Bioanalysis

MACHEREY-NAGEL

User manual

Purification of His-tag proteins

- Protino® Ni-NTA Agarose
- Protino® Ni-NTA Columns 1 mL
- Protino® Ni-NTA Columns 5 mL
- Protino® 96 Ni-NTA

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1 Components

1.1 Kit contents and storage

	Protino® Ni-NTA Agarose		
	745400.25	**745400.100**	**745400.500**
Protino® Ni-NTA Agarose	25 mL	100 mL	500 mL

	Protino® Ni-NTA Columns		
	1 mL	**5 mL**	
REF	**745410.5**	**745415.1**	**745415.5**
Protino® Ni-NTA Columns	5 x 1 mL	1 x 5 mL	5 x 5 mL

	Protino® 96 Ni-NTA	
REF	**745425.1**	**745425.4**
Protino® Ni-NTA Agarose (sufficient for 96 preps using an agarose bed volume of 50 µL for the purification of up to 2 mg polyhistidine tagged protein)	5.5 mL	4 x 5.5 mL
Protino® Purification Plate (proprietary leak-free purification plate for retaining the chromatographic resins)	1	4
MN Wash Plate (one plate is used to minimize cross-contamination, the other plate is used as an adaptor to place the Protino® Purification Plate on a plate shaker)	2	8

Shipping and storage:

The product is shipped at ambient temperature.

Upon receipt Protino® Ni-NTA Agarose or columns should be **stored at 2–8 °C (stable until: see package label)**. Do not freeze.

All other components can be stored at ambient temperature.

1.2 Additional material to be supplied by user

- For the purification under native conditions prepare the following buffers: NPI-10, NPI-20, NPI-250 (see section 4.1)
- For the purification under denaturing conditions prepare the following buffers: NPI-10, DNPI-10, DNPI-20, DNPI-250 (see section 6.1)
- Lysozyme
- Appropriate columns, centrifuge tubes, etc.
- Appropriate centrifuge
- Protino® Ni-NTA Columns
- Liquid chromatography system (MPLC, FPLC™, ÄKTAdesign™, etc.), peristaltic pump, or syringe
- If necessary, appropriate adaptors for connecting the Protino® Ni-NTA Columns to the system of choice. Protino® Ni-NTA Columns are equipped with 10–32 (1/16") inlet and outlet ports. With these ports the columns can easily be connected to standard MPLC/FPLC™ systems (e.g., ÄKTAdesign™). Five adaptor sets are available for connecting the columns to other systems or for using them with a syringe.

Table 1: Adaptor sets

System	Adaptor needed	Adaptor Set
Standard FPLC™ system (e.g., ÄKTAdesign™)	none	none
FPLC™ system, first generation (Pharmacia)	1 x M6 female to 10–32 male 1 x 10–32 female to M6 male	Protino® M6 Adaptor Set, REF 745260
MPLC system (e.g., BioLogic™, BIO-RAD)	1 x 1/4" 28 female to 10–32 male 1 x 10–32 female to 1/4" 28 female	Protino® 1/4–28 Adaptor Set, REF 745261
MPLC system (e.g., Profinia™, BIO-RAD)	1 x Luer female to 10–32 male 1 x 10–32 female to Luer male	Protino® Luer Adaptor Set, REF 745264
Peristaltic pump	1 x 1/16" ID tubing to 10–32 male	Protino® Inlet PP Adaptor Set REF 745263
Syringe	1 x Luer female to 10–32 male	Protino® Inlet Luer Adaptor REF 745262

Protino® 96 Ni-NTA

Plate shaker (optional)

Vacuum manifold system OR suitable centrifuge. Please contact Technical Service for additional information on vacuum or centrifuge processing.

Plate(s) for collecting eluate fraction(s) (e.g. Square-well Block, see ordering information)

1.3 About this user manual

It is recommended to read the instructions of this user manual carefully before use. All technical literature is also available on the internet at ***www.mn-net.com***.

Please contact Technical Service regarding information about changes of the current user manual compared to previous or updated revisions.

2 Product description

2.1 The basic principle

Protino® Ni-NTA products enable fast and convenient purification of recombinant polyhistidine-tagged proteins by immobilized metal ion affinity chromatography (IMAC). Proteins from any expression system can be purified under native or denaturing conditions. Binding of protein is based on the interaction between the polyhistidine tag of the recombinant protein and immobilized Ni^{2+} ions.

Protino® Ni-NTA Agarose consists of the chelating ligand nitrilotriacetic acid (NTA) immobilized on 6 % crosslinked agarose beads that are suitable for batch binding, gravity flow, and FPLC™ columns or high throughput processing. The resin is precharged with Ni^{2+} ions and therefore ready to use. For high throughput purification, Individual amounts of the Protino® Ni-NTA Agarose matrix can be loaded into the wells of the Protino® Purification Plate (included in the Protino® 96 Ni-NTA kit). However, we recommend to use 50 µL bed volume per well. Due to the special designed filter-frit material, the Protino® Purification Plate is leak-free in operation. Therefore, it is possible to perform the entire purification process (equilibration, batch-binding, washing, elution) directly in the wells of the purification plate. Buffer passes through filter-frits only by vacuum or centrifugation. The Protino® Purification Plate enables high well-to-well reproducibility.

The uniquely designed Protino® Purification Plate is also ideal for parallel screening experiments to optimize chromatographic conditions including the sample-to-resin ratio, sample incubation time, wash and elution conditions, buffer additives, etc. Protino® Ni-NTA Agarose uses NTA which represents the most commonly used chelating ligand in IMAC. NTA is a tetradentate chelator which occupies four out of the six binding sites in the coordination sphere of the Ni^{2+} ion. The remaining two coordination sites are usually occupied by water molecules and can be exchanged with histidine residues of the recombinant protein (Figure 1). This formation of coordination sites has turned out to be optimal for purification of polyhistidine-tagged proteins: two available binding sites in the coordination sphere of the Ni^{2+} ion enable tight but reversible selective protein interactions. Chelation of Ni^{2+} ions by NTA through four coordination positions minimizes metal leaching during purification and increases specificity for polyhistidine- tagged proteins.

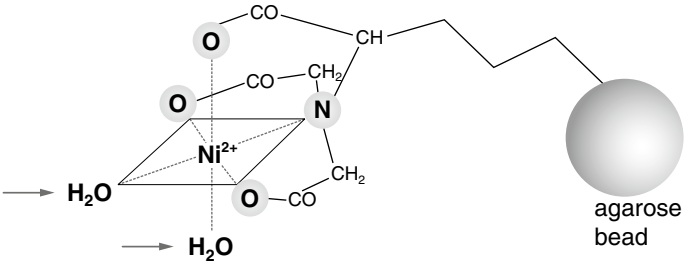


Figure 1 Protino® Ni-NTA Agarose – structure of NTA in complex with Ni^{2+}

2.2 Specifications

Table 2: Specifications Protino® Ni-NTA Agarose	
Application	- Batch binding - Gravity flow column chromatography - MPLC / FPLC™
Form	50 % aqueous suspension containing 30 vol % ethanol, precharged with Ni^{2+}
Support	Crosslinked 6 % beaded agarose
Ligand	Inlet 10–32 (1/16") female Outlet 10–32 (1/16") male
Bead size	4 % beaded agarose
Ligand	Nitrilotriacetic acid (NTA)
Bead size	45–165 µm
Binding capacity[1]	Up to 50 mg/mL settled agarose
Recommended flow rate	1 mL bed volume (column with 6.6 mm inner diameter) 1.0 mL/min 10 mL bed volume (column with 16 mm inner diameter) 5.0 mL/min
Maximum linear flow rate[2]	300 cm/h
Storage temperature	4–8 °C (do not freeze)

Table 3: Specifications Protino® Ni-NTA Columns 1 mL / 5 mL		
Column bed volume	**1 mL**	**5 mL**
System compatibility	- Automated liquid chromatography systems (MPLC, FPLC™, ÄKTAdesign™, etc.) - Peristaltic pump - Syringe	
Column dimensions	0.7 cm inner diameter x 2.5 cm height	1.6 cm inner diameter x 2.5 cm height
Column body material	Polypropylene	
Column ports	Inlet 10–32 (1/16") female Outlet 10–32 (1/16") male	

[1] Binding capacity will vary for each polyhistidine-tagged protein.
[2] High flow rates may reduce binding capacity.

