



NTA vs. IDA: a tale of two ligands

Immobilized metal affinity chromatography (IMAC) is a popular method for protein purification, particularly for recombinant proteins fused to a polyhistidine tag. Transition metal ions immobilized to a matrix through a chelating ligand interact with the polyhistidine tag, effectively sequestering it from a sample. Nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA) are two such ligands commonly used in commercially available resins. In this application note we summarize our knowledge, from publications and in-house experiments, regarding the differences, advantages, and disadvantages of the two ligands. We present a few basic principles that should underly the purchase of a resin to optimize purification results.

Using metals to capture proteins

Immobilized metal affinity chromatography dominates generic single-step protein purification

Since its inception in 1975⁽¹⁾, IMAC or immobilized metal affinity chromatography with its variations and improvements, has been widely used to purify proteins. In its most popular form, a transition metal ion fixed to a matrix via a ligand, interacts with the imidazole rings of a polyhistidine tag (a chain of 3 to 10 histidines) fused to the C- or N-terminus of a peptide. The interaction effectively „sequesters“ the protein from solution, and the bound protein is then dissociated from the metal ion by elution with an imidazole gradient, changes in pH, or metal chelation⁽²⁾.

Initially developed for native proteins with an intrinsic affinity to metal ions, the application of IMAC has expanded to include phosphorylated proteins, antibodies, and a long list of proteins outfitted with a polyhistidine tag. Especially advantageous is the small size and low immunogenicity of

the polyhistidine tag, which generally does not interfere with protein function⁽²⁾. Trivalent and tetravalent ions are commonly used for purification of phosphoproteins, while divalent ions such as Ni^{2+} , Cu^{2+} and Zn^{2+} are used for recombinant his-tagged proteins.

Low cost and functional simplicity account for much of its popularity but IMAC is also very robust, making this purification system ideal for methods that call for protein-specific conditions. For example, the imidazole-metal ion interaction can take place under native and denaturing conditions, as well as under oxidizing or reducing conditions. Furthermore, IMAC is compatible with a broad spectrum of chemicals⁽¹⁾. Finally, the high affinity, specificity and scalability of the system enables very efficient purification of proteins even from crude lysates⁽¹⁾.

More than protein purification

IMAC is a versatile technology with surprising applications. Immobilizing his-tagged antigens to surfaces enhances the sensitivity of ELISA-based diagnostic tools, and protein-protein interaction studies on functionalized surfaces can be mediated by IMAC. In proteomic studies, IMAC is used for pre-separation steps to reduce the complexity of study samples. Finally, in the Chelex method, samples are incubated with a matrix coupled to an unloaded chelating ligand. The ligand depletes metal ion inhibitors of PCR thereby quickly generating a sample ready for amplification.

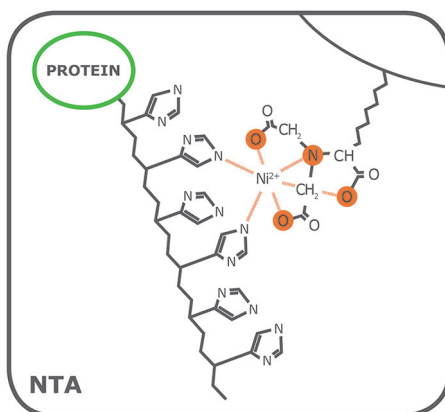
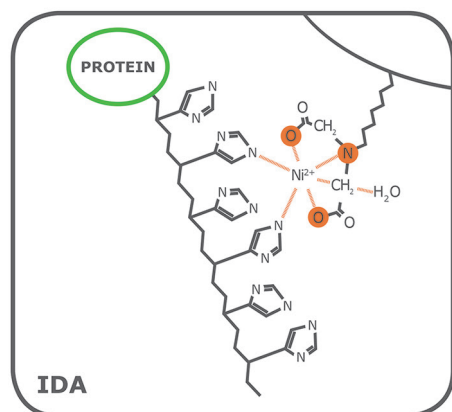
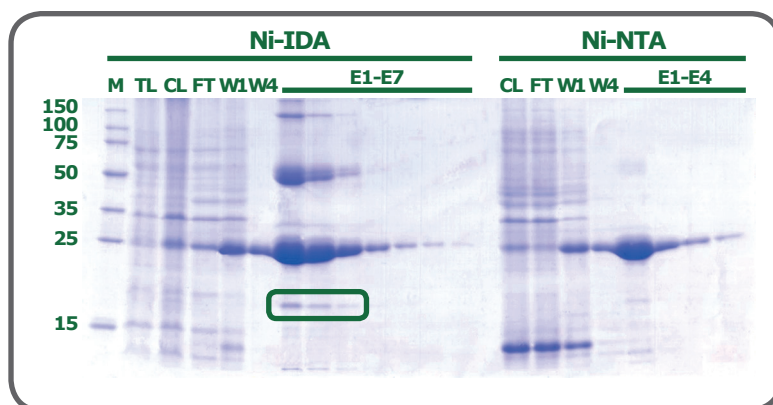


Fig. 1: IMAC chelating ligands IDA and NTA. Iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) are widely used to fix transition metal ions to a matrix, which then serves as an affinity resin. The two ligands differ in the number of valencies with which they coordinate the metal ion (IDA is trivalent, whereas NTA is tetravalent; valencies highlighted in orange), which impacts the quality of the purified protein fraction.

