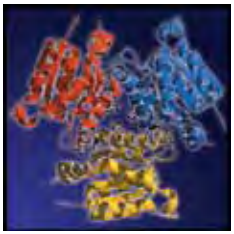


Purification of His-tag proteins

User manual

Protino® Ni-TED/IDA Combi Sample

January 2011 / Rev. 03

MACHEREY-NAGEL

Table of contents

1 Components

1.1 Contents and storage

	Protino® Ni-TED/IDA Combi Sample
	2 preps
REF	745130.Muster
Protino® Ni-TED 1000 Packed Columns	1
Protino® Ni-IDA 1000 Packed Columns	1
8 x LEW Buffer	5 mL
4 x Elution Buffer	5 mL
Plastic Washer	2
User manual	1

Storage conditions

All kit components can be stored at room temperature (18–25 °C) and are stable up to one year.

1.2 Additional materials to be supplied by user

Reagents

- Lysozyme
- Purification under native conditions: Kits already contain buffer stock solutions that have to be prepared according to the instructions, section 5.3.1.

  Purification under denaturing conditions: Denaturing Solubilization Buffer, Denaturing Elution Buffer, additional LEW Buffer (sodium phosphate, sodium chloride, urea, and imidazole). For buffer compositions refer to section 5.1 and 6.1.

Consumables

- Appropriate centrifugation / collection tubes

Equipment

- Appropriate centrifuge, sonicator

2 Introduction

2.1 The basic principle

Protino® Ni-TED/IDA products enable fast and convenient purification of recombinant polyhistidine-tagged proteins by immobilized metal ion affinity chromatography (IMAC). This sample kit contains two kinds of gravity flow columns prepacked with a dry silica-based resin with TED (tris-carboxymethyl ethylene diamine) and IDA (iminodiacetic acid) as a chelating group. Both resins are precharged with Ni^{2+} ions and therefore ready to use. Protino® Ni-IDA is specially suited for screening studies, which require high concentrations of the target protein in the eluate. Protino® Ni-TED yields target protein of excellent purity, furthermore it is compatible to chelating and reducing reagents.

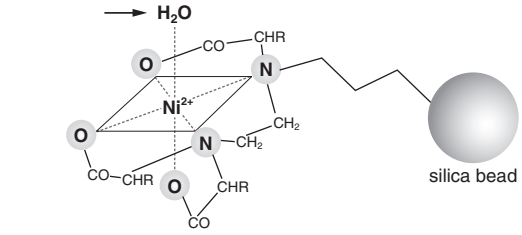
Binding of protein to the IMAC resin is based on the interaction between the polyhistidine tag of the recombinant protein and immobilized Ni^{2+} ions. Ligand **TED** is a strong pentadentate metal chelator, which occupies five of the six binding sites in the coordination sphere of the Ni^{2+} ion. The remaining coordination site of Ni^{2+} is available for protein binding (Figure 1). Other chelating groups such as NTA (nitrilotriacetic acid) have four binding sites available for the Ni^{2+} ion, the remaining two sites of Ni^{2+} are available for protein binding:

TED – (5 bonds) – Ni^{2+} – (1 bond) – Protein

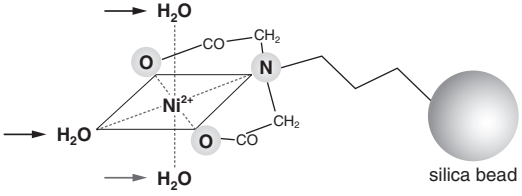
NTA – (4 bonds) – Ni^{2+}– (2 bonds) – Protein

The additional chelation site of TED with Ni^{2+} minimizes metal leaching during purification and increases specificity for polyhistidine-tagged proteins. As a result target protein of excellent purity is eluted from the column.

Ligand **IDA** is a threedentate chelator which occupies three of the six binding sites in the coordination sphere of the Ni^{2+} ion. The remaining three coordination sites are usually occupied by water molecules and can be exchanged with histidine residues of the recombinant protein (Figure 1). IDA enables strong and efficient binding of target protein to the IMAC matrix. In contrast to traditional IDA matrices, Protino® Ni-IDA shows an optimized, low density of IDA ligands, which is created by a special manufacturing process. This non-saturating surface concentration of IDA eliminates non-specific interactions of contaminating proteins with the adsorbent. As a result, Protino® Ni-IDA ensures higher target protein purity.



Ni-TED



Ni-IDA

Figure 1: Structure of TED and IDA in complex with Ni^{2+}

2.2 About this user manual

For quick orientation in this user manual please follow the corresponding cross-reference given below.

Table 1: Protocol guide

Product	Application	Page
Protino® Ni-TED 1000 Packed Columns Protino® Ni-IDA 1000 Packed Columns	Gravity flow column chromatography under native conditions	23
Protino® Ni-TED 1000 Packed Columns Protino® Ni-IDA 1000 Packed Columns	Gravity flow column chromatography under denaturing conditions	29

This manual is organized as follows: All information refers to Protino® Ni-TED as well as Protino® Ni-IDA. Specifications that differ between the two products are indicated with corresponding indices (i.e., XX^{TED} or XX^{IDA}).

Experienced users who are performing the purification of polyhistidine-tagged proteins using **Protino® Ni-TED/IDA 1000 Packed Columns** may refer to the protocol-at-a-glance instead of this user manual (see section 5.3.1). The protocol-at-a-glance is designed to be used only as a supplemental tool for quick referencing while performing the purification procedure. First-time users are strongly advised to read this user manual.

