HisCube Ni-INDIGO

His-tag MagBeads MINI Kit

Protocol by Cube Biotech

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Contents

1 x 5 ml PureCube Ni-INDIGO MagBeads
2 x 50 ml Binding Buffer
2 x 50 ml Wash Buffer
1 x 50 ml Elution Buffer
500 mM EDTA (add 0,866 ml ddH2O)
500 mM DTT (add 1,016 ml ddH2O)
1x Cube MagBead Separator

Required equipment

Pipettes & matching tips
Table centrifuge
1.5 or 2 ml tubes
Syringe + Filter for filtration
End-over-end shaker
Optional: Protease Inhibitor

Storage temperature

4 °C / 39 °F

Online Version

Scan to download the protocol

Support

https://cube-biotech.com/ Live Chat Support
contact@cube-biotech.com

1. Introduction

We are proud to present the HisCube MagBead Kit, the ultimate solution for purifying His-tag proteins. Our INDIGO ligand, developed in-house, guarantees maximum purity and yield in IMAC-based purifications.

The PureCube Ni-INDIGO MagBeads are composed of 6% cross-linked magnetic agarose beads with an average diameter of 30 µm, available in a 25% concentrated suspension. These beads boast a protein binding capacity of over 100 mg/ml of pure beads.

1.1 What sets this kit apart from other His-tag purification kits?

The novel feature that sets this kit apart from traditional His-tag kits is our proprietary INDIGO ligand.
The INDIGO ligand offers a major advantage over other ligands such as NTA or IDA, as it can tolerate up to 20 mM EDTA and 20 mM DTT contained in the purification buffers and has a higher tolerance for broader pH values (pH 6.8 to 8.0).
In contrast, NTA can only tolerate 10 mM DTT and 1 mM EDTA. The benefits of using DTT and EDTA will be explained in section 2.1 of the manual.

2. Contents

The magnetic separator is made of polypropylene with little yet strong neodym magnets designed for use in laboratory research and for applications involving magnetic beads.
The MagBead separators provide a rapid and inexpensive alternative to typical gravity- or pressure-based chromatographic separations.
Our separators are designed for 1.5 and 2 ml reaction tubes.

Buffers: The HisCube Ni-INDIGO MagBeads Kit contains three different buffers. Each buffer is optimized for our Ni-INDIGO Beads. Note that the Elution Buffer contains 250 mM Imidazole, the Wash Buffer 20 mM Imidazole, and the Binding Buffer 10 mM Imidazole respectively. Please read the section "3.2 Protein Quantification" regarding the effect of Imidazole on protein quantification and how to overcome it.

2.1 Why are EDTA & DTT included in this kit?

EDTA & DTT, despite having numerous advantages in many of the buffers used for protein purification, present limitations for traditional NTA and IDA ligands and have prevented their use in His-tag purifications. Despite this, let us examine the benefits they bring.

DTT, when included in buffers, helps to prevent protein aggregation by breaking down unnatural disulfide bonds that can form between proteins during purification, due to the absence of the natural reductive environment of the cytoplasm.

Note

DTT becomes unstable after being dissolved in water. Only add it to the buffers directly prior to its use. Freeze the remaining stocks at -20°C.

Unfortunately, DTT can also reduce the nickel cations of NTA or IDA beads, causing the loss of their functionality.

EDTA chelates cations that are needed in active centers of metallo proteases and is therefore added to many protein purification buffers to prevent protein degradation.
However, it also removes the nickel cation from ligands such as NTA or IDA, causing the purification beads to lose their functionality.

Both EDTA & DTT come lyophilized. Simply add **the given volumes of ddH_2O** to the powders to get a **500 mM stock solution** of each substance. Both their working concentration in the buffers should be around **1 to 5 mM.**

2.2 The Ni-INDIGO MagBeads

The INDIGO ligand is a novel development of Cube Biotech and is the solution to many problems that afflict His-tag protein purifications. As shown in Figure 1, His-tag purifications using the INDIGO ligand are much purer than traditional Ni-NTA.

Ni-INDIGO MagBeads are composed of 6% cross-linked magnetic agarose with an average bead diameter of 30 µm. The MagBeads can be used in magnetic separations as demonstrated in this kit. It can bind up to **100 mg protein per ml** of pure MagBeads used.

