INSTRUCTION MANUAL

SERVA Ni-NTA Magnetic Beads

Magnetic beads for Affinity Purification of His-Tag Fusion Proteins

(Cat. No. 42179)

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Contents

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1. SERVA Ni-NTA Magnetic Beads

1.1. General information

SERVA Ni-NTA Magnetic Beads are optimal for affinity purification of His-tagged fusion proteins. The product is supplied as a suspension of 5 % magnetic beads in 20 % ethanol

1.2. Storage conditions

Store at +2 °C to +8 °C (35 °F to 46 °F). Do not freeze.
If stored at the recommended temperature, the product will be suitable for use until: see label.

2. Affinity purification of proteins under native conditions

The presence of low imidazole concentration in the lysis and binding buffers (≤ 20 mM) will not affect the target fusion protein. In case the His-tagged protein does not bind tot he beads the imidazole concentration should be reducec to 5 – 10 mM.

2.1. Elimination of the preservative

Determine the quantity of agarose resin needed for the purification. The binding capacity will vary for each His-tagged protein. The yield of purified His-tagged protein depends on various parameters, e.g. amino acid composition, structure, molecular weight. The binding capacity of Ni-NTA agarose is > 75 mg/ml suspension (6xHis-GFP).

1 ml suspension corresponds to 50 µl Ni-NTA magnetic beads.

Protein expression level	Amount of His-tagged protein per 10 ml culture	Volume of SERVA Ni-NTA Magnetic Beads suspension per 10 ml culture
< 0.5 mg/L	< 5 µg	10 µl
1 mg/L	10 µg	20 µl
5 mg/L	50 µg	100 µl
10 mg/L	100 µg	200 µl
50 mg/L	500 µg	1 ml

Tab. 1: Required quantity of SERVA Ni-NTA Magnetic Beads depending on protein experssion level. Volumes can be linearly scaled up or down for smaller or larger culture volumes.

For elimination of the preservatives, gently shake the bottle of the magnetic beads to get a homogeneous suspension. Immediately pipette 200 µl of the suspension (corresponds to 10 µl magnetic beads) to an appropriate tube. Place the tube in a magnetic separator or use a magnet to remove the supernatant carefully from the beads. Discard the supernatant.

2.2. Equilibration of the resin

Binding Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0

- Add the binding buffer to the beads (500 µl buffer per 10 µl beads).
- Mix thoroughly to get a homogeneous suspension.
- Place the tube in a magnetic separator.
- Remove the supernatant carefully and discard it.

Binding buffer:
The choice of buffer depends on the particular property of the protein as well as of the type of chelate used. The buffer used most frequently is phosphate (50 mM). The pH of binding buffer generally leads to neutrality (pH 7.0 - 8.0). To avoid ionic interchange, add 0.15 – 0.5 M NaCl.

> **Important:** To increase the selectivity of binding the target protein, in some cases it is necessary to add to the binding buffer a small amount of imidazole (10 - 40 mM). To avoid affecting the O.D. 280 nm it is important to use a high purity imidazole, e.g. SERVA cat. no. 26081. It is also important to avoid the presence of agents like EDTA or citrate at all time.

2.3. Sample application

- Apply sample, e.g. clarified *E. coli* lysate or cell extract.
- Mix the suspension gently either for 30 min at room temperature or 60 min at 4 °C.
- Place the tube in a magnetic separator to sediment the beads.
- Remove the supernatant carefully and discard it.

> **Important:** The binding capacity can be affected by several factors, such as sample concentration, binding buffer or the flow rate during sample application.

2.4. Washing of the resin

Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0

- Add washing buffer to the beads (500 µl buffer per 10 µl beads).
- Mix thoroughly to get a homogeneous suspension.
- Place the tube in a magnetic separator.
- Remove the supernatant carefully and discard it.
- Repeat the washing step twice.

2.5. Elution of the protein

Addition of competitive ligand (generally imidazole), allows the elution of the retained protein.

Protein expression level	Amount of His-tagged protein per 10 ml culture	Volume of SERVA Ni-NTA Magnetic Beads suspension per 10 ml culture	Volume of elution buffer per 10 ml culture
< 0.5 mg/L	< 5 µg	10 µl	25 µl
1 mg/L	10 µg	20 µl	25 µl
5 mg/L	50 µg	100 µl	50 µl
10 mg/L	100 µg	200 µl	100 µl
50 mg/L	500 µg	1 ml	500 µl

Tab. 2: Required quantity of elution buffer depending on protein experssion level. Volumes can be linearly scaled up or down for smaller or larger culture volumes.

Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0

- Add elution buffer to the beads (100 µl buffer per 10 µl beads)
- Mix thoroughly for 10 min at room temperature
- Place the tube in a magnetic separator.
- Remove the supernatant carefully and discard it.
- Remove the supernatant carefully and store it in a new tube on ice
- Repeat the elution step twice or more.
- Collect each fraction in a separate tube.
- Determine the protein concentration of each fraction.

