MoBiTec Ni-IDA Columns

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

1. Features

- Excellent tool for routine purification of recombinant polyhistidine-tagged proteins
  - under native and denaturating conditions
  - starting from diverse expression systems, e.g., *E. coli*, yeast, insect and mammalian cells
- Maximal binding capacity: 90 mg protein per column
- Protein recovery rate > 80%
- Improved target specificity by optimized silica-based Ni^{2+}-IDA matrix
- Imidazol free loading and washing buffer
- Columns are long-term storable when kept dry

2. Introduction

MoBiTec Ni-IDA Columns with silica-based resin provide a fast and convenient routine tool for purification of recombinant polyhistidine-tagged proteins by gravity flow. The form-stable silica matrix is precharged with Ni^{2+} ions and allows purification on the principle of Immobilized Metal Ion Affinity Chromatography (IMAC). Binding of proteins is based on the interaction between the polyhistidine tag of the recombinant protein and immobilized Ni^{2+} ions. The chelating group of the Ni-IDA resin is based on IDA (iminodiacetic acid), which enables strong and efficient binding of target protein onto the IMAC matrix.

In contrast to traditional IDA matrices, MoBiTec Ni-IDA is an optimized matrix with low density of IDA ligands. This non-saturating surface concentration of IDA eliminates almost all non-specific interactions of contaminating host proteins with the adsorbent. As a result, MoBiTec Ni-IDA provides higher target protein purity.
IDA is a tridentate chelator which occupies three of the six binding sites in the coordination sphere of the Ni^{2+} ion. The remaining three coordination sites are usually occupied by water molecules and can be exchanged with histidine residues of the recombinant protein (Fig. 1).

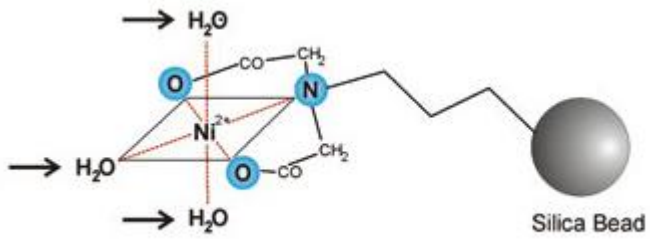

Fig.1: Silica-based Ni-IDA matrix. Schematic drawing of IDA in complex with Ni^{2+}.

3. Product Contents

3.1. Kit components

Order#	Product	Components included
PR-HTK004	MoBiTec Ni-IDA Columns	4 columns with matrix (dry), prepacked; user manual
PR-HTK010	MoBiTec Ni-IDA Columns	10 columns with matrix (dry), prepacked; user manual

3.2. Storage and expiry

MoBiTec Ni-IDA Columns are storable at room temperature for at least 1 year.

3.3. Equipment and materials to be supplied by user

- Microliter pipettes
- Appropriate centrifuge (≥ 10000 x g) and tubes for collection and centrifugation (size is depending on culture volume)
- Sonicator
- Lysozyme
- Optional: Phenylmethylsulfonyl fluoride (PMSF)
  DNase I
  0.45 µm membrane filter
- 500 mM EDTA and 5 mM $MgSO_4$ (only for preparation of periplasmic proteins)
- Buffers according to protocol (composition of all buffers see 3.4.)

3.4. Buffer compositions

Lysis-Equilibration-Wash Buffer (LEW Buffer)

Required for the following protocol: **"6.3.1. Preparation of cleared lysates under native conditions", "6.3.2. Preparation of cleared lysates under denaturing conditions"**

- 50 mM NaH_2PO_4
- 300 mM NaCl
- adjust pH to 8.0 using NaOH

Elution Buffer

Required for the following protocol: **"6.3.1. Preparation of cleared lysates under native conditions"**

- 50 mM NaH_2PO_4
- 300 mM NaCl
- 250 mM imidazole
- adjust pH to 8.0 using NaOH

Denaturing Solubilization Buffer

Required for the following protocol: **"6.3.2. Preparation of cleared lysates under denaturing conditions"**

Please prepare shortly before use!