The **Protino® Ni-TED/IDA Packed Columns** protocols in this manual are organized as follows: The culture volumes and volumes of the respective buffers used for a particular column size are highlighted. Each procedural step is arranged like the following example (taken from section 5.3.2):

---

3 Column equilibration

Equilibrate Protino® Ni TED/IDA Packed Columns with **1 x LEW Buffer**. Allow the column to drain by gravity

2 mL

Protino® Ni 1000 Packed Columns are designed to fit into most 15 mL conical centrifuge tubes (e.g., BD Falcon REF 352097) for convenient fraction collection.

Referring to the a.m. example there has to be used 2 mL of LEW Buffer for column equilibration when using **Protino® TED/IDA 1000 Packed Columns**.

3 Product description

3.1 Specifications

Table 2: Specifications Protino® Ni-TED / IDA

	Protino® Ni-TED 1000 Packed Colums	Protino® Ni-IDA 1000 Packed Colums
Application	Gravity flow chromatography	
Chelating group	TED	IDA
Binding capacity	2.5 mg	5 mg
Matrix	Macroporous silica	
Physical form	Dry matrix, precharged with Ni^{2+}	
Amount of resin per column	250 mg	
Bed volume	500 µL	
pH stability	3–7.5 (≥2 h) 2–3 or 7.5–8.5 (≥2 h)	
Storage	25 °C	
Recommended imidazole concentration for load / wash	0 mM (!)	
Recommended imidazole concentration for elution	250 mM	
Advantages	Target protein of excellent purity is eluted	Concentrated target protein is eluted in the first elution fraction
	Resin is compatible to chelating (e.g., EDTA) and reducing (e.g., mercaptoethanol) reagents	Resin binds target protein efficiently from highly diluted samples

3.2 Purification under native and denaturing conditions

This manual describes methods for the preparation of cell extracts from *E. coli* and procedures for the purification of polyhistidine-tagged recombinant proteins using Protino® Ni-TED and Protino® Ni-IDA.

If recombinant 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 5) as well as insoluble proteins from inclusion bodies (purification under denaturing conditions, see section 6).

In general for purification of polyhistidine-tagged proteins, the bacterial cells are disrupted using lysozyme in combination with sonication. After centrifugation, soluble target protein is found in the supernatant while inclusion bodies remain in the pellet. The clear supernatant can directly be subjected to further purification using Protino® Ni-TED / IDA Packed Columns under native conditions (see section 5). In case of massive formation of inclusion bodies the target protein is extracted from the pellet using a denaturant (8 M urea) and further purified using protocols for the purification under denaturing conditions (see section 6). If the distribution of the recombinant protein is unknown it is recommended to perform SDS-PAGE analysis using the crude cell extract prior to centrifugation and the clear supernatant after centrifugation. While the crude cell extracts will contain both soluble and insoluble target protein, only soluble target protein is found in the supernatant.

3.3 Binding capacity

3.3.1 General information

The binding capacity of Protino® Ni-TED/IDA strongly depends on the **characteristics** of the polyhistidine-tagged protein, for example amino acid composition, molecular weight, 3-D structure, oligomerization properties, etc. 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 and has to be determined for each expression experiment.**

The binding behaviour of any polyhistidine-tagged protein to Protino® Ni-TED/IDA can be examined by calculating the amount of protein that is eluted as a function of the amount of protein that has been loaded (see Figure 2). Please note that the resulting graph will vary in dependence on characteristics and concentration of the individual His-tag protein. The binding curve can be divided in three stages:

1. Stage of maximum recovery. At this stage the loaded protein is bound to the resin nearly quantitatively and can be eluted nearly quantitatively, too (the binding curve is almost linear, see Figure 2, • eluted His-GFPuv).

2. Stage of increasing yield / decreasing recovery. At this stage the binding curve becomes non-linear and finally binding approaches saturation. The protein yield increases with further increasing amount of loaded protein.

3. Stage of maximum yield / minimum recovery. When loading excess protein, the available binding sites of the resin are saturated. The amount of eluted protein reaches a maximum.

The **binding capacity** for each individual protein can be defined as the yield, at which the binding curve changes from the stage of maximum recovery to the stage of increasing yield/decreasing recovery. This point is an optimal compromise between protein load and recovery and will vary for each individual protein.

3.3.2 Binding capacity

The binding capacity of Protino® Ni-TED and Protino® Ni-IDA is exemplified using the green fluorescent protein (6 x His-GFPuv, ~32 kDa) at a concentration of 2 mg/mL.

Please note that different recombinant proteins may show a different binding behaviour.

Figure 2 shows a plot of the amount of eluted 6 x His-GFPuv against the amount of loaded 6 x His-GFPuv. The binding curve can be divided in three stages:

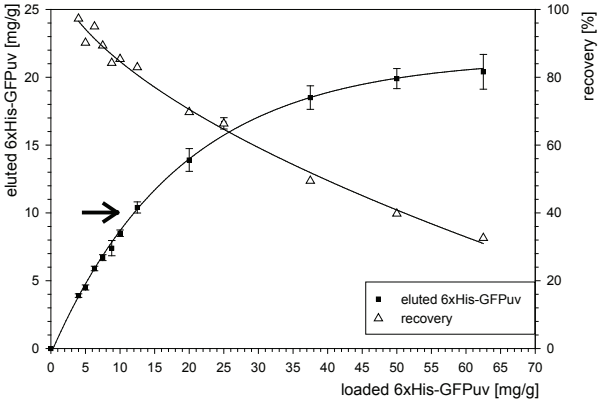
1. Stage of maximum recovery: $<\sim 10^{TED}/20^{IDA}$ mg 6 x His-GFPuv load/g resin
2. Stage of increasing yield/decreasing recovery: $>\sim 10^{TED}/20^{IDA}$ mg 6 x His-GFPuv load/g resin
3. Stage of maximum yield/minimum recovery: $>\sim 60^{TED}/75^{IDA}$ mg 6 x His-GFPuv load/g resin

