The MAT™ Tag System*: A Novel Histidine-based Metal Affinity Tag (MAT) System for Expression, Purification and Detection of Recombinant Fusion Proteins

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Abstract

Immobilized Metal Affinity Chromatography (IMAC) is a widely used method to purify recombinant proteins with histidine-containing affinity tags. It is a simple, inexpensive method for one-step purification of tagged target proteins expressed in a variety of systems. Sigma-Aldrich has developed a unique, seven amino acid metal affinity tag (MAT tag) by screening a fusion tag expression library and selecting metal binding tag sequences that had high affinity to nickel chelate affinity resin. Bacterial and mammalian vectors were developed for cloning and expressing MAT-tagged fusion proteins, which can be easily purified on HIS-Select affinity gels or plates. In addition we have produced a monoclonal antibody that can readily detect N- or C-terminal MAT-tagged proteins with both high sensitivity and specificity.

In this work we describe the development of the MAT tag and demonstrate the utility of the MAT tag technology as a complete system for expression, purification and detection of recombinant proteins. This system allows investigators additional flexibility for studying protein expression, structure, modification, function, and protein-protein interactions.

Introduction

Goal

- To develop a new, IMAC compatible fusion protein tagging and detection system.

Approach

- Utilize HIS-Select™ plates to screen for a novel small polypeptide sequence compatible with IMAC.
- Design and test an *E. coli* expression vector containing the new metal affinity tag (MAT™).
- Develop a monoclonal antibody to detect the MAT tag.
- Test expression, purification and localization of MAT-tagged fusion proteins.

Background

Short peptide tags containing histidine residues allow easy, one-step purification of tagged fusion proteins by IMAC. The HIS-Select nickel chelate IMAC affinity gels and plates were recently developed at Sigma-Aldrich. They contain a proprietary tetradentate nitriloacetic acid (NTA) analog chelate attached to the support matrix by an uncharged, hydrophilic spacer arm **(Fig. 1)**. This chemistry results in a stable, highly selective affinity capture system with low non-specific protein binding.

Our objective was to develop a novel fusion protein tagging system that would be compatible with HIS-Select IMAC affinity gels and plates.

HIS-Select™ Nickel Chelate

Figure 1. Depiction of the HIS-Select nickel chelate IMAC affinity resin structure. A quadradentate NTA chelate group is attached to agarose beads through a proprietary uncharged, hydrophilic spacer arm. This IMAC affinity resin displays less non-specific protein binding than conventional IMAC resins because the spacer arm is uncharged. The atoms of the chelate group that bind the nickel ions are shown in blue.

Screening for MAT Tag

Creation of fusion tag pool for screening for a unique metal affinity tag:

A 27 kD fusion protein coding sequence was amplified by PCR with a partially randomized C-terminal coding sequence primer containing the coding sequence for $_N$His-X-His-X-His-X-His$_C$.

The codon positions marked X were randomized to code for all 20 amino acids except histidine, proline and glutamine.

Primary Screen:

E. coli clones transformed with expression plasmid DNA containing the amplified sequences were screened for expression by staining colony lifts. About 300 clones that showed robust staining with nickel chelate-HRP conjugate were picked and screened by an ELISA assay after capture of the expressed tagged proteins in the wells of HIS-Select HS (high sensitivity) Nickel-Coated 96-Well Plates **(Fig. 2)**.

HIS-Select HS Plate Screening for MAT Tag

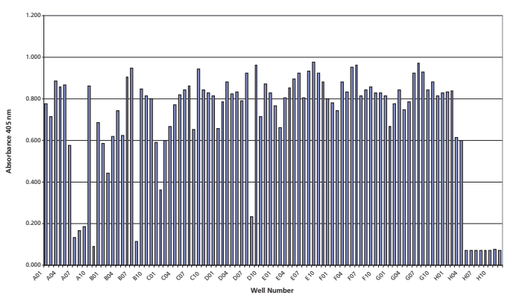

Figure 2. Clones were screened for the ability to bind the nickel chelate in HIS-Select HS Nickel-Coated 96-Well Plates. The bound protein was detected and quantitated by an ELISA assay for the fusion protein. Clones from wells showing high absorbance values were subjected to secondary screening (see below).

Secondary Screen:

Twenty-one *E. coli* clones showing high expression by the ELISA assay were screened for expression by SDS-PAGE analysis of tagged protein captured in the wells of HIS-Select HC (high capacity) Nickel-Coated 96-Well Plates **(Fig. 3)**.

