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One-Step Purification of Histidine-Tagged Fusion Proteins Under High Pressure and High Flow Conditions

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Abstract

Immobilized metal affinity chromatography (IMAC) with a nickel chelate is widely used for purification and identification of histidine-tagged recombinant fusion proteins. One challenge for purification is the time needed to purify a significant amount of HIS-tagged proteins. Fast Protein Liquid Chromatography (FPLC) and other medium pressure systems are often utilized to increase flow rate and significantly decrease the amount of time required for chromatography.

Demonstrated here is a one-step purification of Metal Affinity Tagged (MAT-Tag™) fusion proteins using the HIS-Select™ High Flow (HF) Cartridges. The cartridges are pre-packed with HIS-Select HF Affinity Resin, allowing for high binding capacity and highly specific purification of histidine-tagged proteins. The HF Affinity Gel is a highly cross-linked, 6% beaded agarose allowing higher flow rates and mechanical stability under pressure. The non-charged, hydrophilic linkage of the nickel-nitrilotriacetic acid (Ni-NTA analog) chelate group to the agarose prevents non-specific interactions between the resin and native proteins.

The performance of the HIS-Select Affinity Gel was confirmed via SDS-PAGE, Western Blot, HPLC, and MALDI-ToF Mass Spectrometric Analyses. In addition, it was demonstrated that the HIS-Select HF Cartridge offers high binding capacity and highly specific purification of histidine-tagged proteins. This data clearly demonstrates that the HIS-Select High Flow Cartridge is truly a one-step solution to large volume, high flow purification of histidine-tagged proteins.

Introduction

Purification of histidine-tagged fusion proteins is routinely accomplished by immobilized metal affinity chromatography (IMAC), using nickel as the affinity ligand. The affinity of histidine tags, including the metal affinity tags (MAT-Tag™), to the nickel chelate is generally very high. With many IMAC systems, interactions of non-specific proteins with the chelate can be a disadvantage. The HIS-Select High Flow (HF) Affinity Resin has the nickel chelate attached to an agarose bead matrix utilizing a non-charged linkage chemistry (Figure 1). Due to the chelate linker being hydrophilic, like the surface of most proteins, there is minimal interaction between the resin and non-specific proteins due to polarity, therefore offering superior selectivity. The highly pure tetradentate chelate feature of the resin also aids in reducing non-specific binding and increasing binding capacity. Tetradentate chelates hold the metal ion at four coordination points as opposed to the three binding points exhibited by iminodiacetic acid (IDA) type chelates.

High-throughput purification of proteins is defined by speed. When purifying multiple protein samples or a large volume of any one protein sample, increased throughput is the goal. FPLC applications often involve higher flow rates as a means to increase throughput. The increase in flow rate most often results in pressure increases therefore requiring a certain level of mechanical stability within the resin. The HIS-Select HF Resin is created from highly cross-linked agarose, providing mechanical stability and suitability for use in FPLC applications. The resin performs optimally up to a maximum linear flow rate of 3,000 cm/hr and with minimal compression at pressures up to 200 psi.

The HIS-Select HF Cartridges combine superior selectivity, mechanical stability, and high binding capacity of the HIS-Select HF Resin in a convenient prepacked format to provide truly one-step purification. The luer lock format allows coupling to most liquid chromatography systems. The cartridges were evaluated for specificity, selectivity, and pressure limits.

HIS-Select™ Nickel Chelate

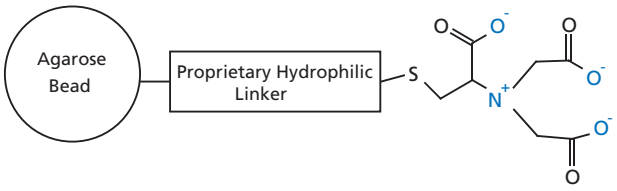


Figure 1. Hydrophilic linker and the highly pure tetradentate NTA chelate aids in the reduction of non-specific protein binding and increases the binding capacity.

- A tetradentate NTA chelate group is attached to agarose beads through a proprietary uncharged, hydrophilic spacer arm. The chelate group atoms that bind the nickel ions are shown in blue
- This technology displays less non-specific protein binding than conventional IMAC resins because the spacer arm (proprietary hydrophilic linker) is uncharged.

