

# Application Note

## HIS-Select™ Affinity Gels and Plates for Affinity Capture of Histidine-Containing Proteins

By Ned Watson, Patricia Lindbloom\*, Kelly Foster, Jodi Zobrist, Michael Scott, Rick Mehig and John Dapron  
Sigma-Aldrich Corporation, St. Louis, MO and  
Purdue University\*, West Lafayette, IN, USA

### Introduction

Affinity-based protein capture methods have become standard techniques for isolation and purification of proteins.<sup>1,2</sup> The development of epitope tagging systems for recombinant proteins and small scale, affinity-based molecular pull-down methods, such as immunoprecipitation, have enabled more rapid and detailed study of expression, modification and interaction of proteins in a wide variety of biological systems.<sup>3,4</sup> One of the most popular and widely used tagging systems involves relatively short peptide epitope tags containing histidine residues. The histidine-containing tags allow easy, one-step purification of the tagged fusion protein by immobilized metal affinity chromatography (IMAC).

IMAC has been a versatile purification tool since first introduced by Porath et al. in 1975.<sup>5,6</sup> The process is based on the potential for protein/peptide amino acid side chains to form coordination bonds with immobilized metals. The amino acid side chains generally will contain oxygen, nitrogen and/or sulfur. Cysteine and histidine residues are the primary points of interaction on proteins. It has been demonstrated that proteins containing clustered histidine residues will interact strongly and with high selectivity for chelated nickel.<sup>6</sup>

The HIS-Select™ nickel chelate is a proprietary tetradentate nitriloacetic acid (NTA) analog chelate and spacer developed at Sigma-Aldrich. This chelate provides strong coordination with nickel while still presenting two nickel coordination sites for efficient complexing with recombinant fusion proteins containing histidine (Figure 1). In addition, the chelate contains an integral, neutral spacer providing for coupling to various matrices by stable uncharged bonding. This chelate chemistry is common throughout the HIS-Select line of products and ensures high selectivity and stable, reproducible performance. In addition, the uncharged spacer results in lower non-specific protein binding than typical IMAC affinity capture products. This new, high performance chelate is used for all the new IMAC affinity products discussed here.

### Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO), unless otherwise stated.

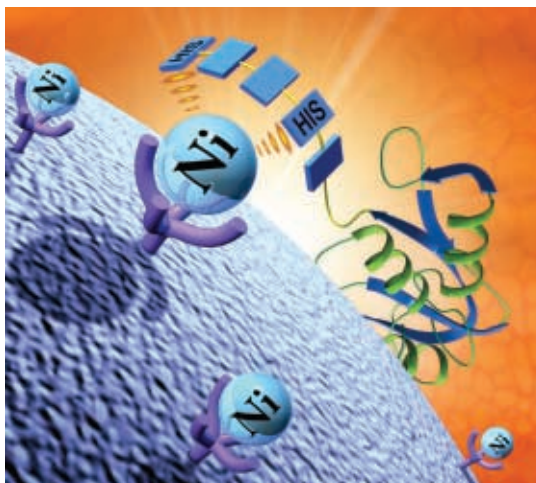


Figure 1. Model of IMAC purification of histidine-tagged proteins on HIS-Select HC Nickel Affinity Gel.