HIS-Select HC Plate Screening for MAT Tag

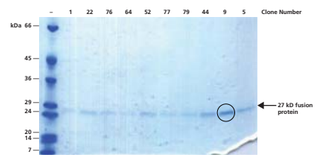

Figure 3. Individual clones showed differential binding and recovery when screened on high capacity HIS-Select plates. Twenty-one clones with the highest ELISA assay values were screened for binding and elution on HIS-Select HC Nickel-Coated 96-Well Plates. Lysates of the clones were incubated in wells with 5 mM imadazole, washed and subsequently eluted with 200 mM imidazole elution buffer. The eluates were analyzed by SDS-PAGE and visualized by EZBlue staining. Clone 9 showed the highest amount of target protein captured and eluted.

MAT Tag Sequence

The plasmid from one of the clones showing high expression in the secondary screen was partially sequenced. The **m**etal **a**ffinity **t**ag amino acid sequence encoded in that plasmid DNA, designated as the **MAT™ tag,** is shown:

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

Expression of FLAG-BAP-MAT

The coding region for bacterial alkaline phosphatase (BAP) was cloned into an expression vector to generate an N-terminal FLAG-tagged and a C-terminal MAT-tagged fusion protein.

Fusion protein expression was induced in *E. coli* and expression of the full-length fusion protein could be detected by SDS-PAGE analysis **(Fig. 4)**.

Induction of FLAG-BAP-MAT Expression in *E. coli*

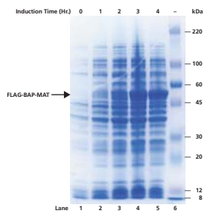

Figure 4. A dual-tagged fusion protein with a C-terminal MAT tag can be expressed in *E. coli.* The coding region for bacterial alkaline phosphatase (BAP) was cloned into an *E. coli* expression plasmid, pT7-FLAG-MAT-1, to generate an N-terminal FLAG tag and a C-terminal MAT tag. *E. coli* strain BL21 was transformed with this plasmid, grown to mid-log phase and induced with 1 mM IPTG. Samples of the culture were taken before and during induction **(lanes 1-5)** and were analyzed by SDS-PAGE. The position of the FLAG-BAP-MAT fusion protein (50 kDa) is indicated compared to ColorBurst markers **(lane 6)**.

Anti-MAT Monoclonal Antibody

The Anti-MAT monoclonal antibody was purified from ascites fluid generated by a hybridoma (MAT 1-87), which was produced by fusion of NS1 mouse myeloma cells (NS1) and splenocytes from BALB/c mice immunized with synthetic MAT peptide (HNHRHKHGGGC) conjugated to KLH via the C-terminal cysteine.

The Anti-MAT monoclonal antibody isotype is mouse IgG2a, as determined by isotyping kits listed in the "Materials" section.

Western Blot Immunostaining with Anti-MAT mAb

Anti-FLAG and Anti-MAT monoclonal antibodies specifically recognized full-length FLAG-BAP-MAT fusion protein by immunostaining of Western blots of samples from induced *E. coli* cell lysates **(Fig. 5, lanes 3 and 6)**. No protein was detected in samples of lysates of uninduced cells **(Fig. 5, lanes 2 and 5)**.

Tag-Specific Immunostaining of FLAG-BAP-MAT

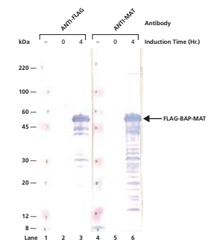

Figure 5. Full-length FLAG-BAP-MAT fusion protein is detected on Western blots by monoclonal antibodies to the FLAG and MAT tags. Lysates from uninduced **(lanes 2 and 5)** and induced **(lanes 3 and 6)** *E. coli* cultures, described in Fig. 3, and ColorBurst Markers **(lanes 1 and 4)** were separated by SDS-PAGE and blotted to nitrocellulose. The resulting blot was immunostained using either ANTI-FLAG M2-HRP conjugate **(lanes 1-3)** or Anti-MAT monoclonal antibody (0.5 µg/ml) followed by Rabbit-Anti-Mouse IgG-HRP conjugate **(lanes 4-6)**. The blots were developed and visualized with TMB Substrate.

