BIOTOOLOMICS

IMAC SepFast Large Beads

Data and Instructions

1. Introduction

Immobilised metal affinity chromatography (IMAC) using Nitrilotriacetic acid (NTA) has been widely employed as a powerful separation approach in the purification of a broad range of proteins and peptides. It is based on the specific interactions between certain transitional metal ions, mostly Ni^{2+}, Zn^{2+} and Co^{2+} to the exposed amino acid surface chains containing histidine (or cysteine and tryptophane). The presence of several adjacent histidines such as $(His)_6$-tag increases the affinity to immobilised metal ions. Increasingly, IMAC resins are employed for the purification of histidine-tagged recombinant proteins expressed in bacteria, yeast and mammalian cells. There are other applications of IMAC resins to purification of certain native non-tagged proteins as well, such as interferons, lectins, antibodies, serum and plasma proteins, peptides and peptide hormones.

Metal ions are immobilised to the carefully designed porous polysaccharide polymer supports via covalently attached strong chelating groups.

2. Product characteristics

IMAC SepFast Large Beads is specifically designed and fabricated for purification of proteins from viscous and/or cell-containing crude samples in batch (stirred tank), gravity flow, or packed column mode. The base matrix is made of heavily cross-linked agarose.

Metal ions can be charged to the covalently attached chelating groups on the solid support. Leakage of metal ions is negligible.

Table 1. Product characteristics

Particle size	150 – 350 μm (>80%)
Base matrix	Cross-linked 6% agarose
Ligand	Nitrilotriacetic acid (NTA)
Metal ion capacity	> 15 μmol / ml resin*
Protein binding capacity	Depends on the type of proteins and binding conditions; could be > 40 mg / ml resin*
Chemical stability**	Stable in 0.1M HCl and 1% SDS tested for 30 mins; 0.5 M NaOH and 30% acetic acid tested for overnight; 0.01M HCl, 0.1M NaOH and 0.2M acetic acid tested for one week.
pH stability**	2-14 (<2 h) 3-12 (up to one week)
Storage	20% ethanol at 4°C

*Tested with nickel ion; **Tested with the absence of metal ions.

IMAC SepFast Large Beads is supplied in non-charged form alone or with precharged metal ions as Ni SepFast Large Beads, Zn SepFast Large Beads and Co SepFast Large Beads.

IMAC SepFast Large beads or its metal charged format (e.g. Ni SepFast Large Beads etc) is highly stable and compatible with a wide range of chemicals commonly experienced in protein purification processes (see Table 2), which means that more flexible operations can be developed for the best performance.

Table 2: Compatibility of reagents with IMAC SepFast Large Beads*

Chelating reagents	EDTA, EGTA	Up to 1 mM, but care should be taken with any chelating reagents. It may be added to the samples rather than directly to the binding buffers.
Denaturing reagents	GuHCl	Up to 6 M
	Urea	Up to 8 M
Detergents	Triton X-100	Up to 2% v/v
	Tween-20	Up to 2% v/v
	NP-40	Up to 2% v/v
	CHAPS	Up to 1%
	SDS	Pre-testing required case to case, 0.1-0.3% might be permitted
Reducing reagents	β-mercaptoethanol	Up to 20 mM
	DTT	Up to 2 mM
	DTE	Up to 2 mM
	Reduced glutathione	Up to 10 mM
Buffer reagents	Sodium phosphate, pH 7.5	Up to 50 mM, commonly recommended
	Tris-HCl, pH 7.5	Up to 100 mM
	Tris-acetate, pH 7.5	Up to 100 mM
	HEPES	Up to 100 mM
	MOPS	Up to 100 mM
	Sodium acetate, pH 4	Up to 100 mM
Other additives	NaCl	Up to 2 M, 0.5 M is recommended as a starting point
	Ethanol	Up to 20%
	Glycerol	Up to 50 %
	Imidazole	Up to 500 mM
	Citrate	Up to 60 mM
	Glycine	Not suggested
	Sodium biocarbonate	Not suggested
	Sodium sulphate	Up to 100 mM

*Tested after Ni^{2+} ion is charged to the resin.