Fig. 2: Higher yield and unspecific binding of GFP purified with Ni-IDA resin compared to Ni-NTA resin. His-tagged recombinant eGFP was expressed in BL2(DE3) competent *E. coli* cells and purified on gravity columns packed with PureCube Ni-IDA or PureCube Ni-NTA agarose. Elution was done with 250 mM imidazole and GFP concentration in the elution fractions was determined spectrophotometrically (488 nm). GFP yield (band at 27 kDa) is visibly higher in the Ni-IDA elution fractions compared to Ni-NTA. In fact, the high concentration led to aggregation giving dimers and tetramers (bands at ~50 and 100 kDa). Additionally, unwanted proteins are present in the elution (boxed at ~20 kDa). The elution fractions collected from the NTA column contain less protein but exhibit significantly reduced unspecific binding. **M:** marker; **TL:** total lysate; **CL:** cleared lysate; **FT:** flow-through; **W1, W4:** wash fractions; **E1-E7:** elution fractions.



Choosing a chelating ligand: which tale to use?

Coordination number of the ligand has an effect on yield and purity of the eluted protein

NTA

- Ⓢ Tetravalent, coordination number 4
- Ⓢ Higher binding specificity
- Ⓢ Generally, lower yield
- Ⓢ Stronger coordination of metal ions
- Ⓢ Lower metal ion leaching

IDA

- Ⓢ Trivalent, coordination number 3
- Ⓢ Generally, more unspecific binding
- Ⓢ Increased metal ion leaching
- Ⓢ Higher metal loading density
- Ⓢ Requires lower eluent imidazole concentration

Porath and colleagues⁽³⁾ developed the first embodiment of an IMAC resin by fixing metal ions to agarose with iminodiacetic acid (IDA). This ligand is still widely used in commercial IMAC resins, along with nitrilotriacetic acid (NTA), first introduced in 1987 by Hochuli and colleagues⁽⁴⁾. Since then, other proprietary chelating ligands have been developed (e.g., TED, TALON[®]), but IDA and NTA represent the most commonly used IMAC resins for affinity purification of proteins.

Structurally, IDA and NTA differ by the presence of an additional carboxymethyl group on NTA. Chemically, the additional functional group makes NTA a stronger coordinator of metal ions because it has 4 valencies available compared to the 3 of IDA (Fig. 1).

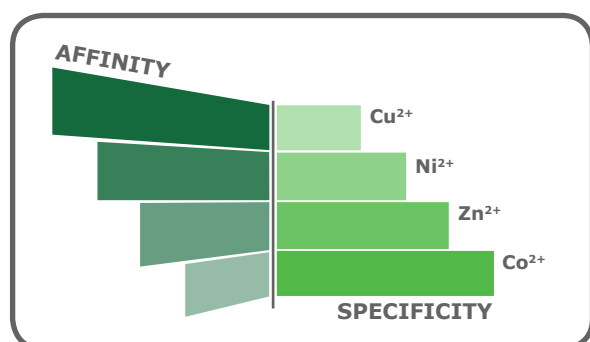
While both ligands have 2 valencies available to interact with the imidazole rings of histidine residues, the coordination number plays a role in the quality of the resulting purified fraction. To begin, as a

trivalent ligand (coordination number 3), IDA exhibits higher leaching of metal ions, particularly in the equilibration and wash steps⁽¹⁾. Elution fractions from NTA-based resins also contain leached metal ions, but substantially less than observed from IDA⁽¹⁾.

Secondly, while some variation is attributable to the nature of the protein being processed, a his-tagged protein eluted from an IDA resin frequently has lower purity compared to that obtained from an NTA resin. Figure 2 shows the SDS-PAGE of eGFP expressed in *E. coli* cells, and purified on PureCube Ni-IDA and PureCube Ni-NTA resins. The first three elution fractions from the IDA resin show distinct bands for lysate protein components other than the target.