Purification of FLAG-GrpE-MAT by IMAC

The coding region for an *E. coli* chaperone protein, GrpE, was cloned into an expression vector to generate an N-terminal FLAG-tagged and a C-terminal MAT-tagged fusion protein.

FLAG-GrpE-MAT fusion protein expression was induced in *E. coli* and the fusion protein was purified from a cell lysate on a 1 ml HIS-Select HF (high flow) Nickel Affinity Gel column. The FLAG-GrpE-MAT purification was analyzed by SDS-PAGE analysis of purification fractions **(Fig. 6)**. About 7 mg of purified FLAG-GrpE-MAT was recovered in the elution fractions.

Western blot immunostaining of purification fractions confirmed the presence of both tags in the full-length FLAG-GrpE-MAT fusion protein **(Fig. 7)**.

HIS-Select IMAC Purification of FLAG-GrpE-MAT from *E. coli*

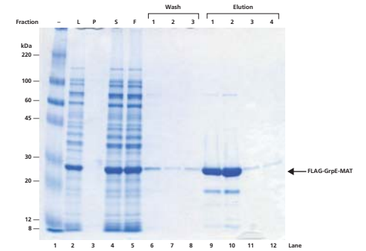

Figure 6. A MAT-tagged fusion protein expressed *E. coli* can be affinity purified by IMAC. The coding region for GrpE was cloned into an *E. coli* expression plasmid, pFLAG-MAC, with an N-terminal FLAG tag and a C-terminal MAT tag. The fusion protein was expressed in *E. coli* strain BL21. A lysate **(L, lane 2)** was made in CelLytic B and separated by centrifugation into a pellet **(P, lane 3)** and a supernatant **(S, lane 4)** fraction. The supernatant was applied to a 1.0 ml HIS-Select HF Nickel Affinity Gel column and the flow-through **(F, lane 5)**, wash **(1-3, lanes 6-8)** and elution **(1-4, lanes 9-12)** fractions were collected. Samples of the fractions and ColorBurst Markers **(lane 1)** were separated by SDS-PAGE and visualized by EZBlue staining.

Tag-Specific Immunostaining of IMAC Fractions from FLAG-GrpE-MAT Purification

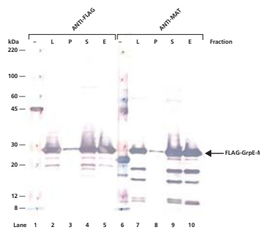

Figure 7. Full length FLAG-GrpE-MAT fusion protein is detected on Western blots by monoclonal antibodies to the FLAG and MAT tags. ColorBurst markers **(lanes 1 and 6)** and samples of the lysate **(L, lanes 2 and 7)**, pellet **(P, lanes 3 and 8)**, supernatant **(S, lanes 4 and 9)** and pooled elution **(E, lanes 5 and 10)** fractions, described in Fig. 6, were separated by SDS-PAGE and blotted to nitrocellulose. The resulting blot was immunostained using either ANTI-FLAG M2-HRP conjugate **(lanes 1-5)** or Anti-MAT monoclonal antibody (0.5 µg/ml) followed by Rabbit-Anti-Mouse IgG-HRP conjugate **(lanes 6-10)**. The blots were developed and visualized with TMB Substrate.

Anti-MAT IP of a 27kD-MAT Fusion Protein

We tested the ability of the Anti-MAT monoclonal antibody to specifically capture a MAT-tagged protein from a mammalian cell lysate. A purified 27 kD fusion protein with a C-terminal MAT tag was spiked into a COS-7 lysate and efficiently captured by immunoprecipitation (IP) with the Anti-MAT monoclonal antibody **(Fig. 8, lane 5)**.

No contaminating non-specific proteins were detected in an IP of the lysate without the spiked target protein **(Fig. 8, lane 4)**.

Immunoprecipitation (IP) of MAT-Tagged Protein with Anti-MAT Monoclonal Antibody

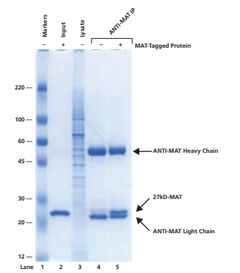

Figure 8. Anti-MAT monoclonal antibody can capture MAT-tagged target protein from a mammalian cell lysate. A purified 27 kD target protein with a C-terminal MAT-tag **(Input, lane 2)** was either spiked into a COS-7 cell lysate (10^7 cells in 1 ml RIPA buffer, **Lysate, lane 3**) at 25 µg/ml (+) or not spiked (–) and captured using Anti-MAT monoclonal antibody (50 µg/ml) and EZview Red Protein G Affinity Gel **(Anti-MAT IP, lanes 4 and 5)**. After washing the affinity gel samples, the bound proteins were eluted and analyzed by SDS-PAGE with EZBlue staining. The amount of lysate shown was only 5% of the amounts of the other samples loaded on the gel.