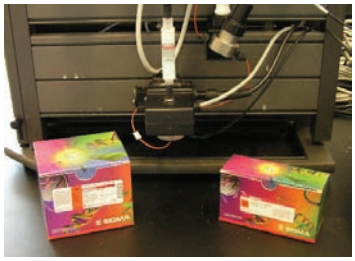
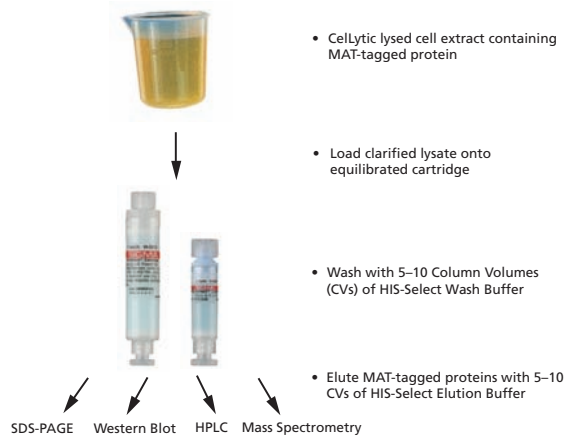


Figure 2. HIS-Select HF Cartridge coupled to a GE Healthcare ÄKTA FPLC chromatography system. Packages containing the HIS-Select HF Cartridges are depicted in the foreground.

Methods



- Glutathione-S-Transferase (GST) was cloned into an expression vector frame with a C-terminal MAT tag and then transfected into BL21 Competent *E. coli*. The MAT-Tag amino acid sequence is:

N-His-Asn-His-Arg-His-Lys-His-C

- The crude *E. coli,* containing the expressed MAT-tagged protein (GST-MAT), was lysed using CelLytic B and Benzonase. The lysate was clarified via centrifugation.
- A HIS-Select HF Cartridge was connected to the GE Healthcare ÄKTA FPLC chromatography system. The storage buffer was removed by rinsing deionized water through the cartridge. The resin was then equilibrated with HIS-Select Wash Buffer.
- The clarified lysate was applied at a flow rate of 1 mL/min. After the extract was applied, unbound proteins were removed by washing with HIS-Select Wash Buffer.
- The GST-MAT protein was eluted from the resin by addition of HIS-Select Elution Buffer.
- SDS-PAGE analysis was performed using 4–20% Tris-Glycine gels. The lysate, flow though, wash, and elution samples were diluted 1:1 in 2× Laemmli Sample Buffer and aliquots (5 µL) were added to each lane. 5 µL of the ColorBurst™ Marker was loaded undiluted. Coomassie staining was performed using EZBlue™ Gel Staining Reagent.
- After separation by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. The membrane containing the MAT-tagged protein was detected with Anti-MAT-Tag™ monoclonal antibody. Anti-Mouse HRP was employed as the secondary antibody and the membrane was developed using 3,3′,5′,5′-tetramethylbenzidine (TMB) substrate.
- In preparation for MALDI-ToF-MS analysis, a 30 mg/mL GST-MAT solution, with 8 M urea, was tryptically digested using the Trypsin Spin Column. The reduced and alkylated sample was diluted with ammonium bicarbonate and applied to the Trypsin Spin Column. The tryptic peptides were eluted with water and acidified with 10% trifluoroacetic acid (TFA).
- Tryptic peptides were desalted using a C18 pipette tip (ZipTip®). The peptides were eluted into an α-cyano-4-hydroxy cinnamic acid matrix solution (70% acetonitrile) for analysis by MALDI-ToF-MS.
- A Supelco Discovery C18 Column (15 cm x 4.6 mm, 5 micron) was used to chromatographically resolve a 3 mg/mL solution of GST-MAT (Waters Alliance 2695 Separations Module, 215 nm).

Materials

Product Name	Cat. No.
Terrific Broth EZMix™ Powder	T9179
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	I6758
Cellytic™ B Cell Lysis Reagent (10×)	C8740
Benzonase	E1014
HIS-Select™ Wash and Elution Buffer Kit	HS0100
ColorBurst™ Marker	C1992
Sample Buffer, 2× Laemmli Concentrate	S3401
EZBlue™ Gel Staining Reagent	G1041
Anti-MAT™ Monoclonal Antibody	M6693
Anti-Mouse IgG-Peroxidase antibody produced from rabbit	A9044
3,3′,5′,5′-tetramethylbenzidine (TMB) substrate	T0565
50 mg/ml Ampicillin Solution (Teknova)	A9525
HIS-Select™ HF Cartridge 1.25 mL	H7788
HIS Select™ HF Cartridge, 6.4 mL	H7163
α-cyano-4-hydroxy cinnamic acid	C8982
Supelco Discovery C18 Column (15 cm x 4.6 mm, 5 micron)	C1815
Trypsin Spin Column Kit	TT0010