Table 3: Specifications Protino® Ni-NTA Columns 1 mL / 5 mL		
Support	Crosslinked 6 % beaded agarose	
Ligand	Nitrilotriacetic acid (NTA)	
Bead size	45–165 µm	
Binding capacity[1]	Up to 50 mg	Up to 250 mg
Maximum back pressure	3 bar (0.3 MPa)	
Recommended flow rates	1 mL/min	5 mL/min
Maximum recommended flow rate[2]	3 bar (0.3 MPa)	
Storage temperature	4–8 °C	
Storage solution	20 % ethanol	

Table 4: Specifications Protino® 96 Ni-NTA	
Purification plate material	**Polypropylene**
Filter-frits	According to special designed filter-frit material, the filter plates are absolutely leak-free. Buffer passes through filter-frits only when the water breakthrough pressure is reached by vacuum or centrifugation
Filter diameter	8.3 mm
Number of wells	96
Volume capacity of well	1.4 mL
Recommended sample volume	Up to 750 µL (at 1,100 rpm on an eppendorf Thermomixer®)
Matrix	Protino® Ni-NTA Agarose, crosslinked 6 % beaded agarose
Form	50 % aqueous suspension containing 30 vol % ethanol, precharged with Ni^{2+}
Ligand	Nitrilotriacetic acid (NTA)
Bead size	45–165 µm
Bed volume	Variable

[1] Binding capacity will vary for each polyhistidine-tagged protein.
[2] High flow rates may reduce binding capacity.

Table 4: Specifications Protino® 96 Ni-NTA	
Recommended bed volume	50 µL
Binding capacity*	Up to 2 mg per well when using 50 µL settled agarose
Recommended vacuum	-0.6 bar (depends on sample properties)
Recommended centrifugation speed	3,000 x *g* (depends on sample properties)

2.3 General information

Binding capacity

- The binding capacity of Protino® Ni-NTA Agarose strongly depends on the characteristics of the polyhistidine-tagged protein (e.g., amino acid composition, molecular weight, 3-D structure, oligomerization properties). Furthermore, the absolute yield also depends on the total amount and concentration of the target protein in the sample which in turn directly correlate with the expression level and the cell density of the expression culture. Therefore binding capacity will vary for each polyhistidine-tagged protein.
- For best results determine the binding behaviour of any polyhistidine-tagged protein prior to attempting large-scale purification.
- A maximum capacity of up to 50 mg/mL was determined for the monomeric green fluorescent protein (6 x His-GFPuv, ~ 32 kDa) expressed in *E. coli*. However, especially

Incubation time (recommendation for Protino® 96 Ni-NTA)

- Especially during sample incubation, sufficient agitation is necessary to disperse the agarose beads. We recommend to use 50 µL of settled Protino® Ni-NTA Agarose per well, an agitation speed of 1,100 rpm (eppendorf Thermomixer®) and a sample volume of up to 750 µL.
- As a starting point, we recommend to shake the suspension for 15–30 min (see Figure Figure 2). However, as the purification plate is absolutely leak-free, longer sample incubation times can be used to achieve maximum yield (see Figure Figure 2). If shorter incubation periods are required (e.g., according to protein stability), incubation can be reduced (e.g., to 5 min), but then yield may decrease (see Figure Figure 2).

* Binding capacity will vary for each polyhistidine-tagged protein and strongly depends on chromatographic conditions.

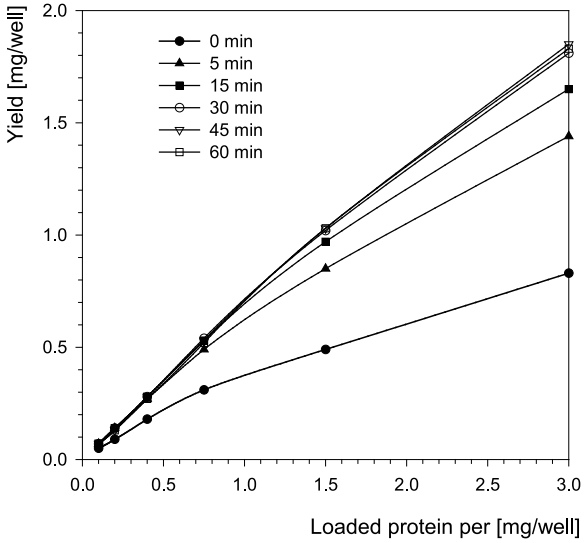


Figure 2 Impact of sample incubation time on protein yield

Protino® Ni-NTA Agarose (50 µL bed volume) was loaded with increasing amounts of His-tagged Green Fluorescent Protein (6 x His GFPuv) in a total volume of 750 µL of NPI-10. After washing with 1.5 mL of NPI-20, the target protein was eluted with 2 x 200 µL of NPI-250. Yields of 6 x His-GFPuv are plotted versus the amount of loaded protein. When wells are loaded with up to 0.8 mg, shaking for 5 min already leads to acceptable yields. However, if larger protein amounts are loaded and maximum yield is required, we recommend to shake the plate for 15 to 30 min

Solubility of the recombinant protein

Protein yield is also dependent on solubility of the recombinant protein. If proteins are expressed in E. coli, ideally the target proteins remain soluble in the cytoplasm. However, especially proteins that are highly expressed accumulate in insoluble aggregates which are called inclusion bodies.

For solubilization of inclusion bodies, buffers containing large amounts of denaturants are used.

This manual includes instructions for isolation of soluble proteins (purification under native conditions, see section 4) as well as insoluble proteins from inclusion bodies (purification under denaturing conditions, see section 5).

Improving purity

Sometimes optimization of purification procedures is necessary to increase purity.

Usually lysis / equilibration buffers and the wash buffer contain 10 mM and 20 mM imidazole, respectively, to suppress binding of contaminating proteins. To improve specificity increase imidazole concentration.

In addition, for more stringent binding and washing conditions the pH may be reduced from pH 8 closer to pH 7 (e.g., pH 7.4) in all buffers

Additives

Avoid high concentration of additives that interact with nickel ions and thus reduce capacity (e.g., chelating agents (EDTA) or reducing agents (DTT, mercaptoethanol)), see compatibility of reagents (section 2.4).