Under the above mentioned conditions the binding capacity of Protino® Ni-TED/IDA for 6 x His-GFPuv is approximately $10^{TED}/20^{IDA}$ mg protein per g of resin (see arrow, Figure 2). At this point the protein recovery is > 80 %. Consequently the following amounts of 6 x His-GFPuv have to be loaded:

TED: For optimal recovery: load ~10 mg protein per 1 g of Protino® Ni-TED Resin, for maximum yield: load ~60 mg protein per 1 g of Protino® Ni-TED Resin.

IDA: For optimal recovery: load ~20 mg protein per 1 g of Protino® Ni-IDA Resin, for maximum yield: load ~75 mg protein per 1 g of Protino® Ni-IDA Resin.

Protino® Ni-TED



Protino® Ni-IDA

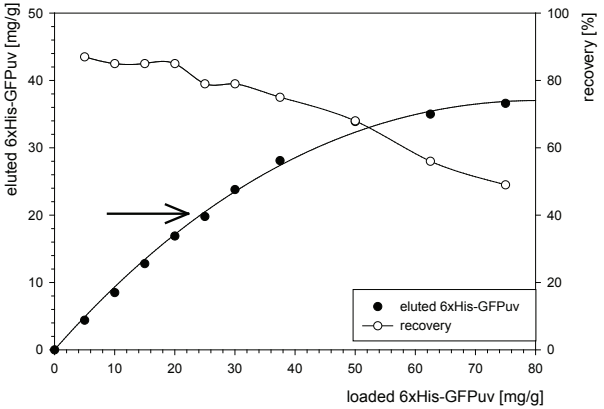


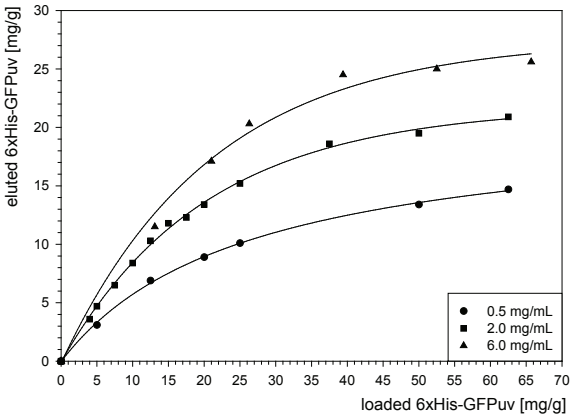
Figure 2: Binding behaviour of 6 x His-GFPuv to Protino® Ni-IDA and Protino® Ni-TED

Gravity flow columns packed with 40 mg of Protino® Ni-IDA Resin were loaded with increasing volumes of an *E. coli* lysate containing 6 x His-GFPuv (protein concentration 2 mg/mL). After washing with 640 µL LEW Buffer the target protein was eluted with 960 µL Elution Buffer. Yield (left axis) and recovery (right axis) of 6 x His-GFPuv are plotted versus the amount of loaded protein. For convenient analysis the values are converted to mg 6 x His-GFPuv per 1 g resin.

Recovery rates and yield can be increased by using samples containing higher concentrated polyhistidine-tagged protein (6 x His-GFPuv). Figure 3 shows that the yield of purified polyhistidine-tagged protein is not only depending on the total amount of target protein loaded on the column (also see Figure 2) but also on its concentration in the lysate. Consequently the concentration of target protein in the sample should be as high as possible.

Please note: The higher the protein concentration in the sample and the higher the total amount of protein loaded on Protino® Ni-TED / IDA Packed Columns or Resin, the higher will be the absolute yields. For example if loading ~60^{TED}/75^{IDA} mg 6 x His-GFPuv (concentration: 6^{TED} / 8^{IDA} mg/mL) per g Protino® Ni-TED / IDA Resin, a maximum yield of ~25^{TED} / 50^{IDA} mg/g can be obtained.

Protino® Ni-TED



Protino® Ni-TIDA

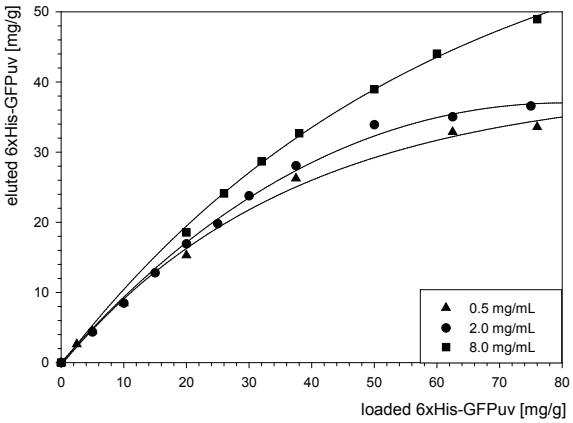


Figure 3: Binding behaviour of 6 x His-GFPuv to Protino® Ni-TED and Protino® Ni-IDA at different concentrations of the polyhistidine-tagged protein in the sample

Recombinant 6 x His-GFPuv was expressed in *E. coli*. The concentration of the target protein in the culture reached 100 mg/L. 1 g cells were lysed in 2 mL LEW Buffer according to section 3.4 in order to obtain a highly concentrated lysate. The concentration of 6 x His-GFPuv in the lysate was 8 mg/mL. Gravity flow columns packed with 40 mg of Protino® Ni-TED/IDA Resin were loaded with increasing volumes (amounts) of the lysate. After washing, the target protein was eluted with Elution buffer. The yield of 6 x His-GFPuv is plotted against the amount of loaded target protein. The same test was performed using diluted lysates with concentrations of 0.5 and 2 mg/mL. For convenient analysis the values are converted to mg 6 x His-GFPuv per 1 g resin.