However, IDA has also advantages. Resins constructed with this ligand are generally less expensive. Additionally, the tridentate ligand requires a lower imidazole concentration to elute protein than tetradentate NTA. Lastly, IDA is a smaller molecule which can be coupled to the matrix at a higher density resulting in a higher metal loading capacity. Generally, low loading density enhances the quality of the purification but results in lower target protein recovery, whereas high loading density generates greater yields but with higher unspecific binding. By combining low loading density of IDA or NTA with the affinity and specificity of different metal ions (Fig. 3), it is possible to optimize the purity and yield of purification for a particular protein.

Fig. 3: Correlated affinity and specificity of metal ions commonly used in IMAC. At one end of the spectrum, copper has a high affinity generating higher protein recovery, but low specificity. Cobalt, at the other end, has low affinity but highly specific binding, reducing the presence of unwanted proteins in the eluate.



Ligand	IL-1 β -His	JNK1-His	GFP-His
Ni-IDA	21 mg/mL	100 mg/mL	100 mg/mL
Ni-NTA	22 mg/mL	61 mg/mL	80 mg/mL
Co-NTA	15 mg/mL	30 mg/mL	19 mg/mL

Table 1: Binding capacity of an IMAC resin is strongly dependent on the metal ion and on the nature of the protein being purified. Standard quality control tests of the PureCube His Affinity resins are carried out with different proteins. Proteins like GFP and JNK1 tend to give robust yields, whereas other proteins like interleukin 1 β result in smaller recoveries. However, the ion loaded to the resin also impacts binding capacity. First, cobalt has a higher binding specificity for proteins than nickel, leading to overall lower recovery. Second, cobalt interacts better with JNK1 than with GFP, whereas the opposite is true for nickel.

Let your protein determine your ligand and metal ion

When asked, most researchers cannot explain why they use one ligand over another for their his-tagged protein purification. However, it may be advantageous to explore different ligands because binding capacity of a given IMAC resin is very dependent on the ion metal used and the nature of the protein being purified. A more optimized purification might be achieved with an alternate ligand-metal combination. For example, typically IDA has a higher metal ion capacity than NTA (>25 $\mu\text{mol Ni}^{2+}$ or Cu^{2+} per mL resin for PureCube IDA Agarose and >15 $\mu\text{mol/mL}$ for PureCube NTA Agarose). Theoretically, IDA avails more binding sites for a target protein resulting in greater capacity, as apparent in Table 1 with JNK1 and GFP.

However, binding capacity varies from one protein to another and can be significantly lower, such as with interleukin-1 β .

A further consideration is that coupling different metal ions to a resin leads to different affinities for a given his-tagged protein. Cobalt exhibits high binding specificity but low affinity (Fig. 3), and thus we see decreased yields with cobalt-based resins (Table 1). Nickel trades off specificity for higher affinity, which accounts for the observed higher recoveries. One can optimize the purification of a his-tagged protein by exploring different metal ion and ligand combinations. It is important to bear in mind, however, that the described trends do not hold across the board for all resins or all proteins.

What else is in your sample? It can affect the bottomline.

The presence of specific compounds in study samples impacts the binding capacity of both IDA and NTA-based resins. DTT is a reducing agent used to stabilize proteins with free sulfhydryl groups and disrupt disulfid bonds in preparation for SDS-PAGE. DTT reduces the metal ions of an IMAC resin, often changing the resin color to brown. In a comparative study, PureCube Ni-IDA Agarose and PureCube Ni-NTA Agarose were exposed to different concentrations of DTT. Binding capacity of both resins dropped non-linearly, but Ni-NTA showed a shallower average decay rate of 8.0% with each increase in DTT concentration, compared to 11.1% for Ni-IDA. The overall decrease in binding capacity at 10 mM DTT was 22% and 30% for Ni-NTA and Ni-IDA, respectively (Fig. 4A).

EDTA has a more dramatic impact on binding capacity. This hexadentate chelating agent is found in many buffers and sequesters metal ions. Our comparative study shows that Ni-NTA is more robust than Ni-IDA at higher concentrations of EDTA. Ni-NTA had a non-linear overall drop in binding capacity of 46%, with slightly steeper decay after 1 mM EDTA. Up to 1 mM EDTA, the binding capacity of Ni-IDA decays similar to that of Ni-NTA. Thereafter, the binding capacity drops dramatically (overall decrease: 65%; Fig. 4B).