Results

Resin Compression Comparison

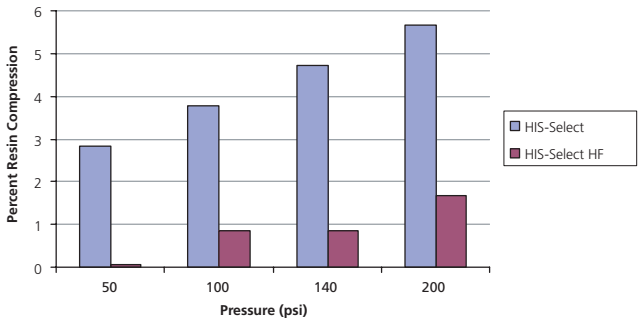


Figure 3. Compression Comparison. The HF affinity gel shares the unique nickel chelate chemistry of the HIS-Select line of products, while providing increased structural rigidity suitable for FPLC applications.

- Both resin types were evaluated by packing 1.0 mL of resin into a 1.2 mL FPLC Column (5 mm × 75 mm).
- The initial flow rate was adjusted to give a net pressure of 50 psi. Pressure and compression were monitored every 5 minutes until stabilized at 100, 140, and 200 psi.
- Columns were run using 50 mM Sodium Phosphate, 200 mM Imidazole, 0.3 M Sodium Chloride, pH 8.0.

System Pressure as a Function of Flow Rates

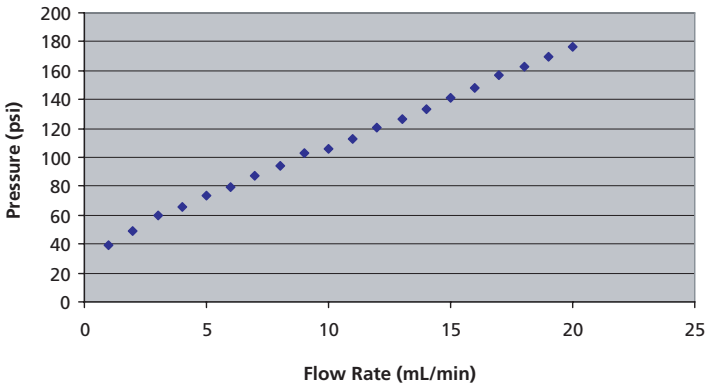


Figure 4. The HIS-Select HF Resin can withstand up to 200 psi without compaction due to the mechanical stability of the agarose.

- Pressure test occurred using the HIS-Select HF 6.4 mL Cartridge coupled to the GE Healthcare ÄKTA FPLC System.
- The resin performs optimally up to a maximum linear flow rate of 3,000 cm/hr and with minimal compression at pressures up to 200 psi.

Chromatograph of the MAT-Tag Protein Elution on an FPLC System

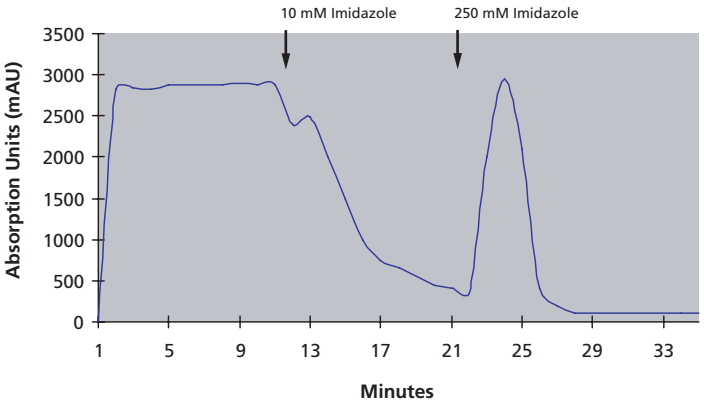


Figure 5. The captured MAT-tagged protein elutes sharply from the HIS-Select Cartridge Cat. No. H7788, generating a concentrated protein sample.

- Lysate was charged onto the column without the presence of imidazole.
- Non-specific proteins were washed from the cartridge in the presence of 10 mM imidazole.
- Target protein was eluted with the addition of 250 mM imidazole. The protein concentration from the elution samples yielded 41 mg/mL (51 mg of total purified protein) according to Bradford Protein Assay.
- The flow rate was 1 mL per minute.