3. Purification procedures

Please read Section 4 before a purification experiment is designed.

After the choice of metal ion (Ni^{2+}, Zn^{2+} and Co^{2+}) is charged to the resin, the target protein can be directly purified from unclarified or clarified cell lysates no matter if it is by batch or gravity operation. As IMAC SepFast Large Beads is compatible with most of the commonly used reagents in biological systems, cell lysates generated by the commercial cell lysing reagents / kits etc in the market place can be directly used with IMAC SepFast Large Beads without extra treatment (note: for precaution it is recommended to test in small scale first). Recombinant proteins expressed as inclusion bodies can be directly purified (and refolded if necessary) after dissolving in denaturing reagents e.g. 6 M GuHCl or 8 M urea.

3.1 Choice of the operation modes
The ***purification mode***, i.e. batch adsorption, gravity column adsorption, purification with magnetic resin, centrifugal adsorption or packed column adsorption etc, can be selected according to the guidance set in the ***Section 4.2***.

3.2 Preparations before protein purification
Protein expression and cell culturing are referred to in the well-established protocols in the literature.

Harvest the cells and / or broth after the culture is finished. For intracellularly expressed proteins, the pelleted cells are generally resuspended in PBS, tris-HCl buffer or other suitable buffers for following cell disruption such as freeze-thaw, ultrasonication, homogenisation and bead milling etc. Or the pelleted cells are directly suspended into a self-made or commercial cell lysing solution for releasing of the target protein (refer to the well established protocols in literatures). Proteins expressed as inclusion bodies can be dissolved in denaturing reagents such as GuHCl and urea first. Clarified or unclarified protein samples can be purified directly. If an unclarified sample is loaded, a treatment with DNAse I (e.g. 5 μg/ml of Benzonase with 1 mM Mg^{2+} for 10-15 mins in ice-bath) may be required to reduce the sample viscosity. Pre-conditioning of the cell lysates, such as pH adjustment, addition of 0.5 M NaCl and low concentration of imidazole (e.g. 20 mM) etc, can be done in this step. ***Note***: imidazole and NaCl of the same final concentrations should be added to the lysate and the binding buffer; cell lysis and addition of imidazole will change the sample pH so adjustment of pH before sample loading is essential.

Charging of metal ion of choice to IMAC SepFast Large Beads (if necessary) can be done according to the protocol set in the ***Section 5***.

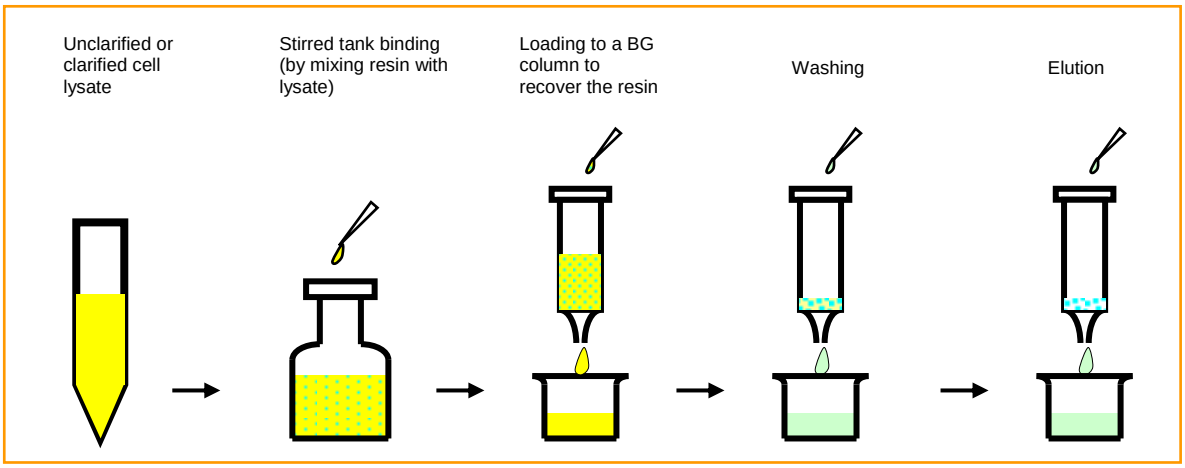
Equilibration / binding buffer is recommended as: 20 mM sodium phosphate + 0.5 M (or up to 1.0 M) NaCl, pH 7.4. For the purification of his-tagged proteins, the presence of low concentration of imidazole is recommended. The exact concentration is protein and metal ion dependent with a guided range of 10 – 50 mM. See the ***Section 4.3*** for more information.

