INSTRUCTION MANUAL

SERVA Ni-NTA Agarose Resin

Agarose Resin for Affinity Purification of His-Tag Fusion Proteins

(Cat. No. 42139)

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1. SERVA Ni-NTA Agarose Resin

1.1. General information

SERVA Ni-NTA Agarose Resin is optimal for affinity purification of His-tagged fusion proteins expressed in baculovirus, yeast, pro- and eukaryotic cells by batch or column purification. The agarose resin is suitable for both, affinity purification under native and denaturing conditions.

1.2. Storage conditions

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

2. Batch purification of proteins under native conditions

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 50 mg/ml resin (6xHis-GFPuv, approx. 32 kDa).

1 ml agarose resin corresponds to 2 ml of 50 % (v/v) Ni-NTA Agarose suspension.

For elimination of the preservatives, there are two possibilities:

A. Gently shake the bottle of agarose resin to get a homogeneous suspension. Immediately pipette the suspension to an appropriate tube. Sediment the resin by centrifugation at 500 x g for 5 min. Remove the supernatant carefully and discard it.

B. Gently shake the bottle of agarose resin to get a homogeneous suspension. Invert the bottle of resin several times and then filter the resin (Fig.1). Put the resin in a container.

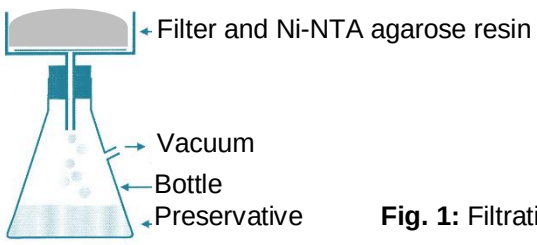

Fig. 1: Filtration of the Agarose suspension

2.2. Equilibration of the resin

Binding Buffer: 50 mM NaH_2PO_4, 300 mMNaCl, 10 mM imidazole, pH 8.0

- Add 10 bed volumes of binding buffer
- Mix thoroughly to get a homogeneous suspension
- Centrifugation at 500 x g for 5 min
- Remove the supernatant carefully and discard it

Binding buffer:
The choice of buffer depends on the particular properties 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:** In some cases to increase the selectivity of the binding of target protein 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 times.

2.3. Sample application

- Apply sample, e.g. *E. coli* lysate or cell extract
- Mix the suspension gently for 30 – 60 min* at room temperature
- Centrifuge the suspension at 500 x g for 5 min to sediment the resin
- Remove the supernatant carefully and discard it

*If necessary increase the contact time to enhance binding.

> **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 10 bed volumes of washing buffer to the resin
- Mix thoroughly to get a homogeneous suspension
- Centrifugation at 500 x g for 5 min
- Remove the supernatant carefully and discard it
- Repeat the washing step twice

The washing can be checked by O.D. 280 nm measurement until the washing buffer reaches the baseline level. Then, the elution of the protein can be performed.

2.5. Elution of the protein

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

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

- Add 1 bed volume of elution buffer to the resin
- Mix thoroughly for 10 min at room temperature
- Centrifugation at 500 x g for 5 min
- Remove the supernatant carefully and store it in a new tube on ice
- Repeat the elution step twice or more, pool the fractions containing the purified His-tagged protein

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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. Gravity purification of proteins under native conditions

Besides the batch purification, protein can also be purified by gravity flow columns. Depending on the total volume necessary the following empty columns can be used:

Cat. No.	Product	Resin volume	Total volume
42173	Mini Columns	0.1- 0.25 ml	1.5 ml
42174	Midi Columns	0.5 – 2 ml	12 ml
42175	Maxi Columns	2 – 6 ml	35 ml

3.1. Column packing and elimination of 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 50 mg/ml resin (6xHis-GFPuv, approx. 32 kDa).

1 ml agarose resin corresponds to 2 ml of 50 % (v/v) Ni-NTA Agarose suspension.

- Gently shake the bottle of resin to get a homogeneous suspension
- Immediately, pipette sufficient suspension in the empty column
- Close lower cap of the column
- Remove the lower cap to allow elimination of the preservative by gravity flow

3.2. Column equilibration

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

- Close the lower cap
- Add 5 bed volumes of binding buffer into the upper part of the column and make sure no air has been trapped
- Close the upper cap and mix manually by inverting the column
- Open the upper and the lower cap to allow elimination of the equilibration buffer
- Discard the flow through, repeat the equilibration step twice

3.3. Sample application

- Close the lower cap
- Add the sample on top of the column
- Close the upper cap
- Keep the sample and resin in contact for 30 – 60 min* at room temperature by inverting the column before removing the upper and lower cap
- Discard the flow through

*If necessary increase the contact time to enhance binding.

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

3.4. Washing of the resin

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

- Close the column outlet with the cap
- Add the washing buffer (10 bed volumes) on top to eliminate all the proteins that have not been retained in the matrix
- Close the column inlet with a cap, mix manually by inverting the column
- Remove the upper and lower cap
- Discard the flow through.
- Repeat the washing step twice

The washing can be checked by O.D. 280 nm measurement until the washing buffer reaches the baseline level. Then, the elution of the protein can be performed.