2.4 Compatibility of reagents

Table 5: Reagent compatibility chart

Reagent	Effect	Comments
Sodium phosphate	Used in buffers 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, HEPES, MOPS	Coordinates with Ni^{2+} ions, causing a decrease in capacity	Up to 100 mM may be used, sodium phosphate buffer is recommended
Sodium Chloride	Prevents ionic interactions and therefore unspecific binding	Up to 2 M can be used, at least 0.3 M should be used
Imidazole	Binds to immobilized Ni^{2+} ions and competes with the polyhistidine-tagged proteins	Is used at low concentration to reduce non specific binding (20 mM) and to elute the target protein (> 100 mM)
Urea	Solubilizes protein	Use 8 M for purification under denaturing conditions
GuHCl	Solubilizes protein	Up to 6 M can be used
ß-mercaptoethanol	Prevents formation of disulfide bonds; can reduce Ni^{2+} ions at higher concentrations	Up to 20 mM in samples has been used successfully in some cases
DTT, DTE	Can reduce Ni^{2+} ions at higher concentrations	Up to 10 mM in samples has been used successfully in some cases
Glutathione reduced	Can reduce Ni^{2+} ions at higher concentrations	Up to 30 mM in samples has been used successfully in some cases
Glycerol	Prevents hydrophobic interactions between protein	Up to 50 % can be used

Table 5: Reagent compatibility chart

Reagent	Effect	Comments
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
SDS	Interacts with Ni2+ ions, causing a decrease in capacity	Not recommended, but up to 0.3 % in samples has been used successfully in some cases
Nonionic detergents: Triton, Tween, etc.	Removes background proteins	Up to 2 % can be used

3 Safety instructions

When working with the **Protino® Ni-NTA** products wear suitable protective clothing (e.g., lab coat, disposable gloves, and protective goggles). For more information consult the appropriate Material Safety Data Sheets (MSDS available online at ***www.mn-net.com/msds***).

The waste generated with the **Protino® Ni-NTA** products has not been tested for residual infectious material. Therefore, liquid waste must be considered infectious and should be handled and discarded according local safety regulations.

3.1 Disposal

Dispose hazardous, infectious, biologically or GMO-contaminated materials in a safe and acceptable manner and in accordance with all local and regulatory requirements.

4 Purification of polyhistidine-tagged proteins under native conditions

4.1 Preparation of buffers for purification under native conditions

NPI-10 / lysis & equilibration buffer (1 liter):		
50 mM NaH_2PO_4	7.80 g $NaH_2PO_4 \bullet 2\ H_2O$	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
10 mM imidazole	0.68 g imidazole	M_r = 68.08 g/mol
Adjust pH to 8.0 using NaOH		
NPI-20 / wash buffer (1 liter):		
50 mM NaH_2PO_4	7.80 g $NaH_2PO_4 \bullet 2\ H_2O$	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
20 mM imidazole	1.36 g imidazole	M_r = 68.08 g/mol
Adjust pH to 8.0 using NaOH		
NPI-250 / elution buffer (1 liter):		
50 mM NaH_2PO_4	7.80 g $NaH_2PO_4 \bullet 2\ H_2O$	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
250 mM imidazole	17.00 g imidazole	M_r = 68.08 g/mol
Adjust pH to 8.0 using NaOH		

Protino® 96 Ni-NTA: Buffer volumes needed for 96 preps using 50 µL of Protino® Ni-NTA Agarose per well.

Step	Buffer	Volume / well	Exact volume / plate	Recommended volume / plate
Equilibration	NPI-10	1 x 0.5 mL	48 mL	60 mL
Washing	NPI-20	3 x 0.5 mL	144 mL	150 mL
Elution	NPI-250	2 x 0.2 mL	38.4 mL	50 mL

Please note that additional NPI-10 buffer may be needed for the preparation of protein extracts.

4.2 Preparation of cleared *E. coli* lysates under native conditions

Cultivate and harvest cells

- Harvest cells from an *E.coli* expression culture by centrifugation at 4,500–6,000 x *g* for 15 min at 4 °C. Remove supernatant.
- To wash the cells resuspend in NPI-10 and centrifuge again. Remove supernatant.
- Cell pellets may be stored at -20 °C or -80 °C until needed.

Resuspend bacteria cells

- Thaw the cell pellet from an *E.coli* expression culture on ice (if frozen).
- Resuspend 1 g of pelleted, wet cells in 2–5 mL NPI-10. Pipette up and down, or stir until complete resuspension without visible cell aggregates. Perform this step on ice.

Lyse cells

- Add lysozyme to a final concentration of 1 mg/mL.
- Stir the solution on ice for 30 min.
- Sonicate the suspension on ice according to the instructions provided by the manufacturer (e.g., use 10 x 15 s bursts with a 15 s cooling period between each burst).
- 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 15 min.

Clarify lysate

- Centrifuge the crude lysate at 10,000 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 (e.g., cellulose acetate).
- Store supernatant on ice.

Proceed to section

4.3 for batch purification

4.4 for semi-batch purification

4.5 for Spin column purification

4.6 for gravity flow purification

4.7 for FPLC™ purification using self-packed columns or

4.8 for FPLC™ purification using Protino® Ni-NTA Columns.

Protino® 96 Ni-NTA: Preparation of lysate in 96-well plates

1 Cultivate and harvest cells

- *E. coli* cells may be cultivated in 96-well plates (1 mL culture volume), 24-well plates (5 mL culture volume), or any other appropriate cultivation vessel, if larger culture volumes are required.
- Harvest cells by centrifugation at 2,000 x *g* for 15 min at 4 °C.
- Store cell pellets at -20 °C or -70 °C for at least 1 h.

2 Prepare cell extracts

Use standard procedures for the preparation of cell extracts, such as lysozyme treatment, sonication, or detergent treatment. Note that optimal sample preparation steps have to be determined empirically depending on the characteristics of the of the polyhistidine-tagged protein and host organism.

For preparation of cell extracts from up to 5 mL *E. coli* expression culture, we recommend the following protocol as a starting point for further optimization:

- Thaw cell pellets at room temperature.
- Resuspend each pellet in 1 mL of 1 x NPI-10 Buffer containing 0.2 mg/mL lysozyme.
- Incubate at room temperature for 30 min in a shaker. If the lysate is still viscous, add 15 U of Benzonase® per well, mix, and incubate at room temperature for 30 min. Benzonase® reduces lysate viscosity by rapidly hydrolysing DNA and RNA.
- Centrifuge the crude lysate at 5,000 x *g* for 30 min at 4 °C to remove cellular debris. If the supernatant is not clear, centrifuge a second time to avoid clogging of the Protino® Purification Plate with insoluble material.
- Store supernatant on ice.

4.3 Batch purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

- Resuspend Protino® Ni-NTA Agarose by mixing thoroughly to achieve a homogeneous suspension.
- Immediately transfer an appropriate amount of suspension to an appropriate tube.
- Pipette 2 mL of the original 50 % suspension per 1 mL of bed volume required.
- Sediment the gel by centrifugation at 500 x *g* for 5 minutes. Carefully decant the supernatant (storage solution) and discard it.
- Add 10 bed volumes of NPI-10 to equilibrate the gel. Invert to mix.
- Sediment the gel by centrifugation at 500 x *g* for 5 minutes. Carefully decant the supernatant and discard it.