Washing buffer can be the same as the binding buffer or may contain additional reagents (e.g. detergents, alcohol and increased imidazole concentration etc) or have low pH value etc, in order to remove as much weakly bound impurities as possible. Refer to the ***Section 4.4*** for more information.

Elution solution should be prepared according to the guidance set in the ***Section 4.5***. The standard one can be 250 mM – 500 mM of imidazole in 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4.

Water and chemicals used for the protein purification process should be of high purity.

3.3 Protein purification in a batch stirred tank mode



1. Depending on the quantity of target protein in the cell lysate, the amount of IMAC SepFast Large Beads is estimated at a binding capacity of 15 mg/ml resin (the actual capacity could be much higher).

2. Fully shake the resin bottle to re-slurry the resin. Take out the required volume (Note: shake the bottle to reslurry the resin immediately before pipetting to avoid settling. Seal the bottle and store at 4°C soon after the resin is taken out.)

3. Hold the column in a vertical position in a proper stand. Wash the resin with at least 10 resin volumes of the equilibration buffer under gravity until the liquid is fully passed through. Note: gently tap the sinter side of the column if no liquid comes out.

4. If the volume of protein sample is less than the working volume of the column, put on the bottom lid and load the protein sample into the column. Otherwise go to step 5. Close the top lid and place the column on a suitable roller mixer for batch binding. Be sure the resin is fully mixed with the protein sample. Depending on the nature and size of the target protein, the binding time varies from a few minutes to up to a few hours. Generally speaking, 10 – 30 minutes is sufficient to utilise most of the resin capacity.

5. If the volume of protein sample is beyond the working volume of the column, re-slurry the resin with a few mls of the binding buffer and pour it into a suitable bottle in which the protein sample is loaded for batch stirred tank binding. Place the bottle on a proper roller mixer. Depending on the nature and size of the target protein, the binding time varies from a few minutes to up to a few hours. Generally speaking, 10 – 30 minutes is sufficient to utilise most of the resin capacity.

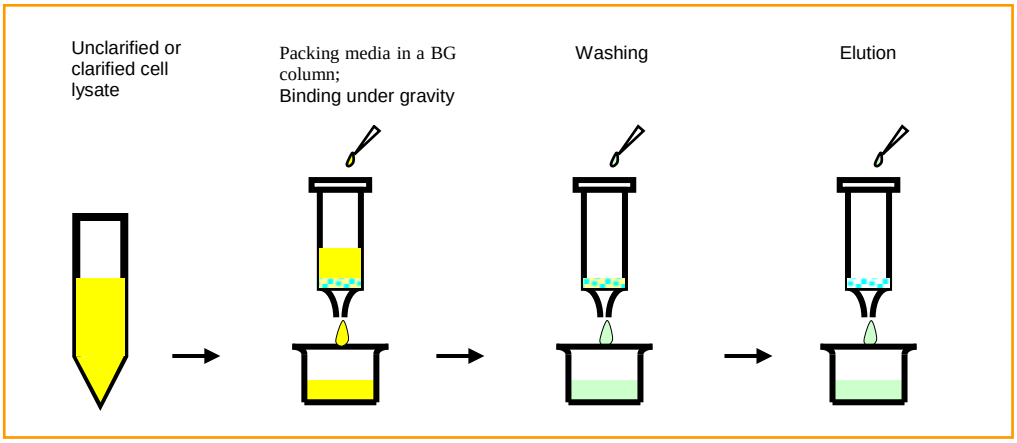
6. After the batch binding is finished, place the column in a suitable stand in a vertical position. Remove the top lid then the bottom lid. In the case of the sample being processed via Step 5, it is poured into the column to recover the resin. The cell lysate (clarified or unclarified) is discharged by gravity or by suction through a syringe (supplied; connecting the bottom side of the column to the rubber tubing of the syringe). It is recommended to use this suction approach as it is a much quicker way to remove the liquid.

