INSTRUCTION MANUAL

SERVA IDA HL Agarose

Agarose for Affinity Purification of His-Tag Fusion Proteins

(Cat. No. 42144, 42145, 42146, 42147)

SERVA Electrophoresis GmbH - Carl-Benz-Str. 7 - 69115 Heidelberg
Phone +49-6221-138400, Fax +49-6221-1384010
e-mail: info@serva.de -http://www.serva.de

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Ver 08/13

1. SERVA IDA LD Agarose

1.1. General information

SERVA IDA LD (low density) Agarose with high selectivity is for optimal affinity purification of His-tagged fusion proteins by batch or column purification.

1.2. Storage conditions

Store at +2 °C bis +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. Affinity purification of soluble proteins

Please note that these resins are adapted to work mainly in native conditions.

2.1. Elimination of the preservatives

Wash the agarose beads with 5 - 10 column volumes of distilled water to remove the preservative (20 % v/v ethanol).

Note: In case of metal-free agarose (Cat. No. 42144), the corresponding metal must be previously added before use as described below.

- **Metal Adsorption:** Prepare acidic or neutral solution of 100 mM salt (chloride or sulfate) of the required cations (Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}) by adding 5 column volumes of this salt solution to the column.
- **Elimination of non-retained metal ion:** Wash the beads with 5-10 column volumes of distilled water to eliminate the non-retained cations.

2.2. Equilibration of the resin

Equilibrate the column with 5-10 column volumes of binding buffer.

Binding buffer:
The choice of buffer depends on the particular properties of the protein as well as of the type of chelate used. The buffers used most frequently are acetate (50 mM) or phosphate (10 - 150 mM). The pH of binding buffer generally leads to neutrality (pH 7.0 - 8.0), but can vary over the range 5.5 – 8.5. 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 imidazolee (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

Once the resin is equilibrated, the sample containing the fusion protein for purification is applied. In some cases a slight increase of contact time may facilitate binding.

2.4. Washing of the resin

It will be washed with the binding buffer until O.D. 280 nm reaches the baseline level.

2.5. Elution of the fusion protein

The elution of the protein can be done in different ways:

2.5.1. Addition of competitive ligand

Addition of competitive ligand (generally imidazole), allows the elution of the retained protein. In general, 500 mM imidazole are enough to elute the protein. It is also possible to use concentration gradients of this reagent (0 – 500 mM). Most proteins are eluted with concentrations around 250 mM. Other reagents that can be used as competitive ligands are histidine (His) and ammonium chloride.

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

2.5.2. Reduction of the pH

Reduction of pH (with or without gradient), also allows the elution of the desired protein (pH 3.0 – 4.0).

2.5.3. Addition of other chelating reagents

A more drastic method uses reagents like EDTA or EGTA (50 mM), which causes the elution of both the fusion protein and chelating metal.

Note: For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is necessary 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 forming inclusion bodies

The recombinant proteins often form insoluble inclusion bodies. Purification under denaturing conditions, using for example urea or guanidine chloride at relevant stages may increase protein solubility. Please find below data of the chemical compatibility of the agarose beads.

	Reagents	
Chemical stability	10 mM HCl 100 mM NaOH 20 % (v/v) Ethanol 100 mM Sodium acetate, pH 4.0	2 % (w/v) SDS 30 % (v/v) 2-Propanol 1 M NaOH 70 % (v/v) Acetic acid
Denaturing agents	8 M Urea	6 M Guanidine-HCl
Detergents	2 % (w/v) Triton® X-100 2 % (w/v) Tween® 20	1 % (w/v) CHAPS
Additives	2 M Imidazole 20 % (v/v) Ethanol + 50 % (w/v) Glycerol 100 mM Na_2SO_4 1.5 M NaCl	1 mM EDTA 1 mM EDTA + 10 mM $MgCl_2$ 60 mM Citrate 60 mM Citrate + 80 mM $MgCl_2$
Reducing agents	10 mM Glutathion, reduced 20 mM 2-Mercaptoethanol	5 mM Dithioerythritol (DTE) 5 mM Dithiothreitol (DTT)
Buffers	50 mM Na_2HPO_4, pH 7.5 100 mM Tris-HCl, pH 7.5 100 mM MOPS, pH7.5	100 mM Tris-Acetate, pH 7.5 100 mM HEPES, pH 7.5

4. Column packing

- Shake the bottle manually to obtain a homogenous suspension of agarose beads/preservative.
- Place a funnel in the head of the column and slowly run the suspension down the walls of the column.

  Note: It is advisable to make the addition slowly to avoid the formation of bubbles. The beads as well as all buffers used may be degassed before added to the column.
- Decant the resin and discard most of the leftover liquid, leaving 1cm above the column head to prevent drying out. This is done by passingit through the column, or pipetting it from the top of the column.
- Repeat previous steps until the desired column height is obtained.
- Insert the adapter gently in the column head until it begins to displace the liquid. Make sure no air is trapped under the net.

- Add distilled water to the purification steam until a constant height (corresponding to the height of the column) is achieved. If the desired height is not achieved, repeat the previous steps.
- When a constant height has been obtained, maintain the flow with the addition of 5 volumes of distilled water to completely eliminate the preservative.
- Equilibrate the column with 5 to 10 column volumes of binding buffer.

Recommended Working Conditions:

Flow rate: 0.5 – 1.0 ml/min

max. Pressure: 0.18 bar (2.6 psi)

5. Regeneration procedure

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

- Elimination of the metal from the resin: It is necessary to wash the resin with 5 column volumes of the following buffer:
  20 mM Sodium phosphate
  500 mM NaCl
  50 mM EDTA
  pH 7.0
- Elimination of the excess EDTA: In order to eliminate the residual EDTA before reloading the resin with the corresponding metal, the column should be washed with 5 column volumes of distilled water.
- Load the column with the corresponding metal: Once the excess EDTA has been eliminated, add 5 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 the excess of metal: Wash with 5 column volumes of distilled water.
- Preparation of the column: Add 5 column volumes of the binding buffer. If the resin is not going to be used for a while it is recommended to replace the last step by the addition of the preservative (20 % (v/v) ethanol).

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 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

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 in 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 expresseion	Optimization of th 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

6.3. Elution

Observation	Causes	Recommendation
High amount of co-eluted proteins	Insufficient washing stage	Increase volume of washing buffer Add imidazole (5-10 mM)
	Inadequate adsorptionsconditions	Check pH Add NaCl to avoid unspecific interactions Addition of non-ionic detergents, ethylenglycol or glycerol Increase imidazole concentration in the binding buffer
	Column too large	Reduce resin quantity
	Low selectivity of the column	Imidazole concentration gradient

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

6.4. 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

7. 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