3.4 Culture size

As outlined above, the protein yield depends on various parameters. However, some recommendations on protein load and culture size can be given as a starting point.

Note that yield and protein load are exemplified for the 6 x His-GFPuv (~32 kDa) and may vary from protein to protein.

- Use rather high concentrations of the target protein in the sample.
- For **maximum yield** use an excess amount of polyhistidine-tagged protein in the loaded sample. For example apply up to $60^{TED}/75^{IDA}$ mg of anticipated 6 x His-GFPuv per 1 g of Protino® Ni-IDA Resin.
- For **maximum recovery** use up to $10^{TED}/25^{IDA}$ mg of 6 x His-GFPuv per 1 g of Protino® Ni-TED/IDA Resin.

The concentration of the polyhistidine-tagged protein in the culture may vary from <1 mg/L up to 200 mg/L depending on cell density and expression level. It is recommended to determine the protein concentration for each expression experiment, for example via SDS-PAGE. On average, 250 mL of culture will produce approximately 1 g of pelleted, wet cells.

- Transfer the cell lysate from a $100–600^{TED}/200–750^{IDA}$ mL (high expression at 100 mg/L) or $1000–6000^{TED}/2000–7500^{IDA}$ mL (low expression at 10 mg/L) *E. coli* culture to 1 g of Protino® Ni-TED/IDA Resin.
- In order to obtain highly concentrated lysates, lyse wet cells in 2–5 mL LEW Buffer per 1 g wet mass. The volume of LEW Buffer should be adjusted according to the amount of polyhistidine-tagged protein in the culture. For example, 1 g cells may be resuspended in 2–5 mL LEW Buffer if a protein is expressed at 50–200 mg/L. For cultures with lower target protein content 1 g cells should be resuspended in 2 mL of LEW Buffer.

For recovering polyhistidine-tagged protein from *E. coli* cultures we recommend treatment with lysozyme in combination with sonication. If you are purifying recombinant protein from eukaryotic cells, treat the cells with an appropriate buffer containing a mild detergent (Sambrook *et al.*, 1989).

Table 3: Determination of culture and buffer volume requirements

	Concentration of HisTag protein in the culture	Results in	Amount of protein load [mg]	Recommended *E. coli* culture volume [mL]	Recommended *E. coli* pellet wet mass[1] [g]
Protino® Ni-TED 1000 Packed Columns (250 mg Resin)	high, ~100 mg/L	$Recovery_{max}$	2.5	25	0.1
		$Yield_{max}$	15	150	0.6
	low, ~10 mg/L	$Recovery_{max}$	2.5	250	1
		$Yield_{max}$	15	1500	6
Protino® Ni-IDA 1000 Packed Columns (250 mg Resin)	high, ~100 mg/L	$Recovery_{max}$	5	50	0.2
		$Yield_{max}$	18.8	188	0.75
	low, ~10 mg/L	$Recovery_{max}$	5	500	2
		$Yield_{max}$	18.8	1880	7.5

	Native conditions	Denaturing conditions		
	LEW Buffer[2] (resuspension) [mL]	LEW Buffer[2] (resuspension) [mL]	LEW Buffer[1] (washing of IB[4]) [mL]	DS Buffer[3] (lysis of IB[4]) [mL]
Protino® Ni-TED 1000 Packed Columns (250 mg Resin)	0.5	0.5	1	0.2
	3	3	6	1.2
	2	5	10	2
	12	30	60	12
Protino® Ni-IDA 1000 Packed Columns (250 mg Resin)	1	1	2	0.4
	15	15	7.5	2.4
	4	4	20	4
	24	24	75	24

[1] On average, 250 mL of culture will produce approximately 1 g of pelleted, wet cells.