3.5. Elution of the protein

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

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

- Close the column outlet with the cap
- Add 1 bed volume of elution buffer to the column
- Close the column inlet with the cap
- Mix thoroughly for 10 min at room temperature
- Remove the upper and lower cap
- Collect the eluate in a new tube and store on ice
- Repeat the elution step twice and pool the collected fractions

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.

4. Spin column purification of proteins under native conditions

The affinity purification an also be performed with spin columns. The following empty columns can be used:

Cat. No.	Product	Resin volume	Total volume
42176	Mini Spin Columns	50 – 100 µl	800 µl

4.1. Column packing and elimination of 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 50 mg/ml resin (6xHis-GFPuv, approx. 32 kDa).

100 µl of 50 % (v/v) Ni-NTA Agarose suspension correspond to 50 µl agarose resin.

- Gently shake the bottle of resin to get a homogeneous suspension
- First remove the upper inlet cap
- Add 100 µl of suspension into the empty column
- Remove the lower cap and put the spin column in a collecting tube
- Centrifugation: 30 s at 500 x g
- Discard the flow through

4.2. Column equilibration

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

- Open the upper cap
- Equilibrate the spin column with 500 µl binding buffer
- Close the upper cap and mix manually
- Remove the lower cap and put the spin column in a collecting tube
- Centrifugation: 30 s at 500 x g
- Discard the flow through

4.3. Sample application

- Add the sample onto top of the column
- Close the upper cap
- Keep the sample and resin in contact for 30 – 60 min* at room temperature by inverting the column
- Remove the lower cap and put the spin column in a collecting tube

- Centrifugation: 30 s at 500 x g
- Discard the flow through

*If necessary increase the contact time to enhance binding.

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

4.4. Washing of the resin

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

- Close the column outlet with the cap
- Add the 500 µl washing buffer on the top to eliminate all the proteins that have not been retained in the matrix
- Close the column inlet with a cap
- Mix manually by inverting the column
- Remove the lower cap and put the spin column in a collecting tube
- Centrifugation: 30 s at 500 x g
- Repeat the washing step twice

The washing can be checked by O.D. 280 nm measurement until the washing buffer reaches the baseline level. Then, the elution of the protein can be performed.

4.5. Elution of the protein

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

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

- Close the column outlet with the cap
- Add 500 µl elution buffer to the column
- Close the column inlet with the cap
- Mix thoroughly for 10 min at room temperature
- Remove the upper and lower cap and put the spin column in a collecting tube
- Centrifuge: 30 s at 500 x g, collect the flow through and store on ice
- Repeat the elution step twice and pool the collected fractions

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.

5. Affinity purification of proteins 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.

5.1. 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

5.2. Solubilisation of the *inclusion bodies*

- Resuspend the pellet in 10 ml 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole.
- 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

- 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 further purification procedure is similar to the protocol for native conditions except that samples and buffers loaded on the column 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

5.3. 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

6. Regeneration of the agarose resin

In general, column 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 dist. Water (10 bed volumes)
- For direct use wash the resin with 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0 (10 bed volumes)
- For storage wash with 30 % (v/v) ethanol (2 bed volumes)

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

- Wash the resin with dist. water (10 bed volumes)
- Removal of the metal ions: Wash with 100 mM EDTA, pH 8.0 (10 bed volumes)
- Removal of excess EDTA: Wash resin with dist. water (10 bed volumes)
- Load resin with metal ions: Once the excess EDTA has been eliminated, add 2 column volumes of 100 mM salt (chloride or sulfate) of these cations (Zn^{2+},Ni^{2+},Cu^{2+},Co^{2+}) to the resin.
- Elimination of excess of metal: Wash with dist. Water (10 bed volumes)
- Add 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0 (10 bed volumes)

7. Troubleshooting

7.1. 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 Batch format purification
Highly diluted or concentrated sample	Highly diluted sample	Sample concentration prior to column application Batch format purification
	Highly concentrated sample	Dilution of the sample

7.2. 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 matrix or not
Target protein binds only partially to the column	Column capacity is exceeded	Apply less protein Regeneration of the column
	Loss of chelating metal	Regeneration of the column 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
	Formation of channels within the column	Re-pack column
	Low binding capacity	Use cation with higher binding capacity

7.3. Elution

Observation	Causes	Recommendation
High amount of co-eluted proteins	Insufficient washing stage	Increase volume of washing buffer Add imidazole (5 - 10 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
	Column too large	Reduce resin quantity

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 Reduce flow rate 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 Batch format of binding and elution
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 - +8 °C
	Precipitations of proteins and/or lipids	Regeneration of the resin
	Variation of pH and/or ionic forces	Prepare new buffers
	Loss of binding capacity	Regeneration of the resin

7.4. Changes in the resin

Observation	Causes	Recommendation
Loss of color	Chelating agents in the sample	Purification of the sample and regeneration of the resin
Change of color	Reducing agents in the sample	Purification of the sample and regeneration of the resin

8. Ordering information

Columns					
Product	**Frit Pore Size**	**Resin Volume**	**Capacity**	**Cat. No.**	**Size**
Mini Columns	20 µm	100 - 250 µl	1.5 ml	42173.01 42173.02	25 columns 100 columns
Midi Columns	20 µm	0.5 – 2 ml	12 ml	42174.01	50 columns
Maxi Columns	20 µm	2 – 6 ml	35 ml	42175.01	50 columns
Mini Spin Columns	35 µm	50 - 100 µl	0.8 ml	42176.01	25 columns