2 Batch binding

- Add the clarified *E.coli* lysate or protein extract to the equilibrated gel.
- Mix the suspension gently for 30–60 min.
- Sediment the gel by centrifugation at 500 x *g* for 5 minutes. Carefully decant the supernatant and discard it.

3 Washing

- Wash the gel by adding 10 bed volumes of NPI-20. Invert to mix.
- Sediment the gel by centrifugation at 500 x *g* for 5 minutes. Carefully decant the supernatant and discard it.
- Repeat the washing step (total wash 2 x 10 bed volumes of NPI-20).

4 Elution

- Add 1 bed volume of NPI-250 to the sedimented gel.
- Mix the suspension gently for 2 min at room temperature to liberate the polyhistidine-tagged protein from the gel.
- Sediment the gel by centrifugation at 500 x *g* for 5 minutes. Carefully decant or pipette the supernatant in a new tube and store eluted protein on ice.
- Repeat the elution step 5 times.
- Analyze fractions for the presence of the target protein. To determine the protein concentration use a Bradford protein assay (quick and easy) or measure the absorbance at 280 nm. Note that imidazole will also absorb at 280 nm.
- Pool fractions containing the majority of the eluted polyhistidine-tagged protein and remove an aliquot for SDS-PAGE analysis.
- Store protein at -70 °C or -20 °C. Note that many proteins irreversibly precipitate out of solution in the presence of imidazole. In this case remove imidazole prior to freezing.
- For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

4.4 Semi-batch purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

- Resuspend Protino® Ni-NTA Agarose by mixing thoroughly to achieve a homogeneous suspension
- Immediately transfer an appropriate amount of suspension to an appropriate chromatography column.
- Pipette 2 mL of the original 50 % suspension per 1 mL of bed volume required.
- Allow the column to drain by gravity.
- Add 10 bed volumes of NPI-10 to equilibrate the gel.
- Allow the column to drain by gravity.

2 Batch binding

- Close column outlet with cap.
- Add the clarified *E. coli* lysate or protein extract to the equilibrated gel.
- Close column inlet with a cap.
- Mix the suspension gently for 30–60 min by slowly inverting the column.
- Install the column in a vertical position.
- Remove bottom and top caps.
- Allow the column to drain by gravity

3 Washing

- Wash the column with 10 bed volumes of NPI-20
- Allow the column to drain by gravity.
- Repeat the washing step (total wash 2 x 10 bed volumes of NPI-20).

4 Elution

- Add 5–10 bed volumes of NPI-250 to the gel.
- Allow the column to drain by gravity and collect the eluate in fractions.
- Store eluted protein on ice.
- Analyze fractions for the presence of the target protein. To determine the protein concentration use a Bradford protein assay (quick and easy) or measure the absorbance at 280 nm. Note that imidazole will also absorb at 280 nm.
- Pool fractions containing the majority of the eluted polyhistidine-tagged protein and remove an aliquot for SDS-PAGE analysis.
- Store protein at -70 °C or -20 °C. Note that many proteins irreversibly precipitate out of solution in the presence of imidazole. In this case remove imidazole prior to freezing.
- For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

4.5 Spin column purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

- Resuspend Protino® Ni-NTA Agarose by mixing thoroughly to achieve a homogeneous 50 % suspension.
- Immediately transfer 50 µL of the suspension to a Receiver Column placed in a collecting tube.
- Centrifuge at 500 x *g* for 30 s.
- To equilibrate the gel add 500 µL of NPI-10.
- Centrifuge at 500 x *g* for 30 s. Discard flowthrough.

2 Binding

- Close column outlet with cap.
- Add up to 700 µL of clarified E. coli lysate to the gel and close the lid.
- Mix the suspension gently for 1 h at room temperature, for example, using an eppendorf thermomixer.
- Remove bottom cap and place Receiver Column in a collecting tube.
- Centrifuge at 500 x *g* for 30 s. Discard flowthrough.

3 Washing

- To wash the gel add 500 µL of NPI-20.
- Centrifuge at 500 x *g* for 30 s. Discard flowthrough.
- Repeat the washing step twice (total wash 3 x 500 µL of NPI-20). Discard flowthrough between washing steps.

4 Elution

- Close column outlet with cap. Add 125–250 µL of NPI-250 and close the lid.
- *Note: The amount of elution buffer required depends on the amount of loaded protein and may vary among fusion proteins. Volumes may have to be adjusted accordingly.*
- Mix the suspension gently for 2 min at room temperature to liberate polyhistidine-tagged proteins from the gel.
- Remove bottom cap and place Receiver Column in a 1.5 or 2 mL micro-centrifuge tube.
- Centrifuge at 500 x *g* for 30 s.
- Repeat the elution step 2 times.
- Store eluted protein on ice.
- Analyze fractions for the presence of the target protein. To determine the protein concentration use a Bradford protein assay (quick and easy) or measure the absorbance at 280 nm. Note that imidazole will also absorb at 280 nm.
- Pool fractions containing the majority of the eluted polyhistidine-tagged protein and remove an aliquot for SDS-PAGE analysis.
- Store protein at -70 °C or -20 °C. Note that many proteins irreversibly precipitate out of solution in the presence of imidazole. In this case remove imidazole prior to freezing.
- For proper storage conditions or downstream application imidazole can be removed by gelfiltration, ultrafiltration or dialysis.

4.6 Gravity flow purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

- Resuspend Protino® Ni-NTA Agarose by mixing thoroughly to achieve a homogeneous suspension.
- Immediately transfer an appropriate amount of suspension to an appropriate chromatography column, which allows slow flow rates of 0.5–1 mL/min.
- Pipette 2 mL of the original 50 % suspension per 1 mL of bed volume required.
- Allow the column to drain by gravity.
- Add 10 bed volumes of NPI-10 to equilibrate the gel.
- Allow the column to drain by gravity.

2 Binding

- Add the clarified *E.coli* lysate or protein extract to the equilibrated gel.
- Allow the column to drain by gravity using a flow rate of 0.5–1 mL/min.
- *Note: If the flow rate is too high polyhistidine-tagged proteins may not bind to the column efficiently. Reduce the flow rate or re-apply the flowthrough to improve binding.*

3 Washing

- Wash the gel by adding 10 bed volumes of NPI-20.
- Allow the column to drain by gravity.
- Repeat the washing step (total wash 2 x 10 bed volumes of NPI-20).

4 Elution

- Add 5–10 bed volumes of NPI-250 to the gel.
- Allow the column to drain by gravity and collect the eluate in fractions.
- Store eluted protein on ice.
- Analyze fractions for the presence of the target protein. To determine the protein concentration use a Bradford protein assay (quick and easy) or measure the absorbance at 280 nm. Note that imidazole will also absorb at 280 nm.
- Pool fractions containing the majority of the eluted polyhistidine-tagged protein and remove an aliquot for SDS-PAGE analysis.
- Store protein at -70 °C or -20 °C. Note that many proteins irreversibly precipitate out of solution in the presence of imidazole. In this case remove imidazole prior to freezing.
- For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

4.7 FPLC™ purification of polyhistidine-tagged proteins under native conditions (self-packed columns)

Prepare buffers according to section 4.1. Filter buffers through a 0.45 µm filter before use. Clear samples by centrifugation and/or pass them through a 0.45 µm filter.

