GENETIX BRAND

HANDBOOK

Nucleo-pore®

Ni-NTA Agarose

☐	Ni-NTA Agarose	NP - 40211	25 ml
☐	Ni-NTA Agarose	NP - 40231	100 ml
☐	Ni-NTA Agarose	NP - 40251	500 ml

genetix Biotech Asia Pvt. Ltd.

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Nucleo-pore®

Contents

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COMPONENTS

Ni-NTA Agarose 25 ml / 100 ml / 500 ml

STORAGE

The product is shipped at room temperature. Upon receipt Ni-NTA Agarose products should be stored at 2- 8°C and are stable up to 1 year. Do not freeze.

MATERIALS NOT PROVIDED WITH THE KIT

- For the purification under native conditions prepare the following buffers:
  NPI-10, NPI-20, NPI-250
- For the purification under denaturing conditions prepare the following buffers:
  NPI-10, DNPI-10, DNPI-20, DNPI-250
  Lysozyme
  Appropriate columns, centrifuge tubes, etc.
  Appropriate centrifuge
  Liquid chromatography system (MPLC, FPLC., AKTAdesign., etc.), peristaltic pump, or syringe . If necessary, appropriate adaptors for connecting the Ni-NTA Columns to the system of choice. 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., AKTAdesign.). Five adaptor sets are available for connecting the columns to other systems or for using them with a syringe.

PRINCIPLE AND PROCEDURE

Ni-NTA products enable efficient and quick purification of recombinant polyhistidine-tagged proteins by immobilized metal ion affinity chromatography (IMAC). Proteins can be purified under native or denaturing conditions from any expression system. Binding is based on the interaction between the polyhistidine tag of the recombinant protein and immobilized Ni2+ ions. 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 Ni2+ ions and therefore ready to use. 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 Ni2+ ion. The remaining two coordination sites are usually occupied by water molecules and can be exchanged with histidine residues of the recombinant protein. 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 Ni2+ ion enable tight but reversible selective protein interactions. Chelation of Ni2+ ions by NTA through four coordination positions minimizes metal leaching during purification and increases specificity for poly-histidine tagged proteins.

PROTOCOLS

1. PURIFICATION OF POLYHISTIDINE-TAGGED PROTEINS UNDER NATIVE CONDITIONS

1a. Buffer Preparation for purification under native conditions

NPI-10 / lysis & equilibration buffer (1 liter):

50 mM NaH_2PO_4	7.80 g NaH_2PO_4 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
10 mM imidazole	0.68 g imidazole	Mr = 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 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
20 mM imidazole	1.36 g imidazole	Mr = 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 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
250 mM imidazole	17.00 g imidazole	Mr = 68.08 g /mol

Adjust pH to 8.0 using NaOH

1b. 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 5µg / ml DNase I and stir on ice for 15 min.

Clear 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 0.45µm membrane (e.g., cellulose acetate). Store supernatant on ice.

See separate sections

for batch purification	-section 1c
for semi-batch purification	-section 1d
for gravity-flow purification	-section 1e
for FPLC. purification using self-packed columns	-section 1f
for FPLC. purification using Ni-NTA Columns	- section 1g

1c. Batch purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

Resuspend 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.

1d. Semi-batch purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

Resuspend 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 ^{0}C or - 20^{0}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.

1e. Gravity-flow purification of polyhistidine-tagged proteins under native conditions

1 Equilibration

Resuspend 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 clear *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: 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 ^{0}C or - 20 ^{0}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.

1f. FPLC. purification of polyhistidine-tagged proteins under native conditions (self-packed columns)

Prepare buffers according to section 1a. 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 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 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.

1g. FPLC. purification of polyhistidine-tagged proteins under native conditions using Ni-NTA Columns 1 ml / 5 ml

Ni-NTA Columns can be operated with liquid chromatography systems (such as AKTAdesign. ystems) via standard 10 - 32 fittings without additional connectors. Prepare buffers according to section 1a. Filter buffers through a 0.45 μm filter before use. Clear samples by centrifugation and / or pass them through a 0.45 μm filter 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

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 ^{0}C or - 20 ^{0}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.

2. 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 Ni-NTA Agarose or Ni-NTA Columns under denaturing conditions.

2a. Buffer Preparation for purification under denaturing conditions

NPI-10 (1 liter):

50 mM NaH_2PO_4	7.80 g NaH_2PO_4 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
10 mM imidazole	0.68 g imidazole	Mr = 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 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
10 mM imidazole	0.68 g imidazole	Mr = 68.08 g /mol
8 M urea	480 g urea	Mr = 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 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
20 mM imidazole	1.36 g imidazole	Mr = 68.08 g /mol
8 M urea	480 g urea	Mr = 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 . 2 H_2O	Mr = 156.01 g /mol
300 mM NaCl	17.54 g NaCl	Mr = 58.44 g /mol
250 mM imidazole	17.00 g imidazole	Mr = 68.08 g /mol
8 M urea	480 g urea	Mr = 60.06 g /mol

Adjust pH to 8.0 using NaOH

2b. 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 ^{0}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^{0}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 ^{0}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 (solubilized protein).

2c. Purification under denaturing conditions using 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.

Proceed to separate sections

for batch purification	-section 1c
for semi-batch purification	-section 1d
for gravity-flow purification	-section 1e
for FPLC. purification using self-packed columns or	-section 1f
for FPLC. purification using Ni-NTA Columns use:	-section 1g,

with the following modifications use —

Supernatant from solubilized protein (section 2b) 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).

3. CLEANING, RECHARGING, AND STORAGE

3a. Cleaning

After use GX- 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 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 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 ^{0}C.

3b. 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)) Ni-NTA Agarose can easily be stripped and recharged with nickel. Wash 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 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^{0}C.