AFFINITY HIS-TAG PURIFICATION

PROCEDURE FOR USE
CHELATING RAPID RUN™ Agarose Bulk Resins

DESCRIPTION

Resins are products that allow batch or column purifications.
This product is supplied as a suspension in 20% ethanol.

INSTRUCTIONS

The resins are adapted to work mainly in native conditions like denaturing.

The following summarized procedure is adapted for the purification of His-tagged proteins under native conditions. The strength of binding of the protein to the resin will depend on: the accessibility of the his-tag, the pH and the buffer composition.

1. Elimination of the Preservative

Wash the beads with 5-10 column volumes of distilled water to eliminate the preservative.

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. The buffers used most frequently are acetate (50 mM) or phosphate (10-150 mM). The pH of binding buffers 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 of NaCl.

Note: In some cases to increase the selectivity of the binding of target protein it is necessary to add to the binding buffer a small concentration of imidazole (10-40 mM). It is important to use high purity imidazole to avoid affecting the O.D. 280 nm. It is important to avoid the presence of agents like EDTA or citrate at all times.

3. Application of the Sample

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

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

4. Washing of the Resin

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

5. Elution of the Pure protein

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

A. Addition of a competitive ligand (generally imidazole), which allows the elution of the retained protein. In general, 0.5 M of imidazole is enough to elute the protein. It is also possible to use concentration gradients of this reagent (0 - 0.5 M). Most proteins are eluted in concentrations around 250 mM. Other reagents that can be used as competitive ligands are histidine and ammonium chloride.

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

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

C. A more drastic method uses reagents like EDTA or EGTA (0.05 M), which causes the elution of both the protein and chelating metal.

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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.

6. Storage

Keep at +2-+8ºC. Do not freeze.

The recombinant proteins often form insoluble inclusion bodies. If so these need to be rendered soluble by a purification under denaturing conditions, using for example urea or guanidine chloride at relevant stages (see table).

STUDIES	REAGENTS	
CHEMICAL STABILITY	HCl 0.01 M NaOH 0.1 M Ethanol 20% Sodium acetate 0,1M pH 4.0	SDS 2% 2-propanol 30% NaOH 1 M HAc 70%
DENATURING AGENTS	Urea 8 M	Guanidine-HCl 6 M
DETERGENTS	Triton X-100 2% Tween 20 2%	Chaps 1%
ADDITIVES	Imidazole 2.0 M Ethanol 20% + glycerol 50% Na_2SO_4 100 Mm NaCl 1.5 M	EDTA 1 mM EDTA 1 mM + $MgCl_2$ 10 mM Citrate 60 mM Citrate 60 mM + $MgCl_2$ 80 mM
REDUCING AGENTS(*)	Reduced glutathione 10 mM ß-mercaptoethanol 20 mM	DTE 5 mM DTT 5 mM
BUFFERS	Na_2HPO_4 50 mM, pH 7.5 Tris-HCl 100 mM, pH 7.5 MOPS 100 mM, pH 7.5	Tris-acetate 100 mM. pH 7.5 HEPES 100 mM, pH 7.5

(*) Note: Under extended treatments with reducing agents, or in processes where high concentrations of these reagents are used, reduction of the metal ion may result – this will affect the binding capacity of the resin, so these agents should be avoided. The reagents described in the table are compatible with Nickel Activated Agarose Beads (Nickel is most commonly used) under the conditions and concentrations indicated in the table. The stability of the Nickel resin has been tested in each of the reagents separately. This resin is manufactured with imminodiacetic acid and in case of buffers that contains reducing agents we recommend to use NTA resins.

There is a very small loss of Nickel with this resin and it only happens in very drastic work circumstances. Given these conditions a slight brown discoloration may appear but does not usually affect performance. The discoloration is due to small particles of Nickel breaking away from the main body of resin and coming into contact with the reducing agents causing the Nickel to reduce and became brown in color. If there is a risk of producing this effect, it can be avoided by pre-treatment of the resin before it has ever been used. This treatment eliminates the cations that are weakly attached to the resin before beginning the process, so the reducing agent doesn't affect the lost Nickel ions.

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Pre-treatment:

1. - Wash the resin with five column volumes of distilled water.
2. - Wash the resin with five column volumes of binding Buffer (without reducing agents in the buffer).
3. - Wash with five column volumes of elution buffer (without reducing agents in the buffer).
4. - Equilibrate with 10 column volumes of binding buffer (without reducing agents).

Once this treatment has been carried out, the resin is ready for the simple purification in such circumstances as described above.

COLUMN PACKAGING

1. Manually shake the bottle to obtain a homogenous suspension of Chelate Rapid Run Agarose Beads/preservative. Place a funnel in the head of 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 product may also be degassed before added to the column.

   Decant the product and discard most of the leftover liquid, leaving 1 cm above the column head to prevent drying out. This is done either by passing it through the column, or pipetting it from the top of the column.