1 Preparing the chromatography system

- Purge the pump with deionized water. Assure that all air is displaced.
- Determine the bed volume of Protino® Ni-NTA Agarose required for your application. Choose an appropriate chromatography column (e.g., from Omnifit or GE Healthcare). If more than 50 % of the column volume is to be packed, equip the column with an extension to hold the complete volume of the agarose suspension.
- Eliminate air from outlet tubing and end piece of the column by injecting deionized water into outlet tubing. Close outlet of column. Leave ~ 1 cm of buffer above the support net or frit.
- Inject deionized water into the inlet tubing of the upper plunger to eliminate air. Place plunger into a beaker containing deionized water until use.

2 Column packing

- Resuspend Protino® Ni-NTA Agarose by mixing thoroughly to achieve a homogeneous 50 % suspension. Immediately transfer the determined volume of suspension to an appropriate vacuum flask and de-gas.
- Pour the entire slurry into the column in one continuous motion along a glass rod held against the inner wall of the column.
- Carefully fill the remaining space with deionized water. Insert the upper plunger into the column without introducing air bubbles. Connect the inlet of the column to a pump.
- Open the column outlet and start the pump. Pass deionized water through the column at a packing flow rate of approximately 300 cm/h until height of gel bed becomes constant. Stop the pump and close the column outlet.
- Position the upper plunger on top of the column bed. Avoid to introduce air bubbles. Open the column outlet and start the pump at a flow rate of approximately 300 cm/h until the bed is stable. Re-position the plunger on the medium surface as necessary.

3 Column equilibration

- Purge the pump with NPI-10.
- Equilibrate the column with 5–10 bed volumes of NPI-10 until the baseline at 280 nm is stable.

4 Binding

- Load the clarified *E. coli* lysate or protein extract onto the column.
- Collect flowthrough and analyze (e.g., by SDS-PAGE) to verify that the polyhistidine-tagged protein has bound. If the fusion protein is found in early fractions of the flowthrough, the flow rate should be decreased. If the fusion protein is absent in early fractions and does appear in late fractions of the flowthrough the column capacity has been exceeded. In this case protein load should be reduced or bed volume should be increased.

5 Washing

- Wash the column with 10–20 bed volumes of NPI-20 or until the baseline at 280 nm is stable.

6 Elution

- Elute the polyhistidine-tagged protein with 5–10 bed volumes of NPI-250 and collect fractions.
- Store eluted protein on ice.
- Analyze fractions for the presence of the target protein. If a 280 nm absorbance flow monitor is not available, determine the protein concentration using a Bradford protein assay (quick and easy) or measure the absorbance at 280 nm. Note that imidazole will also absorb at 280 nm.
- Pool fractions containing the majority of the eluted polyhistidine-tagged protein and remove an aliquot for SDS-PAGE analysis.
- Store protein at -70 °C or -20 °C. Note that many proteins irreversibly precipitate out of solution in the presence of imidazole. In this case remove imidazole prior to freezing.
- For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

4.8 FPLC™ purification of polyhistidine-tagged proteins under native conditions using Protino® Ni-NTA Columns 1 mL / 5 mL

Protino® Ni-NTA Columns can be operated with liquid chromatography systems (such as ÄKTAdesign™ systems) via standard 10–32 fittings without additional connectors.

Prepare buffers according to section 4.1. Filter buffers through a 0.45 µm filter before use. Clear samples by centrifugation and / or pass them through a 0.45 µm filter

		Protino® GST/4B columns	
		1 mL	5 mL
1	**Connect column to the chromatography system**		
	• Purge the pump with PBS. Assure that all air is displaced.		
	• Remove the snap off end at the column outlet and save it for further use.		
	• Remove the upper plug from the column.		
	• Start the pump at a flow rate of approximately 0.3 mL/min.		
	• Fill the inlet port of the column with several drops of PBS to remove air to form a positive meniscus.		
	• Insert the fitting “drop-to-drop” into the column port to avoid introducing air bubbles.		
	Note: The snap off end can be reused as a stop plug for sealing the column outlet for storage.		
2	**Column equilibration**		
	• Equilibrate the column with 5–10 column volumes of NPI-10 until the baseline at 280 nm is stable.		
		5–10 mL	**50–100 mL**
	Use a flow rate up to	1 mL/min	**2.5 mL/min**
3	**Binding**		
	• Load the centrifuged or filtered sample onto the column.		
	Use a flow rate up to	1.0 mL/min	**5 mL/min**
	• Collect flowthrough and analyze (e.g., by SDS-PAGE) to verify that the polyhistidine-tagged protein has bound. If the fusion protein is found in early fractions of the flowthrough, the flow rate should be decreased. If the fusion protein is absent in early fractions and does appear in late fractions of the flowthrough, the column capacity has been exceeded. In this case protein load should be reduced or bed volume should be increased.		

4 Washing

- Wash the column with 10–20 column volumes of NPI-20 or until the baseline at 280 nm is stable.

	10 mL	**50 mL**
Use a flow rate up to	1 mL/min	**5 mL/min**

5 Elution

- Elute the polyhistidine-tagged protein with 5–10 column volumes of NPI-250 and collect fractions.

	10 mL	**50 mL**
Use a flow rate up to	1 mL/min	**5 mL/min**

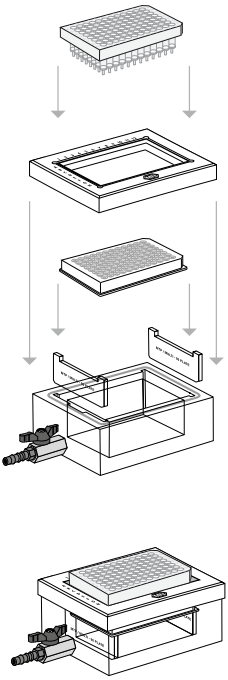
- Store eluted protein on ice.
- Analyze fractions for the presence of the target protein. If a 280 nm absorbance flow monitor is not available,y determine the protein concentration use a Bradford protein assay (quick and easy) or measure the absorbance at 280 nm. Note that imidazole will also absorb at 280 nm.
- Pool fractions containing the majority of the eluted polyhistidine-tagged protein and remove an aliquot for SDS-PAGE analysis.
- Store protein at -70 °C or -20 °C. Note that many proteins irreversibly precipitate out of solution in the presence of imidazole. In this case remove imidazole prior to freezing.
- For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

5 Purification of polyhistidine-tagged proteins under native conditions using Protino® 96 Ni-NTA

Vacuum processing

Setting up the NucleoVac 96 Vacuum Manifold:

Equilibration / Washing steps



Step 4:
Place the Protino® Purification Plate on top of the manifold lid.

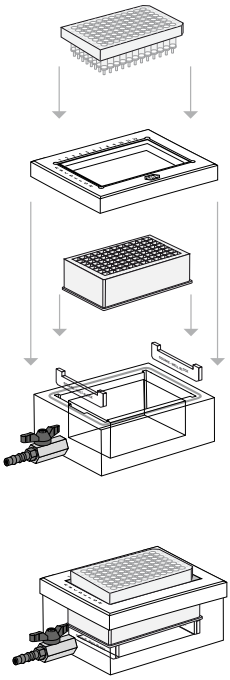
Step 3:
Place the manifold lid on top of the manifold base.