### HIS-Select HC (High Capacity) Nickel Affinity Gel purification of a histidine-tagged protein from *Escherichia coli*

A recombinant clone expressing bacterial alkaline phosphatase protein (BAP) with an N-terminal histidine-containing tag (HAT™ tag, Clontech, Palo Alto, CA; sequence on p. 10) was grown to early log phase with shaking at 37 °C in 50 ml of EZMix™ Terrific Broth (Product Code T 9179). The cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside [IPTG] (Product Code I 6758) and allowed to grow for an additional 5 hours. The cells were harvested by centrifugation at 4 °C at 5,000 x g. The clear supernatant was removed and the cell paste was frozen at -20 °C. About 1.2 g of cell paste of the recombinant *Escherichia coli* expressing the N-terminal HAT-tagged BAP (HAT-BAP) was extracted in 6 ml of 50 mM sodium phosphate, 0.3 M sodium chloride, pH 8.0. The cells were disrupted by sonication for 2 minutes on ice. The extract was clarified by centrifugation for 15 minutes at 20,000 x g at 4 °C. The clear supernatant was recovered and loaded on a 0.5 ml column of HIS-Select HC Nickel Affinity Gel (Product Code P 6611) and allowed to flow through by gravity. The column was washed with 10 ml of equilibration buffer (50 mM sodium phosphate, 0.3 M sodium chloride, 10 mM imidazole, pH 8.0). The column was then eluted with 1.5 ml of 50 mM sodium phosphate, 0.3 M sodium chloride, 200 mM imidazole, pH 8.0. The lysate and column fractions were analyzed on a precast 4-20% tris-glycine gel (Invitrogen, Carlsbad, CA). The proteins were visualized by staining for one hour with EZBlue™ Gel Staining Reagent (Product Code G 1041), followed by destaining overnight with deionized water. Protein concentrations were determined by the Bradford protein assay (Product Code B 6916).

### Preparation of mammalian cell lysates

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Product Code D 6171) containing 10% Fetal Bovine Serum (FBS; Product Code F 2442) in 100 mm diameter tissue culture plates (Product Code C 6546). When the cells were confluent ( $10^7$  cells/plate), the plates were set on ice, the medium was removed, and the adherent cells were washed twice with 10 ml of ice cold Dulbecco's phosphate buffered saline (Product Code D 8537). The cells were lysed directly by adding 1 ml ice cold RIPA lysis buffer [150 mM NaCl, 1.0% Igepal CA-630 (NP-40; Product Code I 3021), 0.5% sodium deoxycholate (Product Code D 6750), 0.1% sodium dodecyl sulfate (SDS; Product Code L 6026), 50 mM Tris, pH 8.0] to each plate and scraping rapidly with a cell scraper. Mammalian Protease Inhibitor Cocktail (100  $\mu$ l; Product Code P 8340) was added to each lysate and the lysates were used immediately or quick-frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until needed.

### Small-scale affinity capture with EZview™ Red HIS-Select HC Nickel Affinity Gel

COS-7 lysates were clarified by centrifugation in a microcentrifuge for 10 minutes at  $8,000 \times g$  at  $4^\circ\text{C}$ . The clear supernatants were recovered and, subsequently, purified HAT-BAP protein (5  $\mu\text{g}/\text{ml}$  lysate) was added in varying amounts. The resulting spiked lysates were incubated with 50  $\mu$ l packed gel volume of standard HIS-Select HC Nickel Affinity Gel or EZview Red HIS-Select HC Nickel Affinity Gel (pre-washed and equilibrated in RIPA buffer; Product Code E 3528) for 1 hour with mixing at  $4^\circ\text{C}$ . The affinity beads were collected by centrifugation for 30 seconds at  $8,000 \times g$  and the supernatants were removed by aspiration. The pellets were washed three times with 1 ml of RIPA buffer per wash and collected by centrifugation as above. After aspirating the final wash supernatants, the affinity bead pellets were each suspended in 25  $\mu$ l of RIPA buffer and 25  $\mu$ l of 2X Laemmli sample buffer (Product Code S 3401) and analyzed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as indicated.