7. Put the bottom lid on, add a few mls of washing solution. Shake to mix the resin with liquid for 10-30 seconds. Remove the liquid by gravity discharge or by syringe suction. Repeat the washing 2 or 3 times. The stringency of washing solution may vary each time to remove as many impurities as possible (see **Section 4: General considerations and optimisations**). The total washing volume should be at least 10 resin volumes. Collect the waste in a suitable container.

8. Elution can be done in gravity flow mode or by batch incubation. Generally speaking, the total eluant volume at 5 – 10 times the resin volume is sufficient to recover the bound protein. For the best recovery yield, incubation of the resin-eluant mixture in a roller mixer for 5 - 10 minutes is recommended. This allows sufficient time for the internal bound protein molecules to diffuse out of the macropores. The eluate is recovered under gravity flow or by syringe suction, and collected to a suitable container. This step may be repeated once or twice to maximise the recovery yield.

9. Buffer exchange and / or desalting might be required to adjust the pH and to remove imidazole and salt in the eluted sample. SuperSpin Desaltor (product code: 210101) can be used as a fast and cost-effective approach. Refer to the product files (www.biotoolomics.com/product) for more details.

3.4 Protein purification in gravity flow mode



1. Depending on the quantity of target protein in the cell lysate, the amount of IMAC SepFast Large Beads is estimated at a binding capacity of 15 mg/ml resin. The actual capacity could be much higher.

2. Use a gravity column with large pore frit. The small chamber under the sintered mesh of the column of choice has to be pre-filled with water and ***should be air bubble free*** (otherwise, the resin may have a lower binding capacity). To do so, fill the column full with water. Remove the bottom lid to let a few drops of liquid come out. In the case that no liquid comes out, tap the column on working benchtop a few times. If air bubble is trapped underneath the sintered mesh, close the top lid and then turn the column upside down. Tap the mesh side to let the trapped bubble escape. Invert the column and open the top lid to let liquid through. When the chamber is fully filled with water and is free of air bubble, put the bottom lid on. Pour out the free water above the mesh. It is ready to accommodate resins.

3. Fully shake the bottle of resin to re-slurry the resin. Take out the required amount of resin, according to the calculation in Step 1.

4. Hold the column in a vertical position in a proper stand. Remove the bottom lid. Equilibrate the resin by gently adding 5 – 10 resin volumes of the equilibration buffer and discharging under gravity until the liquid has fully passed through.

5. Put the bottom lid on. Load the protein sample into the column in the following manner. Add the protein sample slowly to minimise the disturbing of the settled particles. If the protein sample is over the working volume of the column, fill the column full first. Remove the bottom lid to let the feedstock pass through under gravity. The protein sample can be further poured in when more column space is available. Collect the flow through in a suitable container for future analysis.

6. After the above binding process is finished (i.e. the whole protein sample passes through the column), washing solution is gently added to remove the weakly bound impurities. The stringency of washing solution may vary each time to remove as many impurities as possible (see **Section 4: General considerations and optimisations**). At least 5 bed volumes of washing liquid is required. Collect the washing waste in a suitable container for further analysis.

7. Load the eluant to the column in a way that is as gentle as possible to avoid disturbing the settled particles. The total eluant volume at 5 – 10 times the resin volume is sufficient to recover the bound protein. Collect the eluate in a suitable container. The elution may be repeated once or twice to maximise the recovery yield.

8. Buffer exchange and / or desalting might be required to adjust the pH and to remove imidazole and salt in the eluted sample. Depending on the sample volume, SuperSpin Desaltor (product code: 210101) can be used for fast and cost-effective desalting /buffer exchange. Refer to the product files (www.biotoolomics.com/product) for more details.