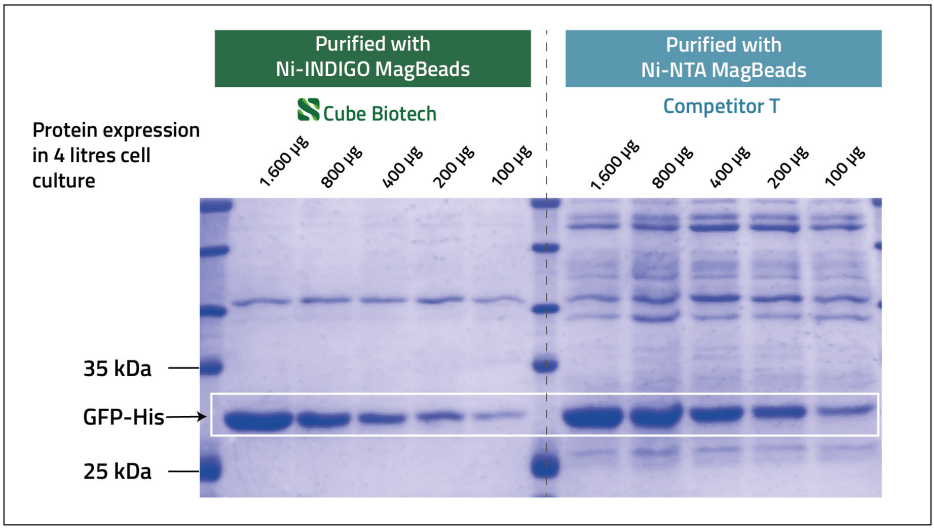

Figure 1: Ni-INDIGO MagBeads (left) compared to Ni-NTA from another competitor (right). This test compared the protein yields and purity of both using controlled amounts of His-tagged GFP in the corresponding cell lysates.

3. Procedure / Protocol

Every protein purification assay starts with the extraction of the protein of interest from the expression cell.
This procedure is dependent on the used expression system. Our Ni-INDIGO MagBeads are compatible with protein lysates from *E. coli*, mammalian cell lines, insect cells, and yeast, to name a few.

Possible mechanical disruption methods are freeze/thaw cycles, sonication, French press, or glass bead lysis (yeast). Please refer to your own lysis protocol.
For small-scale purification, we recommend sonication in Binding Buffer.
The Binding Buffer is essential for pellet resuspension and cell opening.

3.1 Purification steps

Note

The binding capacity of 50 µl pure Indigo MagBeads is up to 5 mg. Depending on the protein's expression level adjust the amount of MagBeads used. The amounts suggested here are for a high expression level.

1. Shake the Ni-INDIGO MagBeads flask well to mix the settled beads with the Storage Buffer. **If** you are using EDTA or DTT add them now to the Binding, Wash, and Elution Buffers.

2. Place your reaction tube of 1.5 or 2 ml in the separator.

3. Pipet 200 µl of the 25% Ni-INDIGO MagBeads slurry into the reaction tube. Let the MagBeads separate from the Storage Buffer until the supernatant is clear. Remove the supernatant.

Note

Step 3 is critical to ensure that all ethanol from the Storage Buffer is removed from the MagBeads to avoid interference with the membrane technology. Ni-INDIGO Storage Buffer contains 20% Ethanol.

4. Resuspend the remaining MagBeads with 1.5 ml Binding Buffer by shaking the reaction tube for at least 30 seconds end-over-end. Then separate the supernatant and discard it.

5. Repeat Step 4 once to remove any residual ethanol.

6. Immediately **before** loading: Re-filter the sample through a 0.2 µm filter (e.g. syringe filter) to remove any solid material that might interfere with later measurements.

6. Load the required volume of filtered sample. The maximum sample volume is 1.8 ml, depending on your chosen reaction tube. Close the lid and vortex for 15 seconds to mix the sample and the MagBeads.

8. Repeat the vortexing of Step 7 every 15 minutes for 1 hour at 4°C or for 30 minutes at room temperature. Some proteins may require longer incubation times. A sign that longer incubation times may be needed is e.g. if much of your protein of interest remains in the flow-through. In this case, choose one of the following alternatives:

   A. Repeat the vortexing every 30 min to 1 hour and take samples regulary over a time span of 3 hours. Analyse these samples e.g via Coomassie Blue staining/ Western Blot to determine the optimal incubation time. You should see a decrease of your protein of interest in the supernatant over time since it binds to the MagBeads.