2. Repeat previous steps until the desired column height is obtained.

3. Insert the adapter gently in the column head until it begins to displace the liquid.

   Note: Make sure no air is trapped under the net.

4. Add distilled water to the purification stream until a constant height (corresponding to the height of the column) is achieved.

   Note: If the desired height is not achieved, repeat steps 1 through 4.

5. When a constant height has been obtained, maintain the flow with the addition of 5 volumes of distilled water to completely eliminate the preservative.

6. Equilibrate the column with 5 to 10 column volumes of binding buffer.

   Note: It is advisable to previously de-gas all the solutions before adding to the column to avoid the formation of bubbles.

FLOW RATE & PRESSURE

MAXIMUM FLOW RATE 100Kpa, 15 cm bed height *	> 600 cm/h
MAX. PRESSURE at 15 cm bed height *	300 kPa

* Data corresponding to the non-activated Rapid Run beads.

CHELATING RAPID RUN RESIN REGENERATION PROCEDURE

During the life of the resin, it may lose binding points because some protein is retained. Hence a loss of the binding capacity may be observed in successive cycles. To return to the starting state, regeneration may be necessary. Regeneration consists of the complete elimination of the metal and therefore of the retained protein.

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

A. Elimination of the metal from the resin: It is necessary to wash the resin with 5 column volumes of a solution 20mM sodium phosphate containing 0.5 M NaCl, 50 mM of EDTA at pH 7.0.

B. 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.

Note: In some special cases it is advisable to make more drastic intermediate stages of regeneration to eliminate denatured proteins or lipids.

Drastic Regeneration Stage: denatured proteins and lipids elimination.

Use when denatured proteins or lipids may have been retained after stage B.

i. Elimination of ionic interactions: Wash in batch for approximately 20 minutes in a solution with 1.5 M NaCl. Later wash with 10 column volumes of distilled water to eliminate ions.

ii. Elimination of precipitated proteins (may be responsible for column pressure changes). Wash in batch at least 2 hours with a solution 1.0 M NaOH. Eliminate the NaOH with 10 column volumes of distilled water.

iii. Elimination of strong hydrophobic interactions: resuspend the resin in batch with a solution of isopropanol 30% for approximately 20 minutes. Then wash with 10 column volumes of distilled water to eliminate the isopropanol.

iv. Wash in batch for 2 hours with a solution 0.5% of non-ionic detergent, acetic acid 0.1 M. Rinse the detergent with ethanol 70% (approximately 10 column volumes). Finally wash with 10 column volumes of distilled water to rinse out the ethanol.

C. Load the column with the corresponding metal: once the excess EDTA has been eliminated, add 5 volumes of the 0.1 M metal solution (normally chlorides or sulphates are used).

D. Elimination of the excess of metal: wash with 5 column volumes of distilled water.

E. Preparation of the column: add 5 column volumes of the binding buffer.

Note: 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.
Also it is recommended to include a regeneration step when beginning to purify a new protein.

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TROUBLESHOOTING GUIDE
Problems and Solutions

Possible causes of problems that could appear during the purification protocol of biomolecules are listed below. The causes described are theoretical and it is always advisable to contact our team with your specific problem.

The table delineates the potential problems at each step in the protocol that might explain poor performance.

1. SAMPLE APPLICATION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
HIGH VISCOSITY SAMPLE	Presence of DNA in the sample.	- Increase sonication time until viscosity is reduced.
	Steric hindrance of the substrate.	- Dilute the sample before its application in the column. In this case, sometimes, it is preferable to carry out the purification in batch format instead of the column format. - Consult “tailor made resins” for high viscosity samples.
HIGHLY DILUTED OR CONCENTRATED SAMPLE	Highly diluted sample.	- It is preferable to concentrate the sample before its purification in the column. - Another solution is to carry out an adsorption step in batch format and pack the column with the resultant resin of the adsorption step.
	Highly concentrated sample.	- It is preferable to make a previous dilution of the sample before its purification in the column.

2. ADSORPTION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
TARGET PROTEIN NOT BOUND TO THE COLUMN	His-tag is not present or has been degraded.	- Check it. If it has been degraded, make the purification at lower temperatures (4ºC) reducing the degradation. Try to reduce the purification step times. Add protease inhibitors. (See chemical compatibility table).
	It is not exposed (inaccessible).	- Purify in denaturing conditions or add the tag in other site (N-terminus, C-terminus, or in both positions).
	Inadequate binding conditions.	- Check the buffer and binding pH. - If the binding has been done in presence of imidazole, reduce its concentration or eliminate it in this step. - Verify if some of the reagents used in the adsorption step interferes with the binding reaction. e.g.: A Zinc resin can lose its metal due to the presence of chelating agents in the sample and therefore, the protein will