[2] Lysis-Equilibration-Wash Buffer

[3] Denaturing Solubilization Buffer

[4] Inclusion Bodies

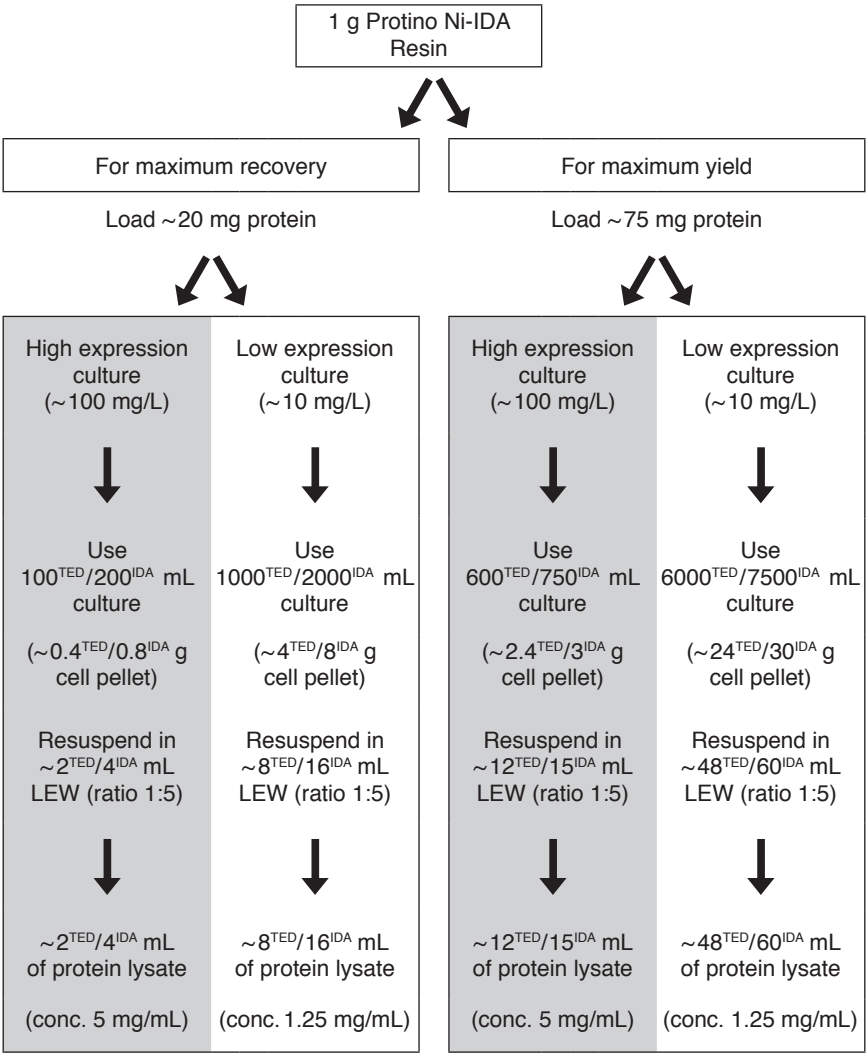


Figure 4: Required culture volumes and LEW Buffer volumes for maximum protein recovery or maximum yield in dependence on protein expression level. **The volumes noted below are only exemplary and are shown for 1 g of Protino® Ni-TED / IDA Resin (purification under native conditions).** Please use these recommendations as a starting point to evaluate optional purification results. Note that purification conditions have to be optimized for each individual polyhistidine-tagged protein.

3.5 Binding, washing, and elution

In comparison to Ni-NTA and classic Ni-IDA, both Protino® Ni-TED and Protino® Ni-IDA are more specific for polyhistidine-tagged proteins (see Figures 5 and 6). Since virtually no contaminating host proteins bind to Protino® Ni-TED/IDA, stringent washing procedures are generally not necessary. Therefore LEW buffer, which is used for lysis, equilibration, and washing, does not contain any imidazole (Ni-NTA and classic Ni-IDA require washing with imidazole-containing buffers). Bound polyhistidine-tagged protein can competitively be eluted by adding imidazole. The recommended elution buffer contains 250 mM imidazole in order to recover even strong binding, multimeric proteins with more than one polyhistidine tag (also see buffer compositions, section 5). However, as shown in Figure 5 and 6, depending on the protein, elution may be equally effective in the presence of much lower imidazole concentrations. If, for example, the stability or integrity of the target protein in 250 mM imidazole is a concern, the concentration of imidazole in the eluent may readily be reduced.

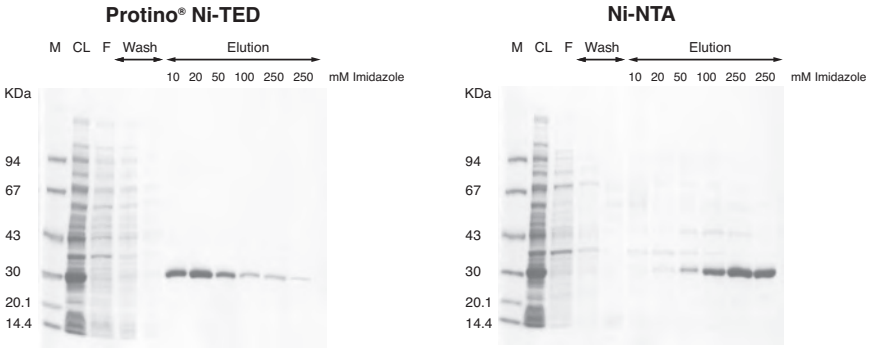


Figure 5: Purification of polyhistidine-tagged GFPuv using Protino® Ni-TED and Ni-NTA

Recombinant GFPuv was expressed in *E. coli*, lysed, loaded onto each gravity flow column, and eluted by a stepwise imidazole gradient. Eluted fractions were analyzed by SDS-PAGE. Pure polyhistidine-tagged protein can be eluted from Protino® Ni-TED (left panel) at much lower imidazole concentrations than from Ni-NTA (right panel). In addition, Ni-NTA releases contaminating proteins from 10 mM to 20 mM imidazole. Therefore, Protino® Ni-TED is more specific for polyhistidine-tagged proteins as no contaminating proteins are visible as shown for the Ni-NTA.
M = Marker proteins, CL = Cleared lysate.