Step 2:
Place the MN Wash Plate in the manifold.

Step 1:
Insert spacers 'MTP/MULTI-96 PLATE' in the manifold base.

Final setup

Elution step



Step 4:
Place the Protino® Purification Plate on top of the manifold lid.

Step 3:
Place the manifold lid on top of the manifold base.

Step 2:
Place the Square-well Block in the manifold.

Step 1:
Insert spacers 'SQUARE-WELL BLOCK' in the manifold base.

Final setup

1 Set up the NucleoVac 96 Vacuum Manifold for the preparation of the Protino® Purification Plate

- Insert spacers labeled 'MTP/MULTI-96 PLATE' notched side up into the grooves located on the short sides of the manifold.
- Insert waste container into manifold base.
- Insert the MN Wash Plate on the spacers (labeled 'MTP/MULTI-96 PLATE') inside the manifold base (here the MN Wash Plate is used to avoid cross-contaminations).
- Close the manifold base with the manifold lid.

2 Prepare Protino® Purification Plate

- Resuspend Protino® Ni-NTA Agarose by mixing thoroughly to achieve a homogeneous suspension.
- Pour the entire contents of one bottle (11 mL of suspension) into a appropriate tray.
- Add 44 mL of deionized water to get a final volume of 55 mL .
- Gently agitate the tray to achieve a homogeneous suspension.

Note: Proper agitation is necessary to make sure the agarose beads are evenly mixed. If the suspension is not mixed well, agarose beads settle to the bottom of the tray and lead to inconsistent filling and finally to poor well-to-well reproducibility!

Use a tray with a flat bottom!

Keep mixing the agarose suspension until all wells of the plate are filled!

The suspension is considered homogeneous if it appears uniform to the eye. No clouds or settled agarose beads should be visible!

- Dispense 500 µL of the suspension into each well of the Protino® Purification Plate (500 µL of 10 % suspension corresponds to 50 µL bed volume).

Note: If you do not use all wells of the plate for purification, seal the top of the empty wells with a foil.

- Place the Protino® Purification Plate on top of the manifold base.
- Apply vacuum of approximately -0.6 bar* for 1 min. If necessary, press down the plate slightly until flow through starts.
- When the diluted storage solution of the agarose has passed the plate, release the vacuum.

*Reduction of atmospheric pressure

3 Equilibration

- Equilibrate Protino® Ni-NTA Agarose by adding 500 µL of Lysis / Equilibration Buffer (NPI-10) to each well.
- Apply vacuum of approximately -0.6 bar* for 1 min.
- Allow the buffer to pass the wells.
- Apply vacuum of approximately -0.8 bar* for a few seconds to remove any residual fluid from the long drip directors.
- Release the vacuum.

4 Batch binding

- Add up to 750 µL of clarified *E. coli* lysate to the preequilibrated wells.
- Put a MN Wash Plate upside down into the plate shaker (here the MN Wash Plate is used as an adaptor to place the Protino® Purification Plate on a plate shaker).
- Place the Protino® Purification Plate on top of the inverted MN Wash Plate (the drips of the Protino® Purification Plate fit right into the holes of the MN Wash Plate).

Note: For robotic applications or more convenient handling, MN provides a special frame to fix the Protino® Purification Plate on plate shakers (see ordering information).

- Depending on the dimension of the shaker, fix the plates properly.
- Shake the plate at 20 °C for 15 min to 30 min.

Note: Adjust the shaking speed so that the agarose remains in suspension and that no liquid comes out of the wells (e.g., 1,100 rpm for an eppendorf Thermomixer®).

5 Lysate removal

- Place the Protino® Purification Plate on top of the manifold base.
- Apply vacuum of approximately -0.6 bar* until all wells have drained. If necessary, press down the plate slightly until flow through starts.
- *Note: The vacuum may have to be adjusted for optimal results.*
- Apply vacuum of approximately -0.8 bar* for a few seconds to remove any residual fluid from the long drip directors.
- When the *E. coli* lysate has passed the plate, release the vacuum.

*Reduction of atmospheric pressure

6 Washing

- Wash Protino® Ni-NTA Agarose by adding 500 µL of Wash Buffer (NPI-20) to each well.
- Apply vacuum of approximately -0.6 bar* for 1 min.
- Allow the buffer to pass the wells.
- Apply vacuum of approximately -0.8 bar* for a few seconds to remove any residual fluid from the long drip directors.
- Release the vacuum.
- Repeat the washing step twice (total wash 3 x 500 µL of NPI-20).
- Remove Protino® Purification Plate from the vacuum manifold.

7 Set up the NucleoVac 96 Vacuum Manifold for Elution

- Remove manifold lid, MN Wash Plate, and waste container from the vacuum manifold.
- Insert spacers labeled 'SQUARE-WELL BLOCK' notched side up into the grooves located on the short sides of the manifold.
- Insert a Square-well Block into manifold base.
- Close the manifold base with the manifold lid.

*Reduction of atmospheric pressure

8 Elution

- Add 200 µL of Elution Buffer (NPI-250) to each well of the Protino® Purification Plate.
- Place the Protino® Purification Plate on a shaker by using the inverted MN Wash Plate from step 4 (batch binding).
- Shake the plate at 20 °C and 1,100 rpm for 1 min (eppendorf Thermomixer®).
- Place the Protino® Purification Plate on top of the manifold base.
- Elute the polyhistidine tagged proteins by applying vacuum of approximately -0.6 bar* for 1 min.
- Apply vacuum of approximately -0.8 bar* for a few seconds to remove any residual fluid from the long drip directors.
- Release the vacuum.
- Repeat the elution step once or until all target protein is removed.
- You may collect the eluates either in the same Square-well Block or in fractions by changing the block between each elution step.
- Store eluted protein on ice.

Note: Prior further analysis, mix the eluate thoroughly.

5.1 Centrifuge processing

- Follow the standard protocol as described in section 4.2. The vacuum steps are substituted by centrifugation of the Protino® Purification Plate at 3,000 x *g* for 2 min at RT.
- During all centrifugation steps, the Protino® Purification Plate should be placed on a Square-well Block (see ordering information, section 8.2) to collect the waste. Omit the MN Wash Plate.
- During the elution step, the Protino® Purification Plate is placed either on top of a Rack of MN Tube Strips (see ordering information, section 8.2) or on a Square-well Block.

*Reduction of atmospheric pressure

6 Purification of polyhistidine-tagged proteins under denaturing conditions

Purification of polyhistidine-tagged proteins under denaturing conditions is similar to purification under native conditions except that the sample and the buffers contain a denaturant (8 M urea or 6 M guanidine hydrochloride).

For the preparation of cell extracts and the equilibration steps, we recommend to use 6 M guanidine hydrochloride, as it is the stronger denaturant. For the washing and elution step, we recommend to switch to 8 M urea, because then eluates can be applied directly to SDS-PAGE analysis.

We recommend this protocol if expression leads to the formation of inclusion bodies. Cells are disrupted under native conditions using lysozyme together with sonication. After centrifugation the polyhistidine-tagged protein is extracted and solubilized from the pellet by using a denaturant (8 M urea). The extract obtained is clarified by centrifugation and applied to Protino® Ni-NTA Agarose or Protino® Ni-NTA Columns under denaturing conditions.