3.5 Protein purification in packed bed mode

Special columns allowing free pass-through of cells and cell debris should be used to pack IMAC SepFast Large Beads. For technical support, please contact BioToolomics.

4. General considerations and optimisations

4.1 Choice of metal ions

IMAC SepFast Large Beads is supplied free of charged metal ion. The pre-charged resins with the most extensively employed metal ions are supplied as Ni SepFast Large Beads, Zn SepFast Large Beads and Co SepFast Large Beads. The metal charging protocol is referred to **Section 5: Charging and regenerating**.

The choice of metal ions mainly depends upon the nature of the target proteins and the specific application requirements. Ni^{2+} is commonly the first choice for purification of histidine-tagged recombinant proteins. As the strength and selectivity of interaction between a target protein and immobilised metal ion is affected by a few factors

including the length and exposed position of the tag, electron distributions of the pair, pH and competitions from other impurities etc, some tagged proteins might be better purified with Zn^{2+}or Co^{2+} rather than Ni^{2+}. The similar considerations apply to purification of untagged proteins as well. A screening of different charged metal ions in combination with the specific application requirement (e.g. purity or yield or both) and binding conditions is recommended.

4.2 Choice of operational mode

There are mainly the following operational modes for consideration: batch stirred tank mode (including magnetic operation with magnetic particles), gravity flow mode, centrifugal (spin) mode and pressurised (packed) column mode.

For laboratories that have no access to expensive and sophisticated process chromatography systems (e.g. packing columns, pumps and valves, detectors and associated computing machinery etc), or to whom protein purification is only a tool rather than their core research activities, the best choice is among batch stirred tank mode, gravity flow mode and centrifugal (spin) mode.

Batch stirred tank operation is the most versatile and flexible approach. Very little training in chromatography is required. It is particularly attractive in cases when the volume of protein sample is large but the concentration is relatively low or the target protein molecule is large (e.g. over 65K Dalton). Longer contact time can be employed in this mode to allow protein molecules diffusing into the pores, which means better utilisation of the whole resin ligands and subsequently higher binding capacity. Also, there is no limitation from the column side. So, large volumes of protein sample can be processed in the same time period as that for small volumes. IMAC SepFast Large Beads is particularly suitable for this operation.

Magnetic IMAC resin such as IMAC SepFast MAG is very suitable for batch stirred tank operation, particularly when the volume of protein sample and the quantity of required resin is small. Solid / liquid separation in each step (e.g. equilibration, binding, washing and elution etc) is readily achieved by using a magnet such as a magnetic stirring bar.

Gravity flow operation is a choice if the volume of protein sample is from a few mls to tens of mls, and the protein molecule isn't very big. More than one column can be easily operated in parallel. IMAC SepFast Large Beads resins possess strong mechanical structure to permit liquid to pass through at an excellent flowrate.

Pressurised (packed) column operation gives the best chromatographic performance. IMAC SepFast Large Beads is particularly designed for such operations. However, expensive and specialised instrument and accessories are required for such kinds of operations. Professional training and expertise in process chromatography is essential in order to gain the most benefit from such operations. Unclarified cell lysates can be directly processed in short packed column fitted with large pore filter mesh.

4.3 Binding conditions

Proteins tagged with one or more 6 x His in either the N-terminus or C-terminus can be strongly bound to the metal charged IMAC SepFast Large Beads. The interaction doesn't depend on the three-dimensional structure of the protein, as long as two or more chelating residuals in the protein can access the immobilised metal ion to form chelating bonds. Host cell proteins (HCP) that contain histidine, cysteine or tryptophan in a close proximity might interact with the resin but the strength is generally much weaker.

Three key factors are commonly optimised for the best binding performance; they are imidazole, pH and salt. The addition of imidazole of a low concentration to the protein feedstock can effectively compete off the HCP binding caused by their chelating residues. Imidazole is usually added to the sample, equilibration buffer and binding buffer at a final concentration of 10 – 50 mM. The exact concentration of imidazole has to be optimised to balance two key parameters (i.e. purity and yield). Sodium chloride must be added to the sample and binding buffer to suppress any non-specific interaction caused by electrostatic charges. Commonly 0.5M of NaCl is used but further optimisation might be required to improve the product purity. In some cases, manipulation of pH (particularly reducing pH value in the sample) can improve product purity, as the chelating residues in HCP can't form chelating bonds after they are charged.