### Affinity capture with HIS-Select HS (High Sensitivity) Nickel-Coated 96-well plate

One gram of *Escherichia coli* cell paste was suspended in 10 ml 50 mM sodium phosphate, 0.3 M sodium chloride, pH 8.0, and lysed by sonication. The lysate was clarified by centrifugation at  $10,000 \times g$  at  $4^\circ\text{C}$  and the clear supernatant was collected. Purified HAT-BAP fusion protein was spiked into the lysate supernatant at various concentrations as indicated. A volume of 200  $\mu$ l of spiked lysate containing a total of 0.25 ng to 32 ng HAT-BAP was added per well in quadruplicate wells of a HIS-Select HS Nickel-Coated 96-well Plate or competitor's plate (Product Code S 5688) and incubated for 3 hours at room temperature. The liquid was removed from the wells by aspiration and each well was washed three times with 300  $\mu$ l Phosphate Buffered Saline containing 0.05% Tween 20 (PBST; Product Code P 3563) to remove unbound material. After aspirating the final wash, the amount of HAT-BAP protein captured per well was detected directly by an enzyme-linked immunoassay. Each ELISA well was incubated for 45 minutes at room temperature with a 1:10,000 dilution of anti-BAP monoclonal antibody (Product Code B 6804). Subsequently, each well was washed three times with 300  $\mu$ l PBST. After the last wash was removed, each well was incubated for 45 minutes

at room temperature with anti-Mouse IgG HRP conjugate (Product Code A 4416). The secondary antibody solution was removed and each well was washed four times as above. Each well was then assayed by incubating with 200  $\mu$ l of TMB substrate (Product Code T 8665) for 40 minutes at room temperature. The reactions were stopped by adding 100  $\mu$ l of 1 M sulfuric acid per well. After mixing, the wells were read at  $A_{450}$  and each set of quadruplicate well values was averaged and corrected for background absorbance in negative control wells.

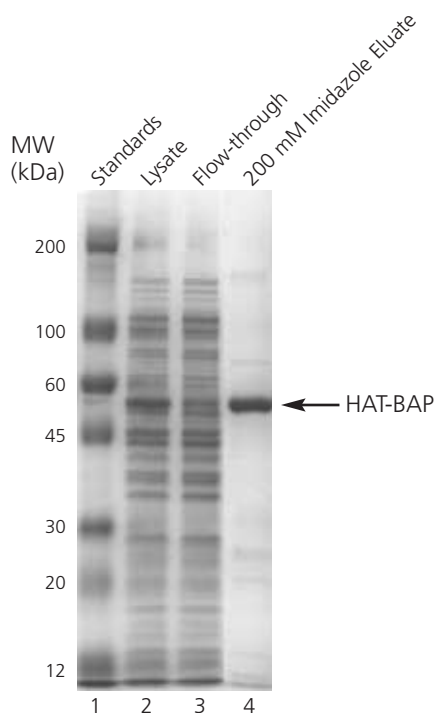
### Affinity capture with HIS-Select HC Nickel Coated 96-well plate

For binding capacity comparisons, an excess of purified HAT-BAP (30  $\mu\text{g}$ ) in 200  $\mu$ l TBS was incubated in triplicate wells of a HIS-Select HC Nickel Coated 96-well Plate (Product Code S 5563) or competitor IMAC affinity plates for 4 hours at room temperature. The samples were removed from the wells by aspiration and each well was washed three times with 300  $\mu$ l Phosphate Buffered Saline containing 0.05% Tween 20 and one time with deionized water to remove residual detergent. After aspirating the final wash, the total protein captured per well was determined directly in the wells by Bicinchoninic Acid protein assay (Product Code BCA-1) and the triplicate well values were averaged.

## Results and Discussion

IMAC has become a popular and powerful means of rapidly purifying recombinant proteins with short poly amino acid tags containing histidine residues. Many current IMAC affinity capture matrices contain positively charged spacer arms that attach the metal chelate affinity group to the support matrix. Although such IMAC affinity capture matrices are widely used, problems with non-specific protein binding can occur due to the nature of the matrix and the charge on the spacer arm. Therefore, we developed a novel, proprietary tetradentate nitriloacetic acid (NTA) analog chelate with an uncharged spacer arm in order to produce high performance IMAC affinity matrices, which are efficient for specifically capturing histidine-tagged proteins with low non-specific protein binding. This improved nickel chelate was used to make both resin and plate-based capture systems for IMAC purification of histidine-tagged recombinant proteins.