6.1 Preparation of buffers for purification under denaturing conditions

NPI-10 (1 liter):		
50 mM NaH_2PO_4	7.80 g $NaH_2PO_4 \bullet 2\ H_2O$	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
10 mM imidazole	0.68 g imidazole	M_r = 68.08 g/mol
Adjust pH to 8.0 using NaOH		
Denaturing Lysis/ Equilibration NPI-10 (1 liter):		
50 mM NaH_2PO_4	7.80 g $NaH_2PO_4 \bullet 2\ H_2O$	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
10 mM imidazole	0.68 g imidazole	M_r = 68.08 g/mol
8 M urea or 6 M guanidine hydrochloride	480 g urea or 572.3 g guanidine hydrochloride	M_r = 60.06 g/mol (urea) M = 95.53 g/mol (guanidine hydrochloride)
Adjust pH to 8.0 using NaOH		
Denaturing Wash buffer NPI-20 (1 liter):		
50 mM NaH_2PO_4	7.80 g $NaH_2PO_4 \bullet 2\ H_2O$	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
20 mM imidazole	7.00 g imidazole	M_r = 68.08 g/mol
8 M urea	480 g urea	M_r = 60.06 g/mol
Adjust pH to 8.0 using NaOH		

Denaturing Elution Buffer-250 (1 liter):

50 mM NaH_2PO_4	7.80 g NaH_2PO_4 • 2 H_2O	M_r = 156.01 g/mol
300 mM NaCl	17.54 g NaCl	M_r = 58.44 g/mol
250 mM imidazole	17.00 g imidazole	M_r = 68.08 g/mol
8 M urea	480 g urea	M_r = 60.06 g/mol
Adjust pH to 8.0 using NaOH		

6.2 Preparation of *E. coli* lysated under denaturing conditions

1 Isolation of inclusion bodies

- Thaw the cell pellet from an *E.coli* expression culture on ice (if frozen). Resuspend 1 g of pelleted, wet cells in 5 mL NPI-10 buffer (without denaturant) on ice. Pipette up and down, or use stirring until complete resuspension without visible cell aggregates.
- Add lysozyme to a final concentration of 1 mg/mL. Stir the solution on ice for 30 min.
- Sonicate the suspension on ice according to the instructions provided by the manufacturer (e.g., use 10 x 15 s bursts with a 15 s cooling period between each burst).
- 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 15 min
- Centrifuge the crude lysate at 10,000 x *g* for 30 min at 4 °C to collect the inclusion bodies. Discard supernatant. Keep pellet on ice.

2 Solubilization of inclusion bodies

- To wash the inclusion bodies resuspend the pellet in 10 mL NPI-10 (without denaturant) per g wet cells.
- Centrifuge suspension at 10,000 x *g* for 30 min at 4 °C. Discard supernatant.
- Resuspend the pellet in 2.0 mL DNPI-10 per g wet cells to solubilize the inclusion bodies. Homogenization or sonication may be necessary to resuspend the pellet. Dissolve the inclusion bodies by stirring on ice for 60 min.
- Centrifuge at 10,000 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.
- f the supernatant is not clear centrifuge a second time or filter through a 0.45 µm membrane (e.g., celluloseacetate) to avoid clogging of the IMAC column with insoluble material.
- Save supernatant (solubilized protein).

6.3 Preparation of *E. coli* lysated under denaturing conditions (96-well format)

1 Cultivate and harvest cells

- *E. coli* cells may be cultivated in 96-well plates (1 mL culture volume), 24-well plates (5 mL culture volume), or any other appropriate cultivation vessel, if larger culture volumes are required.
- Harvest cells by centrifugation at 2,000 x *g* for 15 min at 4 °C. Store cell pellets at -20 °C or -70 °C for at least 1 h.

2 Cell extract preparation

For preparation of cell extracts from up to 5 mL *E. coli* expression culture, we recommend the following protocol as a starting point for further optimization:

- Thaw cell pellets at room temperature. Resuspend each pellet in 0.5–1 mL of 1 x Denaturing Lysis / Equilibration Buffer. Incubate at room temperature for 30 min in a shaker.
- Centrifuge the crude lysate at 5,000 x *g* for 30 min at 4 °C to remove cellular debris. If the supernatant is not clear, centrifuge a second time to avoid clogging of the Protino® Purification Plate with insoluble material.

Note that optimal sample preparation steps have to be determined empirically depending on the characteristics of the of the polyhistidine-tagged protein and host organism.

6.4 Purification under denaturing conditions using Protino® Ni-NTA

Purification of polyhistidine-tagged proteins under denaturing conditions is similar to purification under native conditions except that the sample and buffers loaded on the column contain 8 M urea.

1 Proceed to section

4.3 for batch purification

4.4 for semi-batch purification

4.5 for Spin column purification

4.6 for gravity-flow purification

4.7 for FPLC™ purification using self-packed columns or

4.8 for FPLC™ purification using Protino® Ni-NTA Columns

5 for Protino® 96 Ni-NTA (vacuum processing)

5.1 for Protino® 96 Ni-NTA (centrifuge processing)

with the following modifications – use:

- Supernatant from 5.2 (solubilized protein) as sample or protein extract,
- DNPI-10 instead of NPI-10 (equilibration buffer),
- DNPI-20 instead of NPI-20 (wash buffer),
- DNPI-250 instead of NPI-250 (elution buffer).

7 Cleaning, recharging, and storage

Cleaning

After use Protino® Ni-NTA Agarose should be washed for 30 minutes with 0.5 M NaOH followed by equilibration. We recommend this cleaning procedure if you wish to purify the same His-tag protein. Sodium hydroxide effectively desorbs contaminants originating from the loaded sample, such as unspecifically bound proteins, precipitated proteins and lipoproteins.

- Wash Protino® Ni-NTA Agarose with 15 bed volumes of 0.5 M NaOH for 30 min to solubilize and desorb contaminants.
- When using columns adjust the flow rate accordingly. For example, wash a Protino® Ni-NTA Columns 1 mL by using a flow rate of 0.5 mL/min for 30 min, corresponding to a total volume of 15 mL.
- Remove the NaOH solution by washing with 10 bed volumes of de-ionized water.
- If you are reusing the resin directly, wash with 10 bed volumes of NPI-10 to equilibrate the resin.
- For storage wash with 2 bed volumes of 30 % ethanol. Resuspend the resin in 30 % ethanol and store at 2–8 °C.

Recharging

- Depending on the nature of the sample the cleaning procedure mentioned above may not be satisfactory. In cases, for example when the color of the resin changes (from light blue to white/grey (due to loss of nickel ions) or to brown (due to the reduction of nickel ions)) Protino® Ni-NTA Agarose can easily be stripped and recharged with nickel.

- Wash Protino® Ni-NTA Agarose with 10 bed volumes of de-ionized water.
- Strip of nickel ions by washing with 10 bed volumes of 100 mM EDTA, pH 8.
- Wash resin with 10 bed volumes of de-ionized water.
- Charge resin with 2 bed volumes of 100 mM metal ion aqueous solution (e.g. NiSO4 or NiCl2).
- Other metal ions may be used to increase specificity (e.g., Co2+ or or Zn2+).
- Wash resin with 10 bed volumes of de-ionized water to remove unbound metal ions.
- If you are reusing the resin, directly wash with 10 bed volumes of NPI-10 to equilibrate the resin.
- For storage wash with 2 bed volumes of 30 % ethanol. Resuspend the resin in 30 % ethanol and store at 2–8 °C.