- 50 mM NaH_2PO_4
- 300 mM NaCl

- 8 M urea
- adjust pH to 8.0 using NaOH

Denaturing Elution Buffer
Required for the following protocol: **"6.3.2. Preparation of cleared lysates under denaturing conditions"**
Please prepare shortly before use!
- 50 mM NaH_2PO_4
- 300 mM NaCl
- 8 M urea
- 250 mM imidazole
- adjust to pH to 8.0 using NaOH

Sucrose Buffer
Required for the following protocol: **"6.2. Purification of periplasmic polyhistidine-tagged proteins"**
- 30 mM Tris/HCl
- 20% sucrose

Adjust pH to 8.0

4. Terms and Conditions

For research use only. NOT recommended or intended for diagnosis of disease in humans or animals. Do NOT USE internally or externally in humans or animals. All chemicals should be considered as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products. Suitable protective clothing such as laboratory overalls, safety glasses, and gloves should be worn. Care should be taken to avoid contact with skin or eyes; if contact should occur, wash immediately with water (See Material Safety Data Sheet(s)).
Product warranty is limited to our liability to replacement of this product. All other warranties, expressed or implied, including but not limited to any implied warranties of merchantability or fitness for a particular purpose, are excluded and do not apply. We shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

5. Technical Information

5.1. Protein binding

Proteins containing one or more polyhistidine affinity tags, located at either the amino or carboxyl terminus of the protein, can bind to the Ni-IDA matrix with very high affinity. Even when the tag is not completely accessible (e.g., under native conditions), it will bind as long as more than two histidine residues are available for interaction. In general: the smaller the number of accessible histidine residues, the weaker the binding.

5.2. Binding capacity

The maximal binding capacity of MoBiTec Ni-IDA Columns is 90 mg recombinant protein as tested for histidine-tagged green fluorescent protein (6xHis-GFP, ~32 kDa). The actual obtained yield is depending on the concentration of the histidine-tagged fusion protein and its overall amount in the loaded sample. For a maximal recovery (> 80%) we recommend loading up to 75 mg polyhistidine-tagged protein within 2-3 ml sample volume. To obtain a maximum yield (with lower recovery values) we recommend loading even higher amounts of polyhistidine-tagged protein (up to 100 mg). A high concentration and a high overall amount will result in the highest possible yield.

5.3. Purification of secretory proteins

Producing proteins by secretion can be a great benefit in case of proper folding, disulfide bond formation, and for directing toxic proteins out of the cell. In addition, purification might be easier since the proteins can be purified directly from the corresponding compartment (periplasmic space or culture medium) showing a lower amount of total protein.
Secretory proteins contain a signal peptide that addresses them for the export into the periplasmic space (e.g., *E. coli*) or into the culture medium (e.g., *Bacillus* spec.). Please consider that in case of secretory proteins the polyhistidine-tag must be located C-terminal of the protein. Otherwise, the tag will be cleaved of during signal peptide processing.

5.4. Purification under native and denaturing conditions

The decision whether to purify polyhistidine-tagged proteins under native or denaturing conditions depends on diverse considerations: protein location and solubility, the accessibility of the polyhistidine-tag, and whether biological activity must be retained.
Depending on the expression system and the host, the recombinant proteins will accumulate in the cytoplasm or will be secreted into the periplasmic space or out into the culture medium. Secreted protein is in most cases correctly folded and soluble. Intracellularly accumulated recombinant protein remains either in a soluble form or aggregates as insoluble misfolded protein in inclusion bodies.
In case of soluble protein (purified from cytoplasm, periplasm, or supernatant) with good accessibility of the polyhistidine-tag, the purification can be done under native conditions (see protocol “6.3.1. Preparation of cleared lysates under native conditions”). Native conditions may also be used if co-purification of associated proteins is desired. In case of inclusion body formation the polyhistidine-tagged protein has to be extracted from cell pellet using urea as denaturant (see protocol “6.3.2. Preparation of cleared lysates under denaturing conditions”). Denaturing conditions can also be an option for improving the accessibility of the polyhistidine-tag.