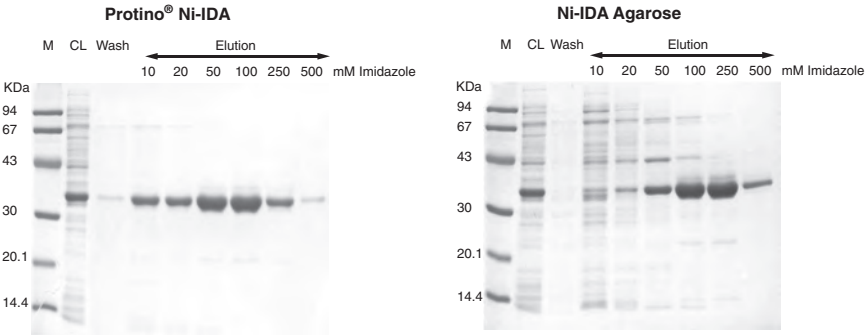


Figure 6: Purification of polyhistidine-tagged GFPuv using Protino® Ni-IDA and Ni-IDA Agarose

Recombinant GFPuv was expressed in *E. coli*, lysed, loaded onto each gravity flow column, and eluted by a stepwise imidazole gradient. Eluted fractions were analyzed by SDS-PAGE. Pure polyhistidine-tagged protein can be eluted from Protino® Ni-IDA (left panel) at much lower imidazole concentrations than from Ni-IDA Agarose (right panel). In addition, Ni-IDA Agarose releases contaminating proteins from 10 mM to 100 mM imidazole.
M = Marker proteins, CL = Cleared lysate.

3.6 Compatibility of reagents

Buffer components that chelate metal ions, such as EDTA and EGTA, should not be used since they strip Ni^{2+} ions from the matrix.

Do not use buffers with pH > 8.4, since silica dissolves in solutions of high pH.

Table 4: Reagent compatibility chart

Reagent	Effect	Comments
Sodium phosphate	Used in LEW and Elution Buffer in order to buffer the solutions at pH 8	$50^{TED/IDA}$ mM is recommended. The pH of any buffer should be adjusted to 8, although in some cases a pH between 7 and 8 can be used
Tris	Coordinates with Ni^{2+} ions, causing a decrease in capacity	$10^{TED/IDA}$ mM may be used, sodium phosphate buffer is recommended
Sodium Chloride	Prevents ionic interactions and therefore unspecific binding	Up to $2^{TED/IDA}$ 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	Should not be included in LEW Buffer
Urea	Solubilizes protein	Use $8^{TED/IDA}$ M for purification under denaturing conditions
GuHCl	Solubilizes protein	Up to $6^{TED/IDA}$ M can be used
ß-mercaptoethanol	Prevents formation of disulfide bonds; Can reduce Ni^{2+} ions at higher concentrations	Up to $50^{TED/IDA}$ mM in samples has been used successfully in some cases
DTT, DTE	Can reduce Ni^{2+} ions at higher concentrations	Up to $10^{TED/IDA}$ mM in samples has been used successfully in some cases
Glutathione reduced	Can reduce Ni^{2+} ions at higher concentrations	Up to $30^{TED/IDA}$ mM in samples has been used successfully in some cases
Glycerol	Prevents hydrophobic interactions between proteins	Up to $50^{TED/IDA}$ % can be used

This table continues on the next page.

Table 5: Reagent compatibility chart		
EDTA	Coordinates with Ni^{2+} ions, causing a decrease in capacity at higher concentrations	Not recommended, but up to $1^{TED}/1^{IDA}$ mM in samples has been used successfully in some cases
Ethanol	Prevents hydrophobic interactions between proteins	Up to $20^{TED/IDA}$ % 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 $0.2^{TED}/0.5^{IDA}$ % in samples has been used successfully in some cases
Triton, Tween	Removes background proteins	Up to $2^{TED/IDA}$ % can be used

4 Safety instructions – risk and safety phrases

The components of the Protino® Ni-TED/IDA Combi Sample kit do not contain hazardous contents.

5 Purification of polyhistidine-tagged proteins from *E. coli* under native conditions

5.1 Preparation of buffers for purification under native conditions

Protino® Ni-TED / IDA Combi Samples *kits contain LEW/Elution Buffer stock solutions that have to be diluted according to the instructions given in the individual protocol (see section 5.3).*

Note that lysis buffer, equilibration buffer, and washing buffer are the same.

Note: Do not include any imidazole in the Lysis-Equilibration-Wash Buffer, since most proteins do not bind to the resin in the presence of even low imidazole concentration!

Lysis-Equilibration-Wash Buffer (1 x LEW Buffer, 1 liter):

- 50 mM NaH_2PO_4 — 7.8 g NaH_2PO_4 x 2 H_2O (MW = 156.01 g/mol)
- 300 mM NaCl — 17.5 g NaCl (MW = 58.44 g/mol)
- Adjust pH to 8.0 using NaOH

Elution Buffer (1 x buffer, 1 liter):

- 50 mM NaH_2PO_4 — 7.8 g NaH_2PO_4 x 2 H_2O (MW = 156.01 g/mol)
- 300 mM NaCl — 17.5 g NaCl (MW = 58.44 g/mol)
- 250 mM imidazole — 17.0 g imidazole (MW = 68.08 g/mol)
- Adjust pH to 8.0 using NaOH

5.2 Preparation of cleared lysates under native conditions

1 **Refer to Table 4, section 3.4 for detailed information on culture and buffer volume requirements**

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 LEW Buffer** (for details see section 3.4). Pipette up and down, or use stirring until complete resuspension without visible cell aggregates. Perform this step on ice.

2 Add **lysozyme** to a final concentration of 1 mg/mL. Stir the solution **on ice** for **30 min**.

3 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 and stir on ice for 15 min.

4 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) to avoid clogging of the IMAC column with insoluble material.

Store supernatant on ice.

Proceed to section 5.3.