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		not bind. Since the presence/ absence of Zinc cannot be visualized by a change of colour, it would difficult to determine this phenomenon. So, in case of doubt, it is advisable to regenerate the column and observe if the target protein is bound to the regenerated resin.
TARGET PROTEIN BINDS ONLY PARTIALLY TO THE COLUMN	Column capacity is exceeded.	- Apply less fused protein to the column.
	The resin has been previously used during several purification cycles without regeneration. This causes a diminution of the binding capacity. This diminution varies in each case and increases with the number of purification cycles of the resin.	- Apply a regeneration step in the column when a decrease of the binding capacities is observed.
	Loss of chelating metal in the resin.	- Apply a regeneration step in the column. Avoid use of reducing and chelating agents.
	Histidine tail is not very exposed.	- Try to use slower flow rates or make the adsorption in batch to allow a better contact between resin and fused protein. Note: a greater exhibition would be obtained working in denaturing conditions.
	Poor protein expression.	- Optimize bacterial expression conditions.
	The fused protein forms inclusion bodies.	- Modify bacterial growth conditions. - Work in denaturing conditions.
	Channels have formed in the column so the sample runs mainly through these undesirable channels.	- Re-pack column.
	The resin shows low binding capacity.	- Try a HIGH DENSITY TEST KIT or with a less selective cation.

3. ELUTION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
HIGH AMOUNT OF CO-ELUTED PROTEINS (CONTAMINANTS)	Insufficient washing stage.	- Increase volume of washing buffer. - Add a low concentration of imidazole (5-10 mM) in the buffer during washing and equilibrating steps.
	Inadequate adsorption conditions.	- Check pH. - Add or increase saline concentration in the binding buffer to avoid non-specific ionic interactions. - Low concentrations of non-ionic detergents can also be added. - Add small quantities of ethyleneglycol or glycerol in the binding

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		buffer to avoid non-specific hydrophobic interactions. - Increase imidazole concentration in the binding buffer. Note: In general, higher imidazole concentrations than 20 mM are not recommended because it can compete with the binding of the target protein. This concentration can be modified with the type of protein to be purified.
HIGH AMOUNT OF CO-ELUTED PROTEINS (CONTAMINANTS)	Column too large.	- Reduce the resin quantity so the fused protein and contaminants will compete for less binding sites, increasing the binding selectivity of the tagged protein.
	The resin used in the purification shows low selectivity to bind the fused protein. In some cases Nickel resin is not as selective as ones loaded with other metals. It may also bind proteins with histidine, cysteine and tryptophan residues.	- Try a LOW DENSITY TEST KIT or a more selective cation (e.g.: Cobalt). - Employ an imidazole concentration gradient to separate the target protein from the rest of retained proteins. Also "Single Step Elution" procedures can be used.
TARGET PROTEIN ELUTES POORLY	Too smooth elution conditions.	- Increase imidazole concentration or reduce pH in the elution step. - Try, if possible, an elution at a higher temperature.
	Sometimes protein binding with chelating metals is too strong. Note: Also the position of the histidine tail can influence the strength of the binding of the target protein.	- Make a elution with a chelating agent such as EDTA. - Make an elution reducing pH (pH 4.0) in the presence of imidazole. - Purify with other chelating resins as the requirements with each cation are different and the binding strength is different with each one. Also, in many cases, using a Low Density resin gives better desorptions of the fused protein. - Increase imidazole concentration up to 1M in the elution buffer. - Reduce the flow in the elution step or make this step in batch format to increase contact time. - Elute in denaturing conditions.
	Fused protein can be precipitated.	- Add solubilizing agents (see compatibilities). - Incubate the column with the elution buffer for 8-10 h and elute with the elution buffer. - Run binding and elution steps in batch format to avoid local concentration of protein and therefore its potential precipitation.
ELUTION PROFILE IS NOT REPRODUCIBLE IN DIFFERENT CYCLES OF PURIFICATION	Sample's nature could have been modified. The histidine tail could have been lost due to protease action.	- It is necessary to prepare a fresh sample. Run the protocol at 2-8ºC. Add protease inhibitors (see chemical compatibilities table).
	Proteins or lipids could have precipitated.	- Regenerate the resin.

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	PH or ionic forces could have been modified.	- Prepare new buffers.
	The sample to apply could be different than the first one.	- Keep all the parameters and same conditions.
	Loss of binding capacity is observed.	- It is recommended to regenerate the column.

4. CHANGES IN THE RESIN

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
LOSS OF COLOUR OF THE RESIN	Presence in the sample of chelating agents that could have caused the diminution of the content of the metal.	- Eliminate the chelating agents in the sample (e.g. by gel filtration) and after regenerate the column. Note: This is easy to see in coloured resins (Cobalt, Nickel or Copper).In other cases such as Zinc the loss of the cation is not so evident by colour changes and could be the cause the non-binding of the protein.
CHANGE OF COLOUR (BROWN) OF THE RESIN	Presence in the sample of reducing agents.	- Eliminate these reducing agents and regenerate the resin.

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