Phosphate buffer is recommended in most cases. Tris-HCl buffer at lower concentration (e.g. 10-50 mM) is normally fine but care should be taken if a higher concentration is employed, as it might affect the binding in the case that the affinity of target protein to the metal ion isn't very strong. Addition of other chelating reagents such as EDTA at very low concentration (e.g. < 1 mM) might improve the product purity in some cases.

Proteins expressed as inclusion bodies can be purified after dissolved with 6 M GuHCl or 8 M urea.

The amount of resin used to purify a given amount of target protein can be considered as well. The addition of an excess amount of resin might promote undesired non-specific binding of HCP, as more free ligands are available to impurities.

4.4 Washing conditions

A stepwise increase of the washing stringency is recommended for the batch or gravity flow operations. In some cases, longer contact time may help to dissociate the bound impurities from the resin. Increased imidazole concentration, increased salt concentration, reduced pH, addition of denaturing reagents like GuHCl and urea, addition of alcohol (e.g. 30%) or glycerol (10 – 50%), addition of detergents like Triton and Tween etc are the commonly used approaches to remove the weakly bound impurities. Reducing reagents can be added in the washing buffer if disulfide bonds between HCP and the target protein may have been formed. A compromise between the final yield and product purity should be considered to develop the best washing conditions.

4.5 Elution conditions

There are mainly three choices of elution approaches: pH, imidazole and EDTA.

When the pH is reduced from neutral to less than 6 (typically 4.2 to 5.5), the histidine residues in the bound protein are protonated. Under this condition, the chelating bond between the metal ion and the histidine residues is

dissociated. As a result, the bound protein is released. Care should be taken to investigate the stability of the target protein under a low pH value like 4.5.

Imidazole at high concentrations (e.g. 100 – 500 mM) can effectively compete off the bound protein as well. The best elution concentration has to be determined case by case. This is the mildest approach among the three mentioned here.

EDTA is a very strong chelating reagent. 100 mM EDTA can fully strip off the immobilised metal ion and therefore release the bound protein as well. It might not be desirable for metal ions to be present in the final protein product.

Ni SuperSpin, Co SuperSpin and Zn SuperSpin are particularly powerful tools for rapid optimisation of the purification (binding, washing and elution) conditions at low cost.

5. Charging and regenerating of IMAC SepFast Large Beads

Charging of metal ion of best choice can be conducted in the following procedure.

1. Transfer the required amount of IMAC SepFast Large Beads into a suitable gravity column its bottom lid on.

2. Place the column to a suitable stand in a vertical position. Remove the bottom lid to drain liquid off by gravity. Add 5 – 10 bed volumes of distilled water (or deionised water) and then drain the liquid off under gravity. Put the bottom lid on.

3. Prepare a solution containing 0.08 M of the metal ion (e.g. Ni^{2+}, Co^{2+} and Zn^{2+} etc) in distilled water. For Zn^{2+} ion, a tiny amount of acid is added to reduce the solution pH to 5.5 or slightly less. Load the same volume of the solution into the column. Close the top lid and fully re-slurry the particles. Leave the mixture for 10 – 30 minutes with periodical shaking. Remove the top lid then the bottom lid to drain the liquid off under gravity.

4. Add 2 - 3 bed volumes of distilled water for washing. Repeat the washing twice.

5. Afterwards, the resin can be equilibrated with 5 - 10 bed volumes of the equilibration buffer under gravity flow.

After the IMAC resin has been used for a few cycles of purification, it may be discarded or regenerated depending on the properties of protein samples and purification conditions etc.

