Pre-packed Ni SepFast™ Columns

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 Ni^{2+}, Co^{2+} and Zn^{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 agarose supports via covalently attached strong chelating groups.

2. Product characteristics

Ni SepFast is packed in the disposable polypropylene / acrylic column (see the Table 1 for details). The resin base matrix is made of heavily cross-linked agarose. Its rough surface and carefully controlled pore structure allows fast access of immobilised metal ligands by target protein molecules. Also, its high mechanical strength permits liquid passing through at decent flow rates. All these translate into high process flexibility and higher protein yield at a shortened purification time.

Table 1. Product characteristics

Particle size	50 – 150 μm
Base matrix	Cross-linked 6% agarose
Metal ion capacity	Approx. 12 – 25 μmol / ml resin*
Protein binding capacity	Depends on the type of proteins and binding conditions; could be > 40 mg / ml resin*
Column material	Polypropylene / acrylic column body, polyethylene filter frits, NBR O-rings
Operational pressure	Up to 4 bars
Operational flowrate	Depends on the nature of the protein sample, normally less than 1 column volume (CV)/min
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; **Tested with the absence of metal ions.

The product 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.

Table 2: Compatibility of reagents with IMAC SepFast BG*

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	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 start 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.

3.1 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 can be 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.

Cell debris needs be removed before the protein sample is loaded to the column.

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.

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 (e.g. 20 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 many 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.

3.2 Protein purification in packed bed mode

1. Set up the chromatography system. Check the tubings and pumps work in the right order.
2. Connect the column to the system.
3. Equilibrate the resin with the binding buffer at a proper flowrate until the signal reaches the baseline or becomes stable.
4. Load the protein sample in the column at an optimised flowrate.
5. Wash the column with the binding buffer until it reaches the baseline.
6. Further wash the column with the washing buffer (if different from the binding buffer) at 3 – 5 column volumes.
7. Elute the bound protein with the elution buffer stepwise or in a linear gradient, at the flowrate the same as above or lower. Collection the elution fractions for further analysis.
8. 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.

4. General considerations and optimisations

4.1 Choice of metal ions
The pre-charged resins with the most extensively employed metal ions are supplied as Ni SepFast, Zn SepFast and Co SepFast. A special pack that contains the above three pre-charged resins (5 ml each type) is supplied for screening purposes.

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+} 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.

Pre-packed resins charged with other metal ions are available on request.

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 BG. 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 a 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.

4.3 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.

4.4 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 a 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.

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. 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.** Increasing the imidazole concentration or further reducing 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 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 a 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) to the sample or washing buffer to disrupt formation of disulfide bond. Try to add detergents or alcohol / glycerol to 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 charged with other metal ions might help.
Column is clogged	1. **The 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. **Column mesh is blocked**. Replace with a new column to work with.

6. Storage

Store the column in 20% ethanol at 4°C - 30°C Seal the column after each use.

7. Further information

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

8. Ordering information

Product	Quantity	Code no.
Pre-packed Ni SepFast columns	1 x 1 ml	180114
	1 x 5 ml	180116
Related products		
Ni SepFast	10 ml	180113
	25 ml	180104
	100 ml	180105
Co SepFast	25 ml	180108
	100 ml	180109
Zn SepFast	25 ml	180110

	100 ml	180111
Screening kit of IMAC SepFast	5 ml each of Ni SepFast BG, Co SepFast BG and Zn SepFast BG, respectively; plus 1 Ready-to-Use His Buffer Kit	180103
IMAC SepFast (non-charged format)	10 ml	180112
	25 ml	180101
	100 ml	180102

Buffer kit and gravity flow columns	**Quantity**	**Code no.**
Ready-to-Use His Buffer Kit	2 x 50 ml phosphate stock solution (0.16 M sodium phosphate + 4 M NaCl, pH 7.4) and 50 ml imidazole stock solution (2 M, pH7.4)	200105
Ni SuperSpin	50	150101
Co SuperSpin	50	150103
Zn SuperSpin	50	150104
SuperSpin Desaltor	50	210101

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