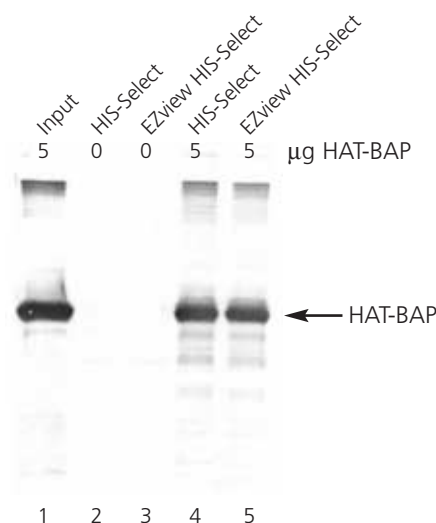
We developed a high capacity agarose-based nickel chelate affinity resin, HIS-Select HC Nickel Affinity Gel, and demonstrated its performance for IMAC purification of a HAT-tagged recombinant protein from *Escherichia coli*. The HAT tag is a 19 amino acid tag, which contains 6 histidine residues interspersed with other amino acids. It is originally derived from a natural protein sequence for chicken lactate dehydrogenase that was known to bind a metal chelate. Recombinant HAT-BAP was expressed in *Escherichia coli* and purified in one step on a small HIS-Select HC Nickel Affinity Gel column. The purified, bound HAT-BAP protein was eluted in the presence of 200 mM imidazole (Figure 2). Analysis of the column fractionation indicated that most of the HAT-BAP protein from the lysate bound the column (compare Figure 2, lanes 2 and 3). The purified, eluted HAT-BAP protein was greater than 95% pure after this one-step purification with little non-specific protein binding observed (Figure 2, lane 4).



**Figure 2. HIS-Select™ HC Nickel Affinity Gel purification of a histidine-tagged fusion protein expressed in *Escherichia coli*.** Cells containing a plasmid encoding a HAT-tagged bacterial alkaline phosphatase (HAT-BAP) fusion protein were grown, induced and lysed as described in Materials and Methods. Fractions were taken during purification of the HAT-BAP fusion protein on a HIS-Select HC Nickel Affinity Gel column and were analyzed by SDS-PAGE followed by colloidal blue staining (EZBlue™ Gel Staining Reagent). Lane 1 contains ColorBurst™ Electrophoresis Marker (Product Code: C 4105) protein gel standards. Purification fraction samples were loaded on the gel as follows: 29 µg of lysate in lane 2; 21 µg of flow-through fraction in lane 3; 3 µg of 200 mM imidazole eluate in lane 4.

Often small-scale affinity capture methods, such as immunoprecipitation, are used to analyze protein expression, post-translational modifications, or protein-protein interactions. A major disadvantage of these small scale affinity capture procedures, as commonly practiced, is that the affinity matrix is difficult to see in microcentrifuge tubes that are used for the complex formation, purification and wash steps. This difficulty in visualization leads to inefficient and tedious manipulations, and often results in loss of material and quantitative variability of results. Therefore, we developed unique, highly visible red colored HIS-Select affinity beads, EZview™ Red HIS-Select Affinity Gel (Product Code E 3528), to be used in small-scale affinity capture of histidine-tagged target proteins.

To compare EZview Red HIS-Select HC Nickel Affinity Gel with standard HIS-Select HC Nickel Affinity Gel for recovery of the HAT-BAP protein from mammalian cell lysates, we spiked purified HAT-BAP into COS-7 cell lysates and performed small-scale affinity capture with both affinity resins. After binding and washing steps, the captured target protein was eluted from the affinity beads subjected to SDS-PAGE and analyzed by immunoblotting. The increased visibility of the EZview resin made washing and supernatant removal manipulations faster and easier during the experiment than with the standard HIS-Select resin. Immunostaining of the Western blots revealed that a similar amount of HAT-BAP protein was recovered from COS-7 lysates with both the standard HIS-Select and the EZview Red HIS-Select HC Nickel Affinity Gel (Figure 3, compare lanes 4 and 5). In addition, no differences in non-specific background protein binding were observed (Figure 3, compare lanes 2 and 3).

