BIOTOOLOMICS

IMAC SepFast HighRes Media

Data and Instructions

1. Introduction

Immobilised metal affinity chromatography (IMAC) 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 Cu^{2+}, 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 such as agarose beads via covalently attached strong chelating groups. The much smaller particle size of IMAC SepFast HighRes allows fast mass transfer of protein molecules within the pores.

2. Product characteristics

IMAC SepFast HighRes is designed and fabricated for high performance chromatographic purification of proteins in packed columns, particularly in FPLC systems. The base matrix is made of heavily cross-linked polysaccharide. Its small spherical shaped particle and carefully controlled pore structure allows superior chromatographic resolution in a packed column operation. Also, its high mechanical strength permits liquid passing through the pressurised column at reasonable flow rates with low to medium pressure drop. All these translate into higher purification performance at shortened purification time.

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

IMAC SepFast HighRes is supplied in 25 ml alone or is supplied with precharged metal ions as Ni SepFast HighRes, Cu SepFast HighRes, Zn SepFast HighRes and Co SepFast HighRes. The common features are listed in Table 1. The choice of charged metal ion could further improve the selectivity to targeted biomolecules.

Table 1. Product characteristics

Particle size	20 – 50 μm
Base matrix	Cross-linked 6% beaded agarose
Metal ion capacity	At least 15 μmol / ml resin*
Protein binding capacity	Depends on the type of proteins and binding conditions; could be > 40 mg / ml resin*
Operational flowrate	Approx. 100 cm/h is recommended
Working pressure	Up to 3 bar
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 - 30 °C

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

IMAC SepFast HighRes or its metal charged format (e.g. Ni SepFast HighRes etc) is highly stable and compatible to 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.

3. Purification procedures

Please read Section 4 before a purification experiment is designed.

IMAC SepFast HighRes is designed and fabricated for high performance packed column purification of proteins.

After the choice of metal ion (among the most popular ones are Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}) is charged to the resin, target protein can be purified from clarified cell lysates. As IMAC SepFast HighRes is compatible to 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 contacted with IMAC SepFast HighRes 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.

Table 2: Compatibility of reagents with IMAC SepFast HighRes*

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

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

3.1 Choice of columns

Most of the chromatography packing columns in the marketplace (e.g. columns supplied by GE Healthcare, Pall and Millipore etc) can be chosen for packing of IMAC SepFast HighRes resin. Attention should be paid to the working pressure of individual column. The standard packing protocols from the column supplier can be adopted without any change.

3.2 Choice of the operation modes

IMAC SepFast HighRes is designed for high performance packed column operations.

3.3 Preparations before protein purification

Protein expression and cell culturing can be referred to the well established protocols in 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. If the sample is too viscous, 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.

For packed column operation, clarification of the protein sample has to been done by centrifugation or filtration or both, as the column might be blocked by cell debris.

Charging of metal ion of choice to IMAC SepFast HighRes (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 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 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.

3.4 Protein purification in a packed column operation

1. Set up the chromatography system. Check the tubings and pumps work in right order.
2. Pack the required amount of resin into the column of right size (refer to established protocols or the column supplier's guidance).
3. Ensure the resin with metal ion of choice (refer to ***Section 5*** for charging of metal ion) being packed properly (e.g. no air bubble trapped somewhere inside the column).
4. Apply 3 – 5 column of distilled water at a flowrate of 100 cm/h to remove any ethanol.
5. Equilibrate the resin with 3 – 5 column of the binding buffer at a flowrate of 100 cm/h. The flowrate can be optimised depending on the nature of the protein.
6. Load the right sample to the column at the same flowrate as above.
7. Wash the column with the binding buffer until it reaches the baseline.
8. Further wash the column with the washing buffer (if different from the binding buffer) at 3 – 5 column volume.
9. Elute the bound protein with the elution buffer stepwise or in a linear gradient, at the flowrate the same as above or lower. Collect the elution fractions for further analysis.
10. Buffer exchange and / or desalting might be required to adjust the pH and to remove imidazole etc in the eluted sample. Depending on the sample volume, SuperSpin Desaltor can be used for fast desalting of small samples.

4. General considerations and optimisations

4.1 Choice of metal ions
IMAC SepFast HighRes is supplied free of charged metal ion. The pre-charged resins with the most extensively employed metal ions are supplied as Ni SepFast HighRes, Cu SepFast HighRes, Zn SepFast HighRes and Co SepFast HighRes. A special pack that contains the above four pre-charged resins (5 ml each type) is supplied as well for the screening purpose. The metal charging protocol is referred to **Section 5: Charging and regenerating**.

The choice of metal ions mainly depends upon the nature of 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+}, Co^{2+} or Cu^{2+} etc rather than Ni^{2+}. The similar considerations apply to purification of untagged proteins as well. Cu^{2+} might be used more often than other metal ions. 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 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 HighRes. The interaction doesn't depend on the three-dimensional structure of the protein, as long as two or more than two 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 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 bond 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 higher concentration is employed, as it might affect the binding in 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.

4.3 Washing conditions
Stepwise increase of the washing stringency is recommended. 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.

4.4 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 low pH value like 4.5.

Imidazole at high concentration (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 stripe off the immobilised metal ion and therefore release the bound protein as well. It might not be desirable for metal ion being present in the final protein product.

5. Charging and regenerating of IMAC SepFast HighRes

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

1. Pack the required amount of IMAC SepFast HighRes into a suitable column.
2. Pump 3-5 bed volume of distilled water at 100 cm/h through the column.
3. Prepare a solution containing 0.08 M of the metal ion (e.g. Ni^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} etc) in distilled water. For Zn^{2+} ion, tiny amount of acid is added to reduce the solution pH to 5.5 or slightly less.
4. Pump 2 bed volume of the above solution through the column at 100 cm/h.
5. Pump 3-5 bed volume of distilled water for washing.
6. Afterwards, the resin can be equilibrated with 5 - 10 bed volume of the equilibration buffer at the same flow rate.

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 pumping 2-3 bed volume of 100 mM EDTA + 0.5 M NaCl, pH 7.5, through the column, the bound metal ion can be fully stripped off. After removing the EDTA solution, wash the resin with 3 bed volume of 0.5 M NaCl in distilled water then 5 bed volume of distilled water. Afterwards, it is ready to recharge the resin according to the protocol described above.

6. Clean-in-place (CIP)

In 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 with concentration up to 2 M can be used to clean the impurities bound by ionic interactions.

The contaminants bound by hydrophobic nature 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 to get 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. Increase the imidazole concentration or further reduce the pH in the elution buffer may help. If hydrophobic interaction is contributed, 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 the change of 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 as it is 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 less 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 bond. 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 inhibitor 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 PC charged with other metal ions might help.
Column is clogged	1. **The protein sample is not clear enough**. Filter the sample with a 0.2 μm filter before loading to the column.

8. Storage

Store the resin in 20% ethanol at 4°C or room temperature.

9. Further information

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

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