Tabel 1: How to find the proper purification conditions: please choose

Native conditions if …	Denaturing conditions if …
protein is soluble	protein aggregates (e.g., inclusion bodies)
polyhistidine-tag is good accessible	the polyhistidine-tag is hardly accessible
co-purification of associated proteins desired	

5.5. Culture size

The recommended culture size volume complies with the concentration of the polyhistidine-tagged protein in the culture. The latter typically varies from <10 mg/l up to 100 mg/l depending on cell density and expression level.

Table 2: Culture size guide

Expression level	Recommended culture volume	Recommended pellet wet weight (*E. coli*)	Estimated amount of poly-histidine-tagged protein in sample
High (~100 mg/l)	0.2-1 l	0.8-4 g	~20-100 mg
Low (~10 mg/l	2-10 l	8-40 g	~20-100 mg

6. Protocols

6.1. Purification of secreted polyhistidine-tagged proteins from the supernatant (e.g. *B. subtilis*)

For preparation of secreted polyhistidine-tagged proteins, cells and supernatant will be separated by centrifugation (15 min, 4500 x g to 6000 x g, 4 °C). The clear supernatant can be directly subjected to MoBiTec Ni-IDA columns. Please follow the protocol “6.3.1. Preparation of cleared lysates under native conditions”

6.2. Purification of periplasmic polyhistidine-tagged proteins

Periplasmic proteins can be separated from cytoplasmic proteins by osmotic shock preparation (see protocol below). The obtained osmotic shock fluid can then be subjected to MoBiTec Ni-IDA columns.

- Harvest the cells from expression culture by centrifugation (15 min, 4500 x g to 6000 x g, 4 °C).
- Resuspend cell pellet in Sucrose Buffer at 80 ml per gram wet weight.
- Keep the cells on ice and add 500 mM EDTA solution dropwise to 1 mM final concentration.
- Incubate the cells on ice for 5-10 min with gentle agitation.
- Centrifuge the cell suspension at 8000 x g for 20 min at 4 °C.
- Remove supernatant completely and resuspend the pellet in the same volume of ice-cold 5 mM $MgSO_4$ solution.
- Shake or stir for 10 min in an ice bath.
- Centrifuge at 8000 x g for 20 min at 4 °C.
- Carefully transfer the supernatant (containing the periplasmic proteins) to a clean tube without disturbing the pellet.
- If the supernatant is not clear, centrifuge a second time or filter through a 0.45 µm membrane to avoid clogging of the Ni-IDA column with insoluble material.
- Dialyze the supernatant extensively against LEW buffer.
- Store supernatant on ice.
- Proceed with the protocol “6.3.1. Preparation of cleared lysates under native conditions”.

6.3. Purification of Intracellular polyhistidine-tagged proteins under native or denaturing conditions

For purification of intracellular polyhistidine-tagged proteins under native conditions, a considerable portion of the protein should be present in a soluble form. If so, please follow the protocol “6.3.1. Preparation of cleared lysates under native conditions”.

High levels of recombinant protein expression might lead to the formation of insoluble aggregates; in *E. coli*, these are known as inclusion bodies.
During preparation of intracellular recombinant protein, cells are disrupted and cell pellets are separated from the soluble fraction by centrifugation. In contrast to soluble protein that remains in the supernatant, the inclusion bodies will sediment with the cell debris, and the polyhistidine-tagged protein has to be extracted from cell pellet using urea as denaturant. 8 M urea completely solubilizes the inclusion bodies and 6xHis-tagged proteins. Under these denaturing conditions, the 6xHis-tag on a protein will be fully exposed so that binding to the Ni-IDA matrix is enabled. If most of the 6xHis-tagged protein is localized within inclusion bodies, please follow the protocol “6.3.2. Preparation of cleared lysates under denaturing conditions”.