For the purpose of regeneration, the resin has to be totally free of any bound metal ion. By incubating the resin in the same volume of 100 mM EDTA + 0.5 M NaCl, pH 7.5, for 10 – 30 minutes with continuous mixing, the bound metal ion can be fully stripped off. This can be done in a gravity column. After removing the EDTA solution, wash the resin with 5 bed volumes of 0.5 M NaCl in distilled water then 5 bed volumes of distilled water. Afterwards, it is ready to recharge the resin according to the protocol described above.

6. Clean-in-place (CIP)

In the case that the resin is severely contaminated by strong ionic substances (e.g. proteins and nucleic acids etc), hydrophobic proteins, lipoproteins and lipids etc, the following clean-in-place approaches can be conducted. Before any CIP is pursued, the immobilised metal ions must be removed according to the procedure described in ***Section 5***.

Salt of a concentration up to 2 M can be used to clean the impurities bound by ionic interactions.

The contaminants bound hydrophobically can be removed by the following reagents: 1 M NaOH, low percentage detergents (e.g. 0.1 – 2%), 70% ethanol or 30% isopropanol in basic or acidic conditions (e.g. in the presence of acetic acid or phosphoric acid). A combination of the above reagents can be explored as well. In general, the incubation time should be longer (e.g. from 30 minutes to overnight) to ensure full dissociation of the contaminants.

7. Trouble shooting

The following tips may help to resolve the possible problems with individual purification process. If you would like further assistance, please contact our technical team or sales representatives for more information.

No target protein in the eluted fractions	1. **Elution condition is too mild to dissociate the bound protein.** Increasing the imidazole concentration or further reducing the pH in the elution buffer may help. If a hydrophobic interaction is contributory, addition of non-ionic detergents (e.g. Tween-20) could improve the recovery yield. Elution with EDTA might be a choice in some cases. 2. **Binding conditions are not correct**. Check pH and composition of all buffers and solutions in each step. It should be pointed out that the addition of some reagents (e.g. imidazole) could cause a change in pH value. The concentration of imidazole in the binding buffer might be too high. 3. **Histidine tag is not present**. Check the protein gene construction is correct and as it was originally designed. 4. **Histidine-tag has been degraded**. Use anti-his antibodies in western blotting to check the location of the tag. 5. **Histidine tag is not sufficiently accessible**. Denaturing reagents such as urea could be added to partially defold the protein. 6. **The target protein has precipitate on the resin**. Try to add detergents (e.g. 0.1 – 1 % v/v) or denaturing reagents (e.g. 4 – 8 M urea). The concentration of NaCl might be reduced. Loading of as smaller amount of protein and reduction of adsorption time can help to minimise such problems.
The target protein is eluted with impurities	1. **Binding and washing conditions not stringent enough**. Refer to Section 4.3 and 4.4 for further consideration. 2. **Impurities are associated with the target protein**. Try to add reducing reagents (e.g. <20 mM β-mercaptoethanol) in the sample or washing buffer to disrupt formation of disulfide bonds. Try to add detergents or alcohol / glycerol in the washing buffer to suppress any non-specific interaction. 3. **Impurities are truncated parts of the target protein**. Check the gene construction and expression conditions to minimise potential mutations. Prevent protein degradation by addition of protease inhibitors and / or reduction of working temperature. 4. **Impurities have higher affinity than the target protein**. Careful optimisation of the binding, washing and elution conditions might allow the recovery of target protein without co-elution of the tightly bound impurities. 5. **Change of metal ion of choice**. IMAC SepFast charged with other metal ions might help.
Column is clogged	1. **The unclarified protein sample is too viscous**. Treatment by ultrasonication or addition of DNase (e.g. 5 μg / ml benzonase + 1 mg / ml of Mg^{2+} in ice-bath for 10-15 mins) can reduce the viscosity. 2. **Air bubble is trapped under the sintered mesh**. Tilt the column (or even invert the column) and tap the mesh side to let the bubble escape. 3. **Column mesh is blocked**. Replace with a new column to work with.

8. Storage

Store the resin in 20% ethanol at 4°C. Seal the bottle soon after each use.

9. Further information

Visit www.biotoolomics.com for further information or contact the technical team or sales representatives.

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