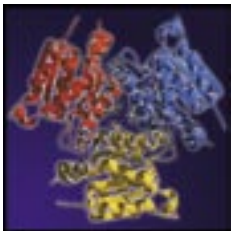

Purification of Polyhistidine-Tagged Proteins

User Manual

Protino® Ni-NTA Agarose

Protino® Ni-NTA Columns 1 mL

Protino® Ni-NTA Columns 5 mL

BIOKÉ
sharing knowledge

Plesmanlaan 1d
2333 BZ Leiden
The Netherlands
T. +31 (0)71 568 10 00
T. Belgium: 0800 71640
F. +31 (0)71 568 10 10
info@bioke.com
www.bioke.com

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MACHEREY-NAGEL

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1 Components

1.1 Contents and storage

	Protino® Ni-NTA Agarose		
REF	**745400.25**	**745400.100**	**745400.500**
Protino® Ni-NTA Agarose	25 mL	100 mL	500 mL
User Manual	1	1	1

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

Shipping and storage

The product is shipped at ambient temperature.

Upon receipt Protino® Ni-NTA Agarose products should be **stored at 2 – 8°C** and are stable up to 1 year. Do not freeze.

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 5.1)
- Lysozyme
- Appropriate columns, centrifuge tubes, etc.
- Appropriate centrifuge

- 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., BioLogic™, 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

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% cross-linked agarose beads that are suitable for batch binding, gravity flow, and FPLC™ columns. The resin is precharged with Ni^{2+} ions and therefore ready to use.

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.

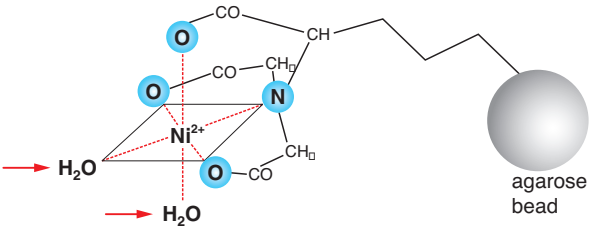

Figure 1: Protino® Ni-NTA Agarose – Structure of NTA in complex with Ni^{2+}

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	Cross-linked 6% 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)

[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		
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	
Support	Cross-linked 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]	4 mL/min	10 mL/min
Storage temperature	4 – 8°C (do not freeze)	
Storage solution	30 vol% ethanol	

[1] Binding capacity will vary for each polyhistidine-tagged protein.

[2] High flow rates may reduce binding capacity.

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*.

Solubility of the recombinant protein

- Protein yield is also dependent upon 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 contain 10 mM and the wash buffer 20 mM imidazole 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 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 4: 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 proteins	Up to 50% can be used
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

Table 4: Reagent compatibility chart		
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 Ni^{2+} 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 – risk and safety phrases

The following components of **Protino® Ni-NTA** products contain hazardous contents.

Component	Hazard contents	Hazard symbol		Risk phrases	Safety phrases
Protino® Ni-NTA Agarose	Ethanol <20%	*	Flammable	R 10	
Protino® Ni-NTA Columns 1 mL/5 mL	Ethanol <20%	*	Flammable	R 10	

Risk phrases

R 10 Flammable

* Hazard labeling not necessary if quantity per bottle below 125 g or mL (certificate of exemption according to 67/548/EEC Art. 25, 1999/45/EC Art. 12 and German GefStoffV § 20 (3) and TRGS 200 7.1). For further information see Material Safety Data Sheet.

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

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 gravity-flow purification 4.6 for FPLC™ purification using self-packed columns or 4.7 for FPLC™ purification using Protino® Ni-NTA Columns.

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 ar 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 ar 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 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 flow-through 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 ar 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.

4.6 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 flow through 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 flow-through, the flow rate should be decreased. If the fusion protein is absent in early fractions and does appear in late fractions of the flow through 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.

4.7 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® Ni-NTA Columns	
	1 mL	5 mL

1 Connect column to the chromatography system

- Purge the pump with NPI-10. 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 NPI-10 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	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

	Protino® Ni-NTA Columns	
	1 mL	5 mL

- Collect flow through 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 flow-through, 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 denaturing conditions

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.

5.1 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

DNPI-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	480 g	urea	M_r = 60.06 g/mol

Adjust pH to 8.0 using NaOH

DNPI-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	1.36 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

DNPI-250 (1 liter):

50 mM	NaH_2PO_4	7.80 g	$Na_2HPO_4 \bullet 2\ H_2O$	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
8 M	urea	480 g	urea	M_r =	60.06 g/mol

Adjust pH to 8.0 using NaOH

5.2 Protein extract preparation 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).

5.3 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.

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1 Proceed to section 4.3 for batch purification
4.4 for semi-batch purification
4.5 for gravity-flow purification
4.6 for FPLC™ purification using self-packed columns or
4.7 for FPLC™ purification using Protino® Ni-NTA Columns

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).

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6 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. $NiSO_4$ or $NiCl_2$).

  Other metal ions may be used to increase specificity (e.g., Co^{2+} or or Zn^{2+}).

- 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.

7 Appendix

7.1 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.
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.

7.2 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® 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 PP Adaptor Set	745263	1
Protino® Inlet Luer Adaptor	745262	1

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

7.3 Product use restriction / warranty

Protino® products were developed, designed, distributed, and sold **FOR RESEARCH PURPOSES ONLY.** They are suitable **FOR *IN–VITRO* USES ONLY**. No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking).

It is rather the responsibility of the user to verify the use of the **Protino® products** for a specific application range as the performance characteristic of this kit has not been verified to a specific organism.

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Please contact:
MACHEREY-NAGEL Germany
Tel.: +49 (0) 24 21 969 270
e-mail: TECH-BIO@mn-net.com

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