6.3.1. Preparation of cleared lysates under native conditions (*E. coli*)

- Prepare the LEW buffer (Lysis-Equilibration-Wash Buffer) and the Elution Buffer as described in “3.4. Buffer compositions”.
- Harvest the cells from expression culture by centrifugation (15 min, 4500 x g to 6000 x g, 4 °C).
- Remove supernatant and store pellet at -20 °C or proceed immediately. Please perform all steps on ice!
- Thoroughly resuspend the pellet in LEW buffer (~1.25-5 mg polyhistidine-tagged protein/ 1 ml LEW, see also table 3 below), by pipetting up and down or vortexing until complete resuspension is reached (no cell aggregates visible anymore). Add lysozyme to a final concentration of 1 mg/ml and optional protease inhibitor (e.g., 0.1 mM PMSF final concentration). Stir the solution on ice for 30 min.
- Sonicate the suspension on ice according to the instructions provided by the manufacturer (e.g. 10 x 15 sec burst with 20 sec rest (amplitude of 20%).
- Carefully check samples appearance after sonication. If the lysate is still viscous from incomplete fragmentation of DNA, add 5 µg/ml DNase I and stir on ice for additional 15 min.
- Centrifuge the crude lysate at 10000 x g for 30 min at 4 °C to remove cellular debris.
- Carefully transfer the supernatant to a clean tube without disturbing the pellet.
- If the supernatant is not clear, centrifuge a second time or filter through a 0.45 µm membrane to avoid clogging of the Ni-IDA column with insoluble material.
- Store supernatant on ice.

Proceed with chapter “6.4. Ni-IDA chromatography under native conditions”.

Table 3: LEW buffer volume guide

Expression level	Recommended culture volume	Recommended pellet wet weight	Ratio pellet weight/LEW volume	Volume of LEW for re-suspension
High (~100 mg/l)	200 ml (minimum)	0.8 g (minimum)	1:5	4 ml
	1 l (maximum)	4 g (maximum)	1:5	20 ml
Low (~10 mg/l)	2 l (minimum)	8 g (minimum)	1:2	16 ml
	10 l (maximum)	40 g (maximum)	1:2	80 ml

6.3.2. Preparation of cleared lysates under denaturing conditions (*E. coli*)

High levels of expression of recombinant proteins in a variety of expression systems can lead to the formation of insoluble aggregates; in *E. coli*, these are known as inclusion bodies. Strong denaturants such as 8 M urea completely solubilize inclusion bodies and 6xHis-tagged proteins. Under these conditions, the 6xHis-tag on a protein will be fully exposed so that binding to the Ni-IDA matrix may be improved.

For preparation of intracellular recombinant proteins, cells are disrupted and cell pellets are separated from the soluble fraction by centrifugation. Whereas inclusion bodies will sediment with the cell debris, soluble recombinant protein remains in the supernatant. Clear supernatant can be directly subjected to MoBiTec Ni-IDA Columns (follow the protocol "6.4. Ni-IDA chromatography under native conditions"). In case of inclusion body formation, the 6xHis-tagged protein has to be extracted from cell pellet using urea as denaturant (follow the protocol below).

Isolation of inclusion bodies

- Harvest the cells from expression culture by centrifugation (15 min, 4500 x g to 6000 x g, 4 °C).
- Remove supernatant and store pellet at -20 °C or proceed immediately. Please perform all steps on ice!
- Thoroughly resuspend the pellet in LEW buffer (~1.25-5 mg polyhistidine-tagged protein/1 ml LEW, see also table 3), by pipetting up and down or vortexing until complete resuspension is reached (no cell aggregates visible anymore). Add lysozyme to a final concentration of 1 mg/ml and optional protease inhibitor (e.g., 0.1 mM PMSF final concentration). Stir the solution on ice for 30 min.
- Sonicate the suspension on ice according to the instructions provided by the manufacturer (e.g., 10 x 15 sec burst with 20 sec rest (amplitude of 20%).
- Carefully check sample appearance after sonication. If the lysate is still viscous from incomplete fragmentation of DNA, add 5 µg/ml DNase I and stir on ice for additional 15 min.
- Centrifuge the crude lysate at 10000 x g for 30 min at 4 °C to collect inclusion bodies. Discard the supernatant and keep the pellet on ice.