5.3 Protino® Ni-IDA Packed Columns – purification under native conditions

5.3.1 Protocol-at-a-glance

		Protino® Ni-TED / IDA Packed Columns
		1000
1	**Cultivate and harvest cells**	4,500– 6,000 x *g* 15 min at 4 °C
2	**Preparation of working solutions (per column)**	
	8 x LEW Buffer	1.5 mL
	+ water	+ 10.5 mL
	= 1 x LEW Buffer	= 12 mL
	4 x Elution Buffer	1.5 mL
	+ water	+ 4.5 mL
	= 1 x Elution Buffer	= 6 mL
3	**Cell extract preparation**	
	Refer to section 5.2.	
4	**Column Equilibration**	
	1 x LEW Buffer	2 mL
5	**Binding**	Load clarified lysate onto the column
6	**Washing**	
	1 x LEW Buffer	2 x 2 mL
7	**Elution**	
	1 x Elution Buffer	3 x 1.5 mL

5.3.2 Procedure

Note: Experienced users may refer to the protocol at a glance, section 5.3.1.

1 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. Store cell pellet at -20 °C if not processed immediately.

2 Preparation of working solutions

Prepare 1 x LEW (Lysis / Equilibration / Wash) Buffer and 1 x Elution Buffer by diluting the supplied stock solutions.

Note: If precipitate is observed in the stock solutions, warm and shake them to dissolve precipitate prior to diluting the buffers.

Mix **8 x LEW Buffer**

1.5 mL

with deionized water

10.5 mL

to get a **final volume** of **1x LEW Buffer** sufficient for one column run.

12 mL

Mix **4 x Elution Buffer**

1.5 mL

with deionized water

4.5 mL

to get a **final volume** of **1x LEW Buffer** sufficient for one column run.

6 mL

3 Cell Extract Preparation

Refer to section 5.2. For detailled information on culture and buffer volumes for cell extract preparation also see Table 3, section 3.4.

4 Column equilibration

Equilibrate Protino® Ni-IDA Packed Columns with **1 x LEW Buffer**. Allow the column to drain by gravity.

2 mL

Protino® Ni-IDA 150/1000 Packed Columns are designed to fit into most 15 mL conical centrifuge tubes (e.g., BD Falcon REF 352097) for convenient fraction collection.

5 Binding

Add the cleared lysate (see section 5.2 to the pre-equilibrated column and allow the column to drain by gravity.

6 Washing

Wash the column with **1 x LEW Buffer**. Allow the column to drain by gravity.

2 x 2 mL

7 Elution

Elute the polyhistidine-tagged protein in a new collecting tube by adding **1 x Elution Buffer**. Allow the column to drain by gravity

3 x 1.5 mL

*Note: Depending on protein characteristics 90 % of the eluted protein can be found in the **first** elution fraction.*

Use protein assay and/or SDS-PAGE analysis to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

6 Purification of polyhistidine-tagged proteins from *E. coli* under denaturing conditions

6.1 Preparation of buffers for purification under denaturating conditions

Protino® Ni-TED/IDA Combi Sample kits *contain stock solutions of LEW Buffer and Elution Buffer for purification under native conditions. For purification under denaturing conditions prepare Denaturing Solubilization Buffer and Denaturing Elution Buffer according to the instruction given in this section. Note that additional volumes of LEW Buffer have to be prepared as well.*

> ***Note: Due to the dissociation of urea, prepare buffers immediately prior to use.***

Lysis-Equilibration-Wash Buffer (1 x LEW Buffer, 1 liter):

- 50 mM NaH_2PO_4 — 7.8 g NaH_2PO_4 x 2 H_2O (MW = 156.01 g/mol)
- 300 mM NaCl — 17.5 g NaCl (MW = 58.44 g/mol)
- Adjust pH to 8.0 using NaOH

Denaturing Solubilization Buffer (1 x buffer, 1 liter):

- 50 mM NaH_2PO_4 — 7.8 g NaH_2PO_4 x 2 H_2O (MW = 156.01 g/mol)
- 300 mM NaCl — 17.5 g NaCl (MW = 58.44 g/mol)
- 8 M urea — 480.5 g (MW = 60.06 g/mol)
- Adjust pH to 8.0 using NaOH

Denaturing Elution Buffer (1 x buffer, 1 liter):

- 50 mM NaH_2PO_4 — 7.8 g NaH_2PO_4 x 2 H_2O (MW = 156.01 g/mol)
- 300 mM NaCl — 17.5 g NaCl (MW = 58.44 g/mol)
- 8 M urea — 480.5 g (MW = 60.06 g/mol)
- 250 mM imidazole — 17.0 g imidazole (MW = 68.08 g/mol)
- Adjust pH to 8.0 using NaOH

6.2 Cell extract preparation 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-TED/IDA Packed Columns under denaturing conditions. Purification of polyhistidine-tagged proteins under denaturing conditions is similar to purification under native conditions except that the cell extract and buffers loaded on the column contain 8 M urea. For buffer compositions see section 5.1 and 6.1.

1 Isolation of inclusion bodies

Refer to Table 3, section 3.4 for detailed information on culture and buffer volume requirements.

Thaw the cell pellet from an *E. coli* expression culture on ice (if frozen). Resuspend **1 g** of **pelleted, wet cells** in **5 mL LEW Buffer** (without denaturant) on ice (also see section 3.4). 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 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

Resuspend the pellet in **10 mL LEW Buffer** per g wet cells to wash the inclusion bodies.

Centrifuge the suspension at **10,000 x *g*** for **30 min at 4 °C**. Discard supernatant.

Resuspend the pellet in **2.0 mL Denaturing Solubilization Buffer 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.

If 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.

Proceed to section 6.3.

6.3 Protino® Ni-TED / IDA 1000 Packed Columns – purification under denaturing conditions

1 Cell extract preparation

Refer to section 6.2. For detailed information on culture and buffer volumes for cell extract preparation also see Table 3, section 3.4.