SDS-PAGE and Western Blot Analysis of Captured GST-MAT

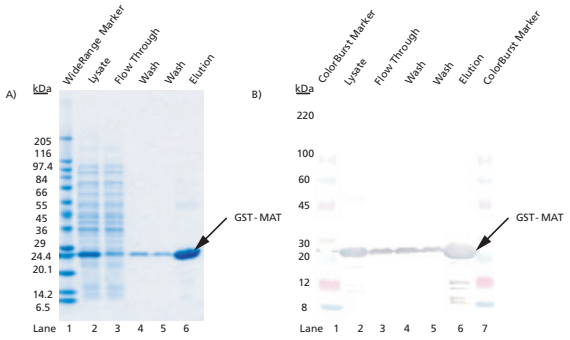
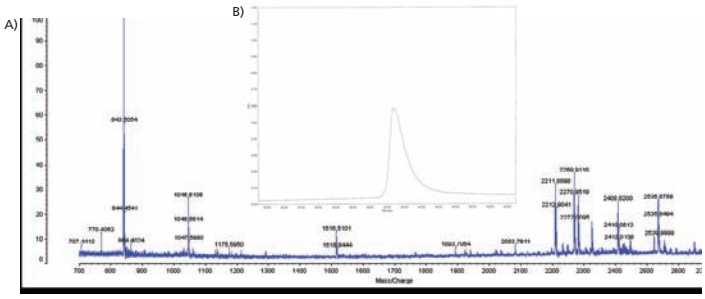


Figure 6. Both SDS-PAGE and Western Blot verify high specificity of the HIS-Select HF Affinity Gel for the MAT-Tag.

- Western Blot demonstrates that the approximately 27 kDa protein captured was GST-MAT.
- Nonspecific capture of other proteins was minimal.

Characterization of the Eluent by MALDI-ToF-MS and HPLC



C)

Observed m/z	Amino Acids (From-To)	Peptide Sequence
707.825	119–124	VDFLSK
769.899	11–17	GLVQPTR
1032.272	64–72	LTQSMAIIR
1138.202	27–34	YEEHLYER
1516.779	89–102	AEISMLEGAVDIR
2269.536	18–34	LLLEYLEEKYEEHLYER
2267.399	27–43	YEEHLYERDEGDKWRNK
2304.848	175–193	LVCFKKRIEAIPQIDKYLK
2326.599	197–217	YIAWPLQGWQATFGGGDHPPK
2357.688	44–63	KFELGLEFPNLPYYIDGDVK
2942.367	40–63	WRNKKFELGLEFPNLPYYIDGDVK
3156.455	18–41	LLLEYLEEKYEEHLYERDEGDKWR
3486.885	35–63	DEGDKWRNKKFE…PNLPYYIDGDVK
3486.885	35–63	DEGDKWRNKKFE…PNLPYYIDGDVK
3962.622	44–77	KFELGLEFPNLP…QSMAIIRYIDK
3980.468	191–224	YLKSSKYIAWPL…DHPPKHNHRHKH

Figure 7. A) MALDI-ToF-MS of digested GST-MAT. B) HPLC Chromatograph of GST-MAT. C) Peptides from GST-MAT; 58% sequence coverage obtained.

- The GST-MAT sample was reduced and alkylated and then trypsinized using a Trypsin Spin Column.
- The samples were desalted utilizing a ZipTip® pipette tip.
- MALDI-ToF-MS data was acquired in the reflectron positive mode using the Axima-CFR+ Mass Spectrometer (Shimadzu Biotech).

Conclusions

- HIS-Select HF Cartridges allow higher flow rates for rapid purification due to mechanical stability of the highly cross-linked, 6% beaded agarose.
- Nickel chelate attached to an agarose bead matrix utilizing a non-charged linkage chemistry allows superior selectivity for the histidine or MAT-tagged protein.
- The HIS-Select HF Cartridges confer high binding capacity of histidine tagged proteins (>15 mg of MAT-tagged protein/mL).
- This technology works with common liquid chromatography systems (e.g. GE Healthcare ÄKTA™).

References

- Porath, J. Immobilized Metal Ion Affinity Chromatography. *Protein Expr. Purif.* **1992**, 3, 263–281.
- Sulkowski, E., Immobilized Metal Ion Affinity Chromatography of Proteins. *In Protein Purification: Micro to Macro*. R. Burgess, (Ed.), pp.149–162 (Alan R. Liss, Inc, New York, 1987).

Acknowledgments

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