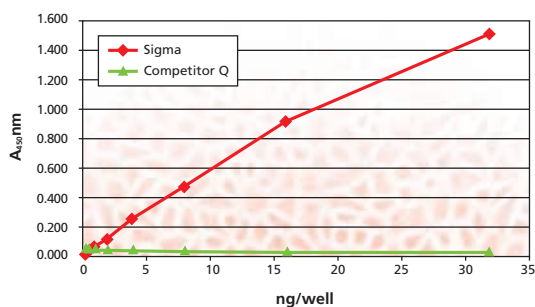


**Figure 3. Affinity capture of HAT-BAP fusion protein from COS-7 cell lysates using HIS-Select and EZview HIS-Select HC Nickel Affinity Gels.** Purified HAT-BAP fusion protein (lane 1) was spiked into COS-7 lysates ( $10^7$  cells in 1 ml RIPA buffer) at 5 µg/ml (lanes 4 and 5) and affinity captured with standard HIS-Select HC Nickel Affinity Gel (lane 4) or EZview™ HIS-Select HC Nickel Affinity Gel (lane 5; Product Code E 3528). Lanes 2 and 3 did not have HAT-BAP spiked in the lysates. The blot shown was probed with anti-HAT antibody followed by goat anti-rabbit alkaline phosphatase conjugate secondary antibody (Product Code: A 3687) and visualized by BCIP/NBT substrate (Product Code: B 1911).

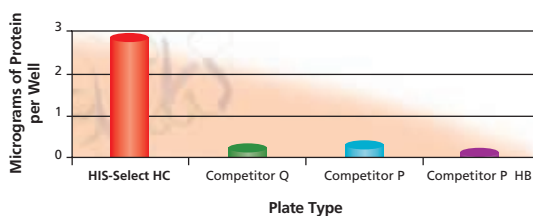
For parallel processing of numerous samples or for other high-throughput applications such as high-throughput protein capture or screening assays, we employed the same unique nickel chelate to make multi-well HIS-Select capture plates. We developed a high sensitivity plate, HIS-Select HS Nickel 96-well plate (Product Code: S 5688), for capture and low level detection of histidine-tagged proteins. The performance of this high sensitivity IMAC plate was compared to a competitor's IMAC affinity plate for the capture of a histidine-tagged target protein, HAT-BAP, from a cell lysate. The HIS-Select HS Nickel 96-well plate captured and allowed detection of spiked HAT-BAP protein from the lysate over a concentration range of 1 to 32 ng/well, as measured in a standard enzyme-linked immunoassay (Figure 4). No protein was detected in this range in the same assay using a competitor's IMAC plate. The lower limit of detection for the HIS-Select HS Nickel 96-well plate was less than 1 ng/well using this standard enzyme-linked assay. Therefore, this plate is an excellent platform for capture and detection of low levels of recombinant fusion proteins with histidine-containing tags.

In addition, novel HIS-Select HC Nickel multiwell plates (96-well plate, Product Code S 5563; 384-well plate, Product Code H 1661) were developed, which have a high capacity for rapid, simultaneous IMAC purification of histidine-containing proteins. The wells of these plates are coated with a proprietary high-density, large pore size, nickel chelate matrix, which provides a much higher capacity for binding proteins with histidine-containing tags. This allows rapid affinity purification of histidine-containing proteins from numerous samples in sufficient quantity per well (typically > 4 µg per well) for analysis by standard biochemical methods. These methods include direct quantitation of captured protein by standard protein assays, such as Bradford or BCA assays, and rapid analysis of the captured histidine-containing protein, or protein complexes, by SDS-PAGE, Western immunoblotting and/or mass spectrometry. The binding capacity of the HIS-Select HC Nickel 96-well plates was compared to plates of competitors using an excess of

