BIOTOOLOMICS

IMAC SepFast MAG 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 magnetic supports via covalently attached strong chelating groups.

2. Product characteristics

IMAC SepFast MAG is specially designed and fabricated for magnetic purification of proteins in batch mode (i.e. stirred tank mode). The base matrix is made of cross-linked polysaccharide encapsulating with fine magnetic particles. Therefore, the resin possesses magnetic property. Removal of liquid after each step such as binding, washing and elution can be readily done by fixing the resin with a magnet.

Note: no magnet is supplied with this product, so customers need to source the suitable magnet.

Four types of IMAC SepFast MAG are supplied in 5 ml format with precharged metal ions as Ni SepFast MAG, Cu SepFast MAG, Zn SepFast MAG and Co SepFast MAG. A special pack that contains the above four pre-charged resins (1 ml each type) is supplied as well for the screening purpose. 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	50 – 150 μm
Base matrix	Cross-linked 6% agarose encapsulating magnetic particles
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*
Chemical stability**	Stable in 0.01M HCl and 1% SDS tested for 30 mins; 0.5 M NaOH tested for overnight
pH stability**	2-14 (<2 h) 4-12 (up to one week)
Storage	20% ethanol at 4°C

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

IMAC SepFast MAG 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

IMAC SepFast MAG is specially designed and fabricated for batch stirred tank purification of small quantity of proteins. After the choice of metal ion (among the most popular ones are Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}) is selected, target protein can be directly purified from unclarified or clarified cell lysates (if it's intracellular protein) or cultured cell broths (if it's extracellular protein). As IMAC SepFast MAG 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 MAG 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 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 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 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.

Table 2: Compatibility of reagents with IMAC SepFast MAG*

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.

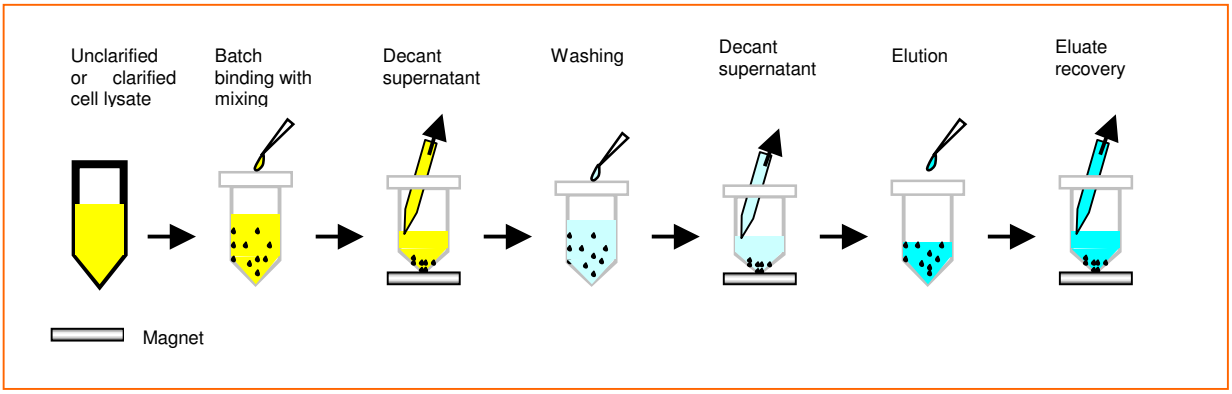
Equilibration / binding buffer is recommended as: 20 mM sodium phosphate + 0.5 – 1.0 M NaCl, pH 7.4. For the purification of his-tagged proteins, the presence of low concentration of imidazole is recommended. The exact concentration is protein and metal ion dependent with a guided range of 10 – 50 mM.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

Washing buffer 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.

3.2 Protein purification procedure



1. Depending on the quantity of target protein in the cell lysate, the amount of IMAC SepFast BG is estimated at a binding capacity of 15 mg/ml resin (the actual capacity could be much higher). This figure is divided by 0.6 to give the volume of resin slurry required, as the resin is supplied at a 60% v/v concentration. For example,

1 ml of cell lysate contains 0.5 mg of his-tagged protein. So 33 μl of the resin is enough, which translate into a slurry volume of 55 μl.

2. Fully shake the bottle to re-slurry the resin. Take out the required amount of the slurry using a 200 μl pipette (Note: the pipette tip should be cut off approx. 3-5 mm from the narrow side to avoid possible tip blockage by the resin particles) and transfer to a suitable container (e.g. a eppendorf centrifuge tube) for batch binding.

3. Wash the resin with 5 - 10 resin volume of the equilibration / binding buffer. After the magnetic particles are settled by a magnet, remove the supernatant by a pipette. Repeat the washing two times more. Be sure the magnet is removed when washing buffer is added and mixed.

4. After washing step, add the protein sample in. Close the container lid and place onto a suitable roller mixer for batch binding. Depending on the nature and size of 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. After the batch binding is finished, drag down the resin with a magnet. Remove the supernatant by a pipette. Depending on the strength of the magnet used, care should be paid not to suck the magnetic particles away. Wash the resin three times (3 – 5 resin volume each time) with the washing buffer. The stringency of washing solution may vary each time to remove as much impurity as possible (see **Section 4: General considerations and optimisations**). Collect the waste to a suitable container.

6. Elution can be done in batch incubation. Generally speaking, the eluent volume at 3 – 5 times of the resin volume is sufficient to recover the bound protein. For the best recovery yield, incubation of the resin-eluent mixture in a roller mixer for 5 - 10 minutes is recommended. This allows sufficient time for the internal bound protein molecules to diffuse out the macropores. The eluate is recovered by pipetting after the particles are dragged down with the magnet. This step may be repeated once or twice to maximise the recovery yield.

7. 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.

4. General considerations and optimisations

4.1 Choice of metal ions

A choice of four different metal ions (i.e. Ni^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+}) is available as Ni SepFast MAG, Cu SepFast MAG, Zn SepFast MAG and Co SepFast MAG. A special pack that contains the above four pre-charged resins 1 ml each type) is supplied as well for the screening purpose.

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 MAG. 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 centration 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.

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

4.4 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.

4.5 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 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. 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 MAG charged with other metal ions might help.

6. Storage

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

7. Further information

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

8. Ordering information

Product	Quantity	Code no.
Ni SepFast MAG	5 ml	190101
Cu SepFast MAG	5 ml	190102
Co SepFast MAG	5 ml	190103
Zn SepFast MAG	5 ml	190104
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

Related products

IMAC SuperSpin	Quantity	Code no.
Ni SuperSpin	50	150101
Cu SuperSpin	50	150102
Co SuperSpin	50	150103
Zn SuperSpin	50	150104
SuperSpin Desaltor	50	210101
Other IMAC resins	**Quantity**	**Code no.**
IMAC SepFast BG	10 ml	180112
	25 ml	180101
	100 ml	180102
Screening kit of IMAC SepFast BG	5 ml each of Ni SepFast BG, Cu SepFast BG, Co SepFast BG and Zn SepFast BG, respectively; plus 1 Ready-to-Use His Buffer kit	180103
Ni SepFast BG	10 ml	180113
	25 ml	180104
	100 ml	180105
Cu SepFast BG	25 ml	180106
	100 ml	180107
Co SepFast BG	25 ml	180108
	100 ml	180109
Zn SepFast BG	25 ml	180110
	100 ml	180111
Columns	**Quantity**	**Code no.**
BG-30 column	20	200101
	50	200102
BG-5 column	20	200103
	50	200104

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