Immunostaining MAT-tagged Protein in Mammalian Cells

MAP kinase (MAPK) was expressed with a FLAG and a MAT tag in 293T cells. Cells were fixed, permeablized, and immunostained with Anti-MAT monoclonal antibody. The MAT-tagged protein was detected in the cytoplasm and the nuclei of transfected cells **(Fig. 9)**.

Immunostaining a MAT-tagged Protein in Mammalian Cells

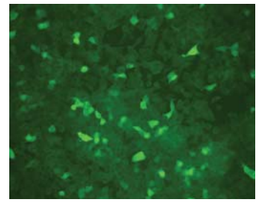

Figure 9. Anti-MAT monoclonal antibody can be used for immunostaining to detect MAT-tagged proteins expressed in mammalian cells. Adherent 293T cells were transfected with a FLAG-MAT-MAPK expression vector. After two days the cells were fixed and permeabilized with 3% paraformaldehyde and 0.5% Triton X-100. The cells were stained with 5 µg/ml Anti-MAT monoclonal antibody and developed with Anti-Mouse IgG (Fab specific)-FITC conjugate at a 1:40 dilution. Staining of the fusion protein can be seen in the cytoplasm and nuclei of the transfected cells. There was no staining of control transfected cells stained without the Anti-MAT antibody (data not shown).

Discussion

1. A novel metal affinity tag, the MAT tag, was discovered by screening a fusion tag expression library using an ELISA assay and by SDS-PAGE analysis of fusion proteins captured with HIS-Select HS and HIS-Select HC Nickel-Coated 96-Well Plates.
2. Vectors were designed for the expression of MAT-tagged fusion proteins in *E. coli.* Expression of MAT-tagged fusion proteins was demonstrated by SDS-PAGE analysis and confirmed by Western blot immunostaining (see below).
3. An Anti-MAT mouse monoclonal antibody (clone 1-87) was developed. The Anti-MAT antibody can specifically detect MAT-tagged fusion proteins on Western blots. The Anti-MAT antibody was also used to capture MAT-tagged target protein efficiently and specifically from a mammalian cell lysate by immunoprecipitation. It can also be used for immunostaining MAT-tagged proteins in cells.
4. We have successfully used the MAT tag system and the anti-MAT monoclonal antibody to express, purify and localize MAT-tagged fusion proteins.

Conclusion

We have developed the MAT tag, a new metal affinity tag, along with MAT-fusion protein expression vectors, and the Anti-MAT monoclonal antibody as a complete protein expression system. We have demonstrated its utility for expression, purification and detection of recombinant proteins.

The novel MAT tag system allows investigators additional flexibility for studying protein expression, structure, modification, function and protein-protein interactions.

Acknowledgements

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References

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Materials

Product Name	Product Number
HIS-Select™ HS Nickel-Coated 96-Well Plates	S 5688
HIS-Select™ HC Nickel-Coated 96-Well Plates	S 5563
HIS-Select™ HF Nickel Affinity Gel	H 0537
EZview™ Red Protein G Affinity Gel	E 3403
CelLytic™ B Cell Lysis Reagent	B 3553
pT7-FLAG-MAT™-1 Expression Vector	E 5280
pFLAG-MAC™ Expression Vector	E 5644
ANTI-FLAG® M2 mAb-HRP Conjugate	A 8592
Anti-MAT™ Monoclonal Antibody	M 6693
Rabbit Anti-Mouse IgG-HRP Conjugate	A 9044
Anti-Mouse IgG (Fab specific)-FTIC Conjugate	F 5262
Sigma ImmunoType™ Kit	ISO-1
Mouse Monoclonal Antibody Isotyping Reagents	ISO-2
ColorBurst™ Marker	C 4105
EZBlue™ Gel Staining Reagent	G 1041
3,3',5,5' Tetramethylbenzidine (TMB) Liquid Substrate for Membranes	T 0565

All other reagents were obtained from Sigma-Aldrich.

***Patent Pending**