4°C**. Keep the beads in suspension by inverting the tube occasionally, or by placing the tube on a roller mixer.

3.2.4. Place the reaction tube in the magnetic separator and carefully remove the supernatant.

3.2.5. Remove tube from the magnet and add **500 µL Denaturing Wash Buffer**. Resuspend carefully and place the reaction tube in the magnetic separator to collect the beads. Remove the supernatant and repeat this step twice.

3.2.6. Elute the His-tagged protein using **100 µL Denaturing Elution Buffer**. Repeat elution once and collect each elution fraction in separate tubes to determine the protein concentration of each fraction.

3.3. Regeneration and storage

- Use **freshly prepared buffers** for all steps.
- All solutions should have a temperature of 4 °C or ambient temperature! Hot sodium hydroxide can harm resin and chromatography material!

3.3.1. Wash magnetic beads with **500 µL** (100 µL per 1 µL magnetic beads) ddH_2O.

3.3.2. Regenerate magnetic beads by washing with **500 µL** 500 mM NaOH (freshly prepared).

3.3.3. Immediately wash magnetic beads with **500 µL** ddH_2O.

3.3.4. Equilibrate the magnetic beads with **500 µL** neutralization buffer.

3.3.5. Wash with **500 µL** ddH_2O.

3.3.6. **For Storage: Equilibrate** magnetic beads in 20% (v/v) ethanol with 10 mM sodium acetate, pH 6.5 and store at 2–8 °C.

4. Optimization

Starting from the standard protocol described in section 3, binding efficiency can be optimized for each individual protein.

- If the protein concentration in the sample is known, adjust the bead volume accordingly. For optimal yield, the total bead binding capacity should be around 5 times the total amount of protein. For example, if the total amount of a 30 kDa protein is 80 µg, which theoretically could be bound by 1 µL of beads, add 5x 1 µL = 5 µL beads and incubate for 1 h to achieve the best yield.
- For samples with a low target protein concentration, a smaller bead volume should be added to reduce contamination of host cell proteins.
- For large proteins >90 kDa, the binding capacity may decline. If a low yield is observed, increase the bead volume.

5. Troubleshooting

No or weak binding to Ni-INDIGO	
pH is not correct	The resin tolerates pH 2-14, while protein binding is stable at pH 5-8. A pH below 5 can lead to a reduced binding capacity.
Imidazole concentration in Binding buffer is too high	Binding buffer contains 10 mM imidazole to prevent binding of untagged proteins. If His-tagged proteins do not bind under these conditions, reduce the imidazole concentration to 1–5 mM.
His-tag is not present.	Add protease inhibitors during cell lysis and work quickly at 2-8 °C. If E. coli is used as expression host, use a protease deficient expression strain.
His-tag is not accessible.	Fuse the tag with the other protein terminus, use a different linker, or increase the number of histidine (His_6 to His_{10}).
His-tag has been degraded.	Check if the tag is associated with a portion of the protein that is processed. If it is the case, change the position of the tag. Avoid purification in discontinuous batch mode. Prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag.
His-tag is partially accessible.	Reduce the washing volume.
Protein degradation and aggregation	The protein of interest is degraded by proteases that can be found inside the cell lysate. These need to be inhibited. As some proteases usually work with cations as co-factors, EDTA will chelate these cations and thus inactivate the proteases. If this is the case, we recommend adding 1 - 5 mM EDTA to the Binding Buffer. It is recommended to combine 5 kinds of different protease inhibitors or utilize a ready-to-use commercial protease inhibitor cocktail. When the protein of interest needs divalent cations to function or fold properly, do not use EDTA.
Protein aggregation	Sometimes proteins can aggregate in a lysate, which prevents proper binding to the INDIGO beads. You should screen the buffer conditions with regard to co-factors,

	detergents and stabilizers such as glycerol. To counter protein aggregation, you can also add around 1 - 5 mM DTT to all buffers, if the target protein contains cysteines.
The magnetic beads are not properly regenerated.	Wash the beads with 100 µL/µL MagBeads 25-50 % acetic acid. Immediately wash with 100 µL/µL MagBeads ddH_2O followed by 100 µL/µL Magbeads 20 mM Tris (pH 7) and ddH_2O, respectively. After the last wash store the magnetic beads in storage buffer (20 mM acetate, 20 % (v/v) ethanol, pH 6.5).
Contaminating proteins	
Contaminants are short forms of the tagged protein	When working with *E. coli*, use protease deficient expression strains. Add protease inhibitors before/after cell lysis. Fuse His-tag with the other protein terminus. Check if internal translation initiation starts (only in case of C- terminal tag) or premature termination sites (only in case of N- terminal tag) are present. Add another tag to the other terminus and use both tags for purification.
Contaminants are covalently linked to the recombinant protein via disulfide bonds.	Add reducing reagents to all buffers necessary for cell lysis and protein purification.
Contaminants are non-covalently linked to the recombinant protein.	Increase the ionic strength of all buffers (up to 5 M NaCl) or add mild detergents (up to 2% Triton X-100, 2% Tween 20, 0.1% CHAPS, etc.).
Contaminants are histidine-rich proteins.	Increase the number of histidine (His_6 to His_{10}) and repeat the wash steps multiple times