2 Solubilization of inclusion bodies

Refer to section 6.2. For detailed information on culture and buffer volumes for cell extract preparation also see Table 3, section 3.4.

3 Column equilibration

Equilibrate Protino® Ni-IDA Packed Columns with **Denaturing Solubilization Buffer**. Allow the column to drain by gravity.

2 mL

Protino® Ni-IDA 150 / 1000 Packed Columns are designed to fit into most 15 mL conical centrifuge tubes (e.g., BD Falcon REF 352097) for convenient fraction collection.

4 Binding

Add solubilized inclusion bodies (cleared lysate, see section 6.2) to the pre-equilibrated column and allow the column to drain by gravity.

5 Washing

Wash the column with **Denaturing Solubilization Buffer**. Allow the column to drain by gravity.

6 Elution

Elute the polyhistidine-tagged protein in a new collecting tube by adding **Denaturing Elution Buffer**. Allow the column to drain by gravity.

3 x 1.5 mL

*Note: Depending on protein characteristics 90 % of the eluted protein can be found in the **first** elution fraction.*

Use protein assay and/or SDS-PAGE analysis to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

7 Cleaning, recharging, and storage

Cleaning

After use, wash resin with 10 bed volumes of LEW Buffer and 10 bed volumes of deionized water. Wash with 2 bed volumes of 20 % ethanol and store resin in 20 % ethanol at 4 °C.

Recharging

Depending on the nature of the sample Protino® Ni-TED/IDA can be reused 3–5 times. Reuse should only be performed with identical polyhistidine-tagged proteins to avoid possible cross-contamination.

After the final elution step wash Protino® Ni-TED/IDA Resin with 10 bed volumes of LEW Buffer. After equilibrating with LEW Buffer the resin is ready for reuse.

Complete regeneration

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

2 bed volumes of	6 M GuHCl, 0.2 M acetic acid
5 bed volumes of	deionized water
3 bed volumes of	2 % SDS
5 bed volumes of	deionized water
5 bed volumes of	100 % EtOH
5 bed volumes of	deionized water
5 bed volumes of	100 mM EDTA pH 8
5 bed volumes of	deionized water
5 bed volumes of	100 mM $NiSO_4$
10 bed volumes of	deionized water

8 Appendix

8.1 Troubleshooting

Problem	Possible cause and suggestions
Sample does not enter column bed	*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 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. *Incorrect binding conditions* • Check composition of buffers and verify pH 7–8. Ensure that there is no chelating or strong reducing reagent or imidazole present.
Protein elutes with wash buffer	*Incorrect buffer composition* • Check composition of buffers and verify pH 7–8. Ensure that there are no chelating or strong reducing agents or imidazole present.
Protein does not elute from column	*Elution conditions are too mild.* • Increase concentration of imidazole.
Unwanted proteins elute with polyhistidine-tagged protein	*Insufficient wash* • Use larger volumes for washing step. *Binding and wash conditions are too mild* • Add small amounts of imidazole (1–5^{TED} / 1–10^{IDA} mM). Verify that the imidazole concentration is low enough to bind the polyhistidine-tagged protein.

Problem	Possible cause and suggestions
Unwanted proteins elute with polyhistidine-tagged protein *(continued)*	*Contaminating proteins and target protein are linked together via disulfide bonds* • Add up to 30 mM ß-mercaptoethanol to reduce disulfide bonds. *Contaminating proteins are proteolytic products of target protein* • Perform cell lysis at 4 °C. • Include protease inhibitors. *Expression is too low* *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.* • Increase expression level. • Increase amount of starting cell material. • Do not exceed recommended lysis volumes.

8.2 Ordering information

Product	REF	Pack of
Protino® Ni-TED Resin	745200.5 745200.30 745200.120 745200.600	5 g 30 g 120 g 600 g
Protino® Ni-TED 150 Packed Columns (contains 40 mg of resin each)	745100.10 745100.50	10 preps 50 preps
Protino® Ni-TED 1000 Packed Columns (contains 250 mg of resin each)	745110.10 745110.50	10 preps 50 preps
Protino® Ni-TED 2000 Packed Columns (contains 500 mg of resin each)	745120.5 745120.25	5 preps 25 preps
Protino® Ni-IDA Resin	745210.5 745210.30 745210.120 745210.600	5 g 30 g 120 g 600 g
Protino® Ni-IDA 150 Packed Columns (contains 40 mg of resin each)	745150.10 745150.50	10 preps 50 preps
Protino® Ni-IDA 1000 Packed Columns (contains 250 mg of resin each)	745160.10 745160.50	10 preps 50 preps
Protino® Ni-IDA 2000 Packed Columns (contains 500 mg of resin each)	745170.5 745170.25	5 preps 25 preps
Protino® Columns 14 mL (empty gravity flow columns for use with e.g. Protino® Ni-TED / IDA Resin)	745250.10	10 columns
Protino® Columns 35 mL (empty gravity flow columns for use with e.g. Protino® Ni-TED / IDA Resin)	745255.10	10 columns
NucleoBond® Rack Small (for Protino® Ni-TED/IDA 150 Packed Columns)	740562	1
NucleoBond® Rack Large (for Protino® Ni-TED/IDA 1000/2000 Packed Columns)	740563	1
Rack of MN Tube Strips (1 rack, 12 strips with 8 tubes each, and 12 Cap Strips)	740637	1 set

8.3 Product use restriction/warranty

Protino® Ni-TED / IDA 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.

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Last updated: 07/2010, Rev. 03

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