Solubilization of inclusion bodies

- Resuspend the pellet in 10 ml LEW buffer to wash the inclusion bodies. Centrifuge the suspension at 10000 x g for 30 min at 4° C and discard the supernatant.
- Resuspend the pellet in 2 ml "Denaturating Solubilization Buffer" to solubilize the inclusion bodies. For complete solubilization it may be necessary to vortex or sonicate the solution. Stir the suspension for further 60 min on ice.
- Centrifuge at 10000 x g for 30 min at 20 °C to remove any remaining insoluble material. Carefully transfer the supernatant to a clean tube without disturbing the pellet.
- If the supernatant is not clear, centrifuge a second time or filter through a 0.45 µm membrane to avoid clogging of the MoBiTec Ni-IDA column with insoluble material.
- Store supernatant at 4°C

Proceed with chapter "6.5. Ni-IDA chromatography under denaturing conditions".

6.4. Ni-IDA chromatography under native conditions

Column equilibration

Equilibrate the column with 8 ml LEW buffer. Allow the column to drain by gravity.

Binding
Add the cleared lysate (at least 3 ml) to the pre-equilibrated column and allow the column to drain by gravity.

Washing
Wash the column three times with 8 ml LEW buffer (3 x 8 ml). Allow the column to drain by gravity.

Elution
Elute the polyhistidine-tagged protein in 3-6 fractions. Add in total 18 ml Elution Buffer divided in a suitable number of fractions and collect separately. Allow the column to drain by gravity.
Note: Commonly, 90% of the eluted protein can be found within the first 6 ml of elution. Use protein assay and/or SDS-PAGE analysis to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

6.5. Ni-IDA chromatography under denaturing conditions

Column Equilibration
Equilibrate the column with 8 ml of "Denaturing Solubilization Buffer". Allow the column to drain by gravity.

Binding
Add the cleared lysate (at least 3 ml) to the pre-equilibrated column and allow the column to drain by gravity.

Washing
Wash the column twice with 16 ml "Denaturing Solubilization Buffer". Allow the column to drain by gravity.

Elution
Elute the polyhistidine-tagged protein in 3-6 fractions. Add in total 18 ml "Denaturing Elution Buffer" divided in a suitable number of fractions and collect separately. Allow the column to drain by gravity. Note: Commonly, 90% of the eluted protein can be found within the first 6 ml of elution. Use protein assay and/or SDS-PAGE analysis to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

6.6. Storage, reuse, and regeneration

6.6.1. Reuse and short-term storage

Depending on the nature of the sample, MoBiTec Ni-IDA Columns can be reused 3-5 times. Reuse should only be performed with identical polyhistidine-tagged proteins to avoid possible cross-contamination.

After the final elution step wash the column with the following solutions:
1. 10 ml 100 mM EDTA, pH 8
2. 10 ml deionized water
3. 10 ml 100 mM $NiSO_4$
4. 10 ml deionized water
5. After equilibrating with LEW buffer or "Denaturing Solubilization Buffer" the column is ready for reuse.

6. For storage overnight we recommend to store the column with LEW buffer at 4 °C. Otherwise follow the protocol for long-term storage below.

6.6.2. Long-term storage

After performing the “reuse protocol” wash column with

1. 20 ml LEW buffer
2. 20 ml deionized water
3. 4 ml 20% ethanol
4. Store column with 20% ethanol at 4 °C
5. After equilibrating with 20 ml LEW buffer the column is ready for reuse.

6.6.3. Complete regeneration

If a complete regeneration is mandatory, wash the column with the following solutions:

1. 4 ml 6 M GuHCl, 0.2 M acetic acid
2. 10 ml deionized water
3. 6 ml 2% SDS
4. 10 ml deionized water
5. 10 ml 100% ethanol
6. 10 ml deionized water
7. 10 ml 100 mM EDTA, pH 8
8. 10 ml deionized water
9. 10 ml 100 mM $NiSO_4$
10. 20 ml deionized water
11. After equilibrating with 20 ml LEW buffer the column is ready for reuse.