purified HAT-BAP protein. Even though the HAT tag is a relatively weak nickel-binding tag, nearly 3 µg HAT-BAP was bound per well for the HIS-Select HC Nickel 96-well plate (Figure 5). The amount of HAT-BAP bound to the wells of the HIS-Select HC Nickel 96-well plate was much greater than the amount bound to wells of the competitor plates, which was at the lower limit of detection. These high capacity IMAC affinity plates are extremely useful for isolation and characterization of histidine-tagged proteins and for parallel purification of large numbers of histidine-tagged proteins for applications such as biochemical and structure/function analysis of multiple deletion and point mutant protein variants.



**Figure 4.** HIS-Select HS Nickel Coated 96-well plate comparison with a competitor's plate for capture of HAT-BAP target protein from cell lysates.



**Figure 5.** Higher binding capacity with HIS-Select HC 96-well plates. An excess (30 µg) of a purified HAT-BAP was loaded into wells of various 96-well plates. After washing, the bound protein in the wells was assayed directly by a BCA protein assay. The average per-well binding capacity was calculated for each plate. HC = High Capacity; HB = High Binding.

#### About the Authors

Ned Watson, Ph.D. and Kelly Foster, B.S. are Senior Scientists; Jodi Zobrist, M.S. and Michael D. Scott, M.S. are Scientists; and Rick Mehigh, Ph.D. and John Dapron, B.S. are Principal Scientists in the Biotechnology R&D Department at Sigma-Aldrich, St. Louis, MO. Patricia Lindbloom is a Sigma Undergraduate Co-op student from the Biology Department at Purdue University, West Lafayette, IN.

## ORDERING INFORMATION

Product Code	Product Description	Unit
P 6611	HIS-Select™ HC Nickel Affinity Gel*	5 ml 25 ml
E 3528	EZview™ Red HIS-Select™ HC Nickel Affinity Gel*	1 ml 5 x 1 ml
S 5688	HIS-Select™ HS Nickel 96-Well Strip Plate*	1 plate 5 plates
S 5563	HIS-Select™ HC Nickel 96-Well Plate*	1 plate 5 plates
H 1661	HIS-Select™ HC Nickel 384-Well Plate*	1 plate 5 plates

\* Patent Pending

Amino acid sequence of the HAT tag

Lys-Asp-His-Leu-Ile-His-Asn-Val-His-Lys-Glu-Glu-His-Ala-His-Ala-His-Asn-Lys

## Conclusions

We developed a unique, stable tetradentate nickel chelate that utilizes a non-charged hydrophilic spacer to reduce non-specific protein binding. We have attached this proprietary chelate to both resin and multi-well plates to provide improved platforms for the purification and analysis of proteins containing histidine tags. The new IMAC affinity matrices are a significant improvement over previous IMAC affinity resins and plates and should be more useful for the capture and analysis of histidine-tagged proteins for a number of different applications in the future.

## Acknowledgements

The authors would like to thank Dr. Bill Kappel of the Protein R&D group at Sigma-Aldrich for helpful discussions and critical reading of the manuscript.

## References

- Scouten, W. H., Affinity Chromatography: Bioselective Absorption on Inert Matrices. John Wiley and Sons, Inc., New York (1981).
- Street, G. (Ed.), Highly Selective Separations in Biotechnology. Chapman and Hall, London (1994).
- Kolodziej, P. A. and Young, R. A., Epitope tagging and protein surveillance. Methods Enzymol. **194**, 508-519 (1991).
- Harlow, E. and Lane, D., Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, p. 423-470 (1988).
- Porath, J., et al., Metal chelate affinity chromatography, a new approach to protein fractionation. Nature, **258**, 598-599 (1975).
- Sulkowski, E., Purification of proteins by IMAC. Trends in Biotechnology, **3**, 1-12 (1985).