Most proteins are eluted with concentrations around 250 mM. Nonetheless, the eluates should be monitored (Bradford assay, SDS PAGE, O.D. 280) to determine the protein yield.

Note:
Generally, the subsequent elimination of imidazole is not necessary. But if required, it may be done by dialysis, precipitation with ammonium sulfate or ultrafiltration.

For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is needed for certain applications such as X-ray crystallography or RMN, where the protein structure is to be determined later. For these purposes, a His-tag is usually spliced to the protein at a protease cleavage site.

3. Affinity purification of proteins under denaturing conditions

The purification under denaturing conditions is similar to the procedure under native conditions except the buffers:

Binding buffer:
100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea, pH 6.3

Washing buffer:
100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea, pH 6.3

Elution buffer:
100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea, pH 4.5

Important: Due to urea dissociation, adjust the pH immediately before use.

4. Affinity purification of inclusion bodies under denaturing conditions

Recombinant proteins often form insoluble inclusion bodies. If so, these need to be rendered soluble by purification under denaturing conditions using for example urea or guanidine chloride.
Cells are disrupted under native conditions using enzymes together with sonication. After centrifugation, the fusion protein is extracted and solubilized using denaturing reagents.

4.1. Isolation of *inclusion bodies*

- Thaw frozen cell pellet on ice
- Resuspend 1g of pelleted, wet cells in 5 ml buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) on ice
- Pipette up and down, or use stirring until complete resuspension without visible cell aggregates
- Add lysozyme (SERVA cat. no. 28262) to a final concentration of 1 mg/ml
- Stir solution on ice for 30 min
- Sonicate the suspension on ice and check the appearance after sonication
- If the lysate is still viscous: Add 5 µg/ml DNase I and stir on ice for 15 min
- Centrifuge the lysate at 10,000 x g for 30 min at 4 °C to collect the inclusion bodies
- Discard the supernatant and keep pellet on ice

4.2. Solubilization of the *inclusion bodies*

- Resuspend the pellet in 10 ml 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0.
- Centrifuge at 10,000 x g 30 min at 4 °C.
- Discard the supernatant.
- Add 2.0 ml (per g wet cells) 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8 M urea, pH 8.0.

  Note: If 8 M urea is not sufficient for solubilzation, it is possible to use 6 M Guanidine-HCl (Glu-HCl) in the lysis buffer, alternatively. Prior to SDS PAGE Glu-HCl has either to be diluted or removed by TCA precipitation of the protein.
- 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 eliminate insoluble material
- Transfer the supernatant to a clean tube
- Centrifuge until the supernatant is clear and save it

The purification procedure is similar to the protocol for native conditions except that samples and buffers contain 8 M urea.

Binding buffer:
50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8 M urea, pH 8.0

Washing buffer:
50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 8 M urea, pH 8.0

Elution buffer:
50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 8 M urea, pH 8.0

4.3. Chemical compatibility

	Reagents	Comments
Denaturing agents	Urea	Solubilisation of proteins, ≤ 8 M
	Guanidine-HCl	Solubilisation of proteins, ≤ 6 M
Detergents	Non-ionic detergents, e.g. Triton® X-100, Tween® 20	Removal of interfering proteins ≤ 2% can be used
Additives	Imidazole	Competes with His-Tag Reduction of unspecific binding (20 mM) Elute His-tagged-Protein (100 mM) Avoids hydrophobic interactions between proteins
	Glycerol	≤ 50 % can be used
	EDTA	Decreases capacity, complexes cations Not recommended, ≤ 1mM may be tolerated
	Ethanol	Avoids hydrophobic interactions between proteins, may precipitate proteins, ≤ 20 % can be used
Reducing agents	Glutathione, reduced	High concentrations may reduce Ni^{2+} ≤ 30 mM may be tolerated
	2-Mercaptoethanol	Avoids formation of disulfide bonds High concentrations may reduce Ni^{2+} ≤ 20 mM may be tolerated
	Dithioerythritol (DTE) Dithiothreitol (DTT)	High concentrations may reduce Ni^{2+} ≤ 10 mM may be tolerated
	SDS	Avoids hydrophobic interactions, decreases capacity, not recommended, ≤ 0.3 % may be tolerated
Buffers	Sodium phosphate	Sodium phosphate buffer 50 mM pH 8.0 is recommended
	Tris, HEPES, MOPS	additional metal ions decrease capacity, ≤100 mM can be used
	Sodium chloride	Avoids unspecific binding

5. Regeneration of the Ni-NTA Magnetic Beads

In general, regeneration is always necessary when changing proteins. When continuing with the same protein, it is recommended to do regeneration when an appreciable diminution in the yield is observed. The frequency of these stages varies with the protein and the conditions used.