8 Appendix

8.1 Troubleshooting

Problem	Possible cause and suggestions
Column is clogged	*Sample / lysate contains insoluble material* • If the sample is not clear use centrifugation or filtration (0.45 µm membrane) to avoid clogging of the IMAC 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.
Wells of the plate have become clogged	• Sample / lysate contains insoluble material • Use centrifugation or filtration to avoid clogging. • Sample / lysate remains viscous from genomic DNA • Add additional DNase
Protein does not bind to the resin	*Problems with vector construction* • Ensure that protein and tag are in frame. • Sometimes the position of the tag influences expression rate and solubility. Evaluate N- and C terminally tagged variants of the protein. • His-Tag is not accessible. • Use denaturing conditions to purify the protein. • Use a C terminal Histag instead of a N terminal tag or vice versa. *Incorrect binding conditions* • Check composition and pH of all buffers. Ensure that all additives are compatible (see compatibility of reagents, 2.4)
Protein elutes with wash buffer	*Incorrect buffer composition* • Check composition and pH of all buffers.
Protein does not elute	*Elution conditions are too mild.* • Increase concentration of imidazole from 250 mM to 500 mM. *Protein has precipitated* • Elute under denaturing conditions.

Problem	Possible cause and suggestions
Unwanted proteins elute with poly-histidine-tagged protein	*Insufficient wash* • Use larger volumes for washing step. • Use NPI-50 for third washing step (containing 50 mL imidazole). *Binding and wash conditions are too mild* • Use 10–20 mM imidazole in the binding and washing buffers. *Contaminating proteins and target protein are linked together via disulfide bonds* • Add up to 20 mM 2-mercaptoethanol to reduce disulfide bonds. *Contaminating proteins are proteolytic products of target protein* • Perform cell lysis at 4 °C. • Include protease inhibitors. *Resin is not saturated with His-tagged protein* *Contaminating host proteins have a better chance to bind to the resin when only small amounts of target protein are present in the lysate. Very low amounts of polyhistidine-tagged protein are not able to replace the majority of contaminating proteins effectively.* • Reduce the amount of Protino® Ni-NTA resin or increase the amount of sample. *Expression is too low* • Increase expression level. Sometimes the position of the tag influences expression rate and solubility. Use a C terminal Histag instead of a N terminal tag or vice versa. • Increase amount of starting cell material. • Do not exceed recommended lysis volumes.

8.2 Ordering information

Product	REF	Pack of
Protino® Ni-NTA Agarose	745400.25 745400.100 745400.500	25 mL 100 mL 500 mL
Protino® Ni-NTA Columns 1 mL	745410.5	5 columns
Protino® Ni-NTA Columns 5 mL	745415.1 745415.5	1 column 5 columns
Protino® 96 Ni-NTA	745425.1 745425.4	1 x 96 preps 4 x 96 preps
Protino® Columns 14 mL (empty gravity flow columns)	745250.10	10 columns
Protino® Columns 35 mL (empty gravity flow columns)	745255.10	10 columns
Protino® M6 Adaptor Set	745260	1
Protino® 1/4–28 Adaptor Set	745261	1
Protino® Luer Adaptor Set	745264	1
Protino® Inlet Luer Adaptor	745263	1
Protino® Inlet Luer Adaptor	745262	1
Square-well Block	740481 740481.24	4 24
Protino® Purification Plate	745426.1 745426.4	1 4
MN Shaker Frame	740489	1
NucleoVac Vacuum Manifold	740681	1
NucleoVac Vacuum Regulator	740641	1

Visit ***www.mn-net.com*** for more detailed product information.

8.3 Product use restriction / warranty

Protino® Ni-NTA products are intended, developed, designed, and sold FOR RESEARCH PURPOSES ONLY, except, however, any other function of the product being expressly described in original MACHEREY-NAGEL product leaflets.

MACHEREY-NAGEL products are intended for GENERAL LABORATORY USE ONLY! MACHEREY-NAGEL products are suited for QUALIFIED PERSONNEL ONLY! MACHEREY-NAGEL products shall in any event only be used wearing adequate PROTECTIVE CLOTHING. For detailed information please refer to the respective Material Safety Data Sheet of the product! MACHEREY-NAGEL products shall exclusively be used in an ADEQUATE TEST ENVIRONMENT. MACHEREY-NAGEL does not assume any responsibility for damages due to improper application of our products in other fields of application. Application on the human body is STRICTLY FORBIDDEN. The respective user is liable for any and all damages resulting from such application.

DNA/RNA/PROTEIN purification products of MACHEREY-NAGEL are suitable for *IN VITRO*-USES ONLY!

ONLY MACHEREY-NAGEL products specially labeled as IVD are also suitable for *IN VITRO*-diagnostic use. Please pay attention to the package of the product. *IN VITRO*-diagnostic products are expressly marked as IVD on the packaging.

IF THERE IS NO IVD SIGN, THE PRODUCT SHALL NOT BE SUITABLE FOR *IN VITRO*-DIAGNOSTIC USE!

ALL OTHER PRODUCTS NOT LABELED AS IVD ARE NOT SUITED FOR ANY CLINICAL USE (INCLUDING, BUT NOT LIMITED TO DIAGNOSTIC, THERAPEUTIC AND/OR PROGNOSTIC USE).

No claim or representations is intended for its use to identify any specific organism or for clinical use (included, but not limited to diagnostic, prognostic, therapeutic, or blood banking). It is rather in the responsibility of the user or - in any case of resale of the products - in the responsibility of the reseller to inspect and assure the use of the DNA/RNA/protein purification products of MACHEREY-NAGEL for a well-defined and specific application.

MACHEREY-NAGEL shall only be responsible for the product specifications and the performance range of MN products according to the specifications of in-house quality control, product documentation and marketing material.

This MACHEREY-NAGEL product is shipped with documentation stating specifications and other technical information. MACHEREY-NAGEL warrants to meet the stated specifications. MACHEREY-NAGEL's sole obligation and the customer's sole remedy is limited to replacement of products free of charge in the event products fail to perform as warranted. Supplementary reference is made to the general business terms and conditions of MACHEREY-NAGEL, which are printed on the price list. Please contact us if you wish to get an extra copy.

There is no warranty for and MACHEREY-NAGEL is not liable for damages or defects arising in shipping and handling (transport insurance for customers excluded), or out of accident or improper or abnormal use of this product; defects in products or components not manufactured by MACHEREY-NAGEL, or damages resulting from such non-MACHEREY-NAGEL components or products.

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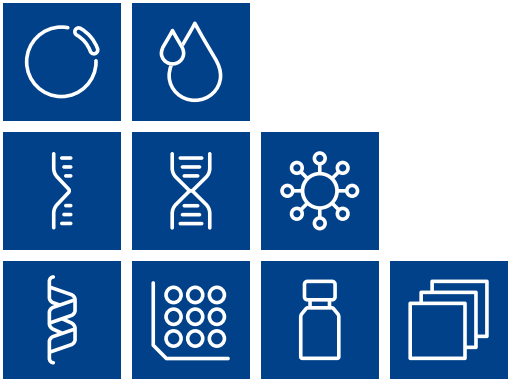

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