   B. Incubate on an end-over-end shaker at room temperature or at 4°C for up to 3 hours, depending on the temperature stability of the protein. Take samples regulary and analyse them as decribed in A.

9. Separate the supernatant from the MagBeads with the magnet. Save the supernatant for later control, this is the flow-through.

10. Load the reaction tube with up to 1.2 ml of Wash Buffer. Vortex or invert the closed tube a couple of times. Then separate the supernatant from the MagBeads with the magnet until you have a clear supernatant. It is best to save the supernatant. This supernatant is called Wash Fraction.

Note

Keep aliquots of the individual wash fractions for subsequent Coomassie stain analysis.

11. Repeat the wash step at least two times to ensure the removal of unspecifically bound protein. If applicable, check the samples for protein content using a UV-spectrophotometer. Absorbance of the undiluted sample at 280 nm should be < 0.1.

Note

You can elute the protein in small volumes of Elution Buffer in order to achieve a high protein concentratin. Elution in small volumes can be advantageous as no concentration step is required afterwards.

12. Elute the target protein by adding up to 100 µl Elution Buffer for 10 to 30 minutes under rotation with occasional vortexing. Then separate it with the magnet. Repeat the elution step up to 5 times. Then separate the eluate from the MagBeads with the magnet.

13. Save each eluate fraction in a separate new tube and determine the protein. (Refer to 3.2 Protein Quantification to determine the protein concentration)

3.2 Protein Quantification

The Elution Buffer in this kit containing 250 mM Imidazole typically has an A_{280} of 0.2-0.4. To assure a reliable quantification of your eluted protein you have several options:

A. Perform a dialysis or size exclusion chromatography against buffer without Imidazole followed by a UV-VIS measurement at 260-280 nm. The removal of Imidazole is advised for future applications.

B. Perform a Bradford Protein assay. This assay is based on the formation of a complex between Coomassie Brillant Blue R250 and your eluted proteins leading to a shift of the absorption maximum from 470 nm to 595 nm after protein binding in acidic medium. The Bradford protein assay is more tolerant towards high Imidazole concentrations than UV-VIS measurement, the Lowry or the Biuret assay.

C. If you have a high concentration of eluted protein it is still possible to measure a UV-VIS spectrum while performing the blank with Elution Buffer. Keep in mind that the measurement will be less accurate compared a UV-VIS measurement with Imidazole free buffer and an Imidazole free sample.

4. Troubleshooting

Before: If your problem cannot be solved by this troubleshooting guide, feel free to state your problem directly to: contact@cube-biotech.com
or visit our webpage www.cube-biotech.com and contact our chat support.

Protein Yield: Problems with the protein yield can be numerous, follow this checklist to avoid the most common ones:

- **Expression:** Always confirm that your protein is indeed expressed. Check your lysate e.g. through a Coomassie stain, to confirm the presence of your protein of interest.
- **Expression II:** Afterwards you should perform a Western Blot with an anti-His antibody (example Cube Biotech product 40040) to exclude the possibility of a false-positive result of the Coomassie stain.
- **Tag Accessibility:** Occasionally the 3D structure of a protein can mask the His-tag and therefore hinder the Ni-INDIGO Bead's access to the tag. There are two ways to prevent this: First, change the terminus of the protein which the His-tag is attached to. Second, add a spacer sequence between the protein's terminus and the His-tag.

Protein Purity: Add additional washing steps, by repeating Step 10 or slightly increasing the Imidazole concentration of the Wash Buffer to get rid of unwanted molecules with less affinity to the MagBeads.

Note

Imidazole affects the pH of your buffer, check upon addition.

MagBeads Residue: Sometimes the separation step is too short. The measurement is falsified by MagBeads that are left over in the supernatant. Remember to follow Step 6 of the protocol and use proper separation times to prevent this.

Protein Aggregates: Sometimes proteins can aggregate in a lysate. This prevents proper binding to the Ni-INDIGO MagBeads. To counter this, add around 1 to 5 mM DTT to all buffers. However, if your protein of interest is not prone to forming disulfide bridges, then adding DTT is not necessary.

Active Proteases: Sometimes the protein of interest is degraded by proteases that can be found inside the cell lysate. These need to be inhibited. As 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 to 5 mM EDTA to at least the Binding Buffer.

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