- Solubilize and desorb contaminants:
  Wash with 500 mM NaOH, 30 min

- Remove NaOH solution:
  Wash with 100 µl dist. Water (10-times bead volume)
- For direct use wash the beads with 100 µl 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0 (10-times bead volume)
- For storage wash with 30 % (v/v) ethanol (2-times bead volumes)

In some cases, the above described procedure may not be enough, e.g. when the color of the beads changes due to loss/reduction of nickel ions.
Regeneration consists of the complete elimination of the metal.

- Wash the resin with 100 µl dist. water (10-times bead volume)
- Removal of the metal ions:
  Wash with 100 µl 100 mM EDTA, pH 8.0 (10-times bead volume)
- Removal of excess EDTA:
  Wash resin with 100 µl dist. water (10-times bead volume)
- Load beads with metal ions: Once the excess EDTA has been eliminated, add 2x 100 µl 100 mM salt ($NiCl_2$ oder $NiSO_4$) to the beads.
- Elimination of excess of metal:
  Wash with 100 µl dist. Water (10-times bead volume)
- Add 100 µl 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0 (10-times bead volume)

6. Troubleshooting

6.1. Sample application

Observation	Causes	Recommendation
High viscosity of the sample	DNA in the sample	Nuclease or sonication treatment
	Steric hindrance of the substrate	Dilution of the sample
Highly diluted or concentrated sample	Highly diluted sample	Sample concentration prior to column application
	Highly concentrated sample	Dilution of the sample

6.2. Adsorption

Observation	Causes	Recommendation
Target protein not bound to the column	His-tag is not present or has been degraded	Use of protease inhibitors Purification performed at + 4 °C
	His-tag is not exposed (inaccessible)	Purification under denaturing conditions Add tag on other site-terminus, or both positions
	Inadequate binding conditions	Check buffer and pH; reduce imidazole concentration Check whether buffer components interact with the beads or not
Target protein binds only partially to the column	Column capacity is exceeded	Apply less protein
	Loss of chelating metal	Avoid use of reducing and chelating agents
	His-tag is not very well exposed	Reduce flow rate Batch format purification

Observation	Causes	Recommendation
Target protein binds only partially to the column	Poor protein expression	Optimization of the expression
	Formation of inclusion bodies	Modification of the bacterial growth Purification under denaturing conditions
	Low binding capacity	Use cation with higher binding capacity

6.3. Elution

Observation	Causes	Recommendation
High amount of co-eluted proteins	Insufficient washing stage	Increase volume of washing buffer Add imidazole (max. 40 mM)
	Inadequate adsorption conditions	Check pH Add NaCl to avoid unspecific interactions Addition of non-ionic detergents, ethylene glycol or glycerol Increase imidazole concentration in the binding buffer

Observation	Causes	Recommendation
Target protein elutes poorly	Too smooth elution conditions	Increase imidazole concentration Reduce pH Elution at higher temperature, if possible
	Too strong interaction between protein and chelating metal	Elution with EDTA Elution at pH 4.0 and with imidazole Using another agarose resin Increase imidazole concentration to 1 M Elution under denaturing conditions
	Precipitation of fusion protein	Add detergents Incubate the column with elution buffer for 8 - 10 h and elute with elution buffer
Elution profile is not reproducible in different cycles of purification	Modification of the sample, e.g. His-tag degradation because of protease activity	Prepare fresh samples Add protease inhibitors Purification at +2 °C to +8 °C
	Precipitations of proteins and/or lipids	Use new beads
	Variation of pH and/or ionic forces	Prepare new buffers

6.4. Changes in the beads

Observation	Causes	Recommendation
Loss of color	Chelating agents in the sample	Purification of the sample and regeneration or use of new beads
Change of color	Reducing agents in the sample	Purification of the sample and regeneration or use of new beads

7. Ordering information

Product	Cat. no.	Size
SERVA Ni-NTA Magnetic Beads	42179.01	2 ml
	42179.02	10 ml
SERVA Ni-NTA Agarose Resin	42139.01	25 ml
	42139.02	100 ml
SERVA IDA Metal-free HD Agarose Resin	42140.01	25 ml
	42140.02	100 ml
SERVA IDA Metal-free LD Agarose Resin	42144.01	25 ml
	42144.02	100 ml
SERVA Ni-IDA HD Agarose Resin	42141.01	25 ml
	42141.02	100 ml
SERVA Ni-IDA LD Agarose Resin	42145.01	25 ml
	42145.02	100 ml
SERVA Zn-IDA HD Agarose Resin	42142.01	25 ml
	42142.02	100 ml
SERVA Zn-IDA LD Agarose Resin	42146.01	25 ml
	42146.02	100 ml
SERVA Co-IDA HD Agarose Resin	42143.01	25 ml
	42143.02	100 ml
SERVA Cu-IDA LD Agarose Resin	42147.01	25 ml
	42147.02	100 ml