6.7. Compatibility of reagents

Buffer components that chelate metal ions, such as EDTA and EGTA, should not be used, since they strip Ni^{2+} ions from the matrix. Do not use buffers with pH > 8.4, since silica dissolves in solutions of high pH.

Reagent	Effect	Comment
β-mercaptoethanol	Prevents formation of disulfide bonds; can reduce Ni^{2+} ions at higher concentrations.	Up to 50 mM in samples has been successfully used in some cases.
DTT, DTE	Can reduce Ni^{2+} ions at higher concentrations.	Up to 10 mM in samples has been successfully used in some cases.
EDTA	Coordinates with Ni^{2+} ions, causing a decrease in capacity at higher concentrations.	Not recommended, but up to 1 mM in samples has been used successfully in some cases.
Ethanol	Prevents hydrophobic interactions between proteins.	Up to 20% can be used; ethanol may precipitate proteins, causing low flow rates and column clogging.
Glutathione reduced	Can reduce Ni^{2+} ions at higher concentrations.	Up to 30 mM in samples has been successfully used in some cases.
Glycerol	Prevents hydrophobic interactions between proteins.	Up to 50% can be used.
GuHCl	Solubilizes proteins.	Up to 6 M can be used.
Imidazole	Binds to immobilized Ni^{2+} ions and competes with the polyhistidine-tagged proteins	Imidazole should not be included in LEW buffer.
SDS	Interacts with Ni^{2+} ions, causing a decrease in capacity.	Not recommended, but up to 0.5% in samples has been used successfully in some cases.
Sodium chloride	Prevents ionic interactions and therefore unspecific binding.	up to 2 M can be used, at least 0.3 M should be used
Sodium phosphate	Used in LEW and Elution Buffer in order to buffer the solutions at pH 8.	50 mM is recommended. The pH of any buffer should be adjusted to 8, although in some cases a pH between 7 and 8 can be used.
Tris	Coordinates with Ni^{2+} ions, causing a decrease in capacity.	10 mM may be used, sodium phosphate buffer is recommended
Triton, Tween	Removes background proteins.	Up to 2% can be used.
Urea	Solubilizes proteins.	Use 8 M urea for purification under denaturing conditions.

6.8. Troubleshooting

Problem	Caused by	Suggestions
Sample does not enter the column bed.	Sample/lysate contains insoluble material.	If sample is not clear, use centrifugation of filtration (0.45 µm membrane) to avoid clogging of the column.
	Sample/lysate contains genomic DNA.	Lysate may remain viscous from incomplete shearing of genomic DNA after sonication. Add 5 µg/ml DNase I and incubate on ice for 10 min.
Protein does not bind to the resin.	Vector construct is not correct.	Check if gene of interest and tag has been cloned in frame.
	Binding conditions are incorrect.	Check composition of buffers and verify pH 7-8. Ensure that there is no chelating or strong reducing reagent or imidazole present.
Protein elutes with wash buffer.	Buffer compositions are incorrect.	Check composition of buffers and verify pH 7-8. Ensure that there is no chelating or strong reducing reagent or imidazole present.
Protein does not elute from column.	Elution conditions are too mild	Increase concentration of imidazole.
Contamination of other proteins within the eluate.	Insufficient washing	Use larger volumes for washing.
	Binding and washing conditions are too mild.	Add small amounts of imidazole (1-10 mM). Take care that the imidazole concentration remains low enough to enable binding of the polyhistidine-tagged proteins.
	Contaminating proteins and the polyhistidine-tagged protein are connected via disulfide bonds.	Add up to 30 mM β-mercapto-ethanol to reduce disulfide bonds.
	Contaminating proteins are degradation products of polyhistidine-tagged protein.	- Perform cell lysis at 4 °C. - Include protease inhibitor.
	Expression is too low; this leads to increased binding of contaminating proteins.	- Increase expression level. - Increase amount of culture volume or cell pellet weight.

7. Order Information, Shipping, and Storage

Order#	Product	Amount
PR-HTK004	MoBiTec Ni-IDA Columns	4 columns
PR-HTK010	MoBiTec Ni-IDA Columns	10 columns
shipped at RT; store columns at RT		

8. Contact and Support

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