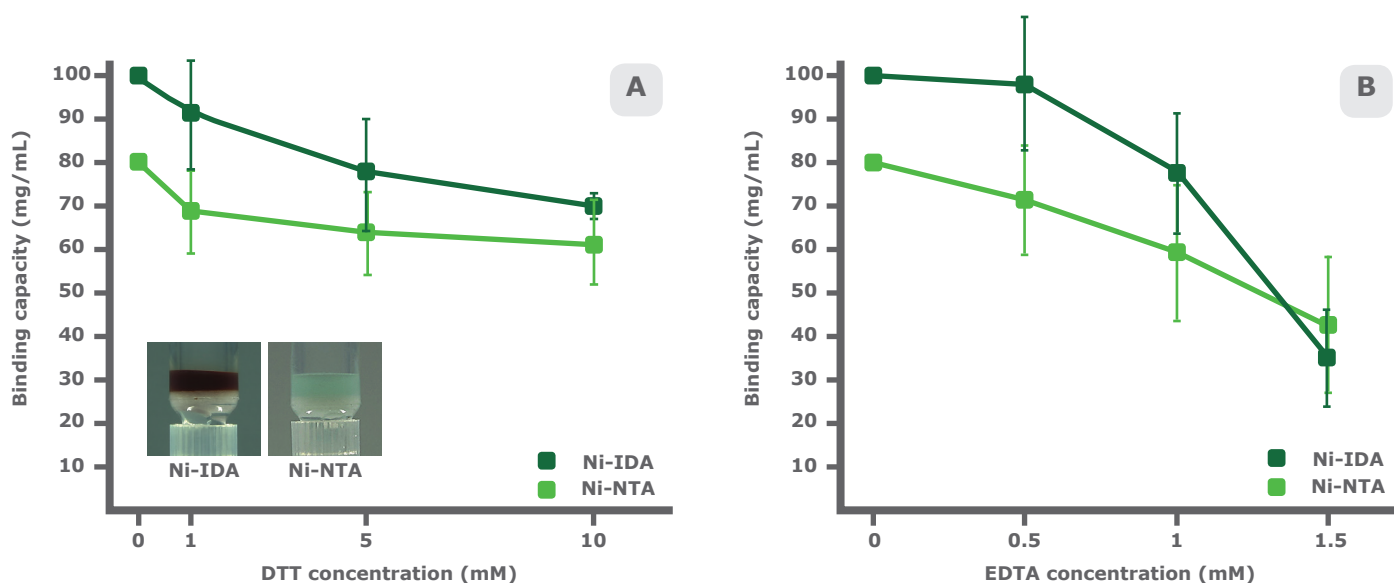


Fig. 4: Ni-NTA is more robust in the presence of reducing and chelating agents than Ni-IDA. GFP-His was purified on gravity columns containing PureCube Ni-NTA or PureCube Ni-IDA Agarose after exposing the resins for 1 h to 3 concentrations of DTT (A) or EDTA (B). Both resins exhibit lower binding capacity, but Ni-NTA has a shallower decay rate in response to both agents, especially at higher concentrations. We have also observed that PureCube Ni-NTA Agarose does not turn brown in presence of DTT (insert of A). Error bars are first standard deviation (n=3).

NTA vs. IDA: making the right choice

In deciding on an IMAC resin for your protein purification needs consider the following questions:

- ❑ How pure does your target protein need to be?
- ❑ What yield of target protein do you need?
- ❑ Does your sample contain reagents like DTT or EDTA?
- ❑ What are your budgetary constraints?
- ❑ What yields do you anticipate from your expression?

If you seek high yields and the purity of the recovered protein is of lesser priority, an IDA-based resin might be the right choice. It is less expensive and is still a robust resin that is easily regenerated and reloaded. To minimize unspecific binding, the use of alternate metal ions such as zinc and cobalt could be explored. For applications where purity of the recovered protein is essential (e.g., for subsequent crystallization), NTA-based resins are the most optimal choice. Specificity can be further improved by loading the resin with a metal ion that exhibits highly specific binding (e.g., cobalt). Another advantage of NTA-based resins is their resilience when exposed to agents such as DTT and EDTA, which accommodates a wider range of sample buffers.

Resin format is also a decision factor. Matrix and purification process can impact final results. Additionally, using excessive resin for a given protein yield can lead to impurities, as binding sites are left exposed.

When working with small amounts of protein, it is recommendable to use magnetic beads as a matrix; these are particularly effective for dilute samples, proteins expressed at low levels, and pull-down experiments. Protein amounts standardly obtained from mid-scale suspension culture (>50 µg) are better processed with agarose in batch or gravity column purification. Agarose-based purifications can also be scaled up to bulk production amounts. Finally, packed chromatography columns enable automated processing of protein yields ranging from 1 to 100 mg (Fig 5).

In the end, optimizing the purification procedure for a His-tagged protein may require testing a couple different affinity resin options, but with a few principles, the examination of available IMAC resins can be a systematic process.

Principles of this tale:

- ❑ IDA-based resins tend to be less expensive
- ❑ NTA-based resins typically generate purer eluates
- ❑ Alternate metal ions can adjust the binding specificity of a resin. Co²⁺ is most specific, then Ni²⁺, and Zn²⁺, followed by Cu²⁺
- ❑ Metal ion leaching is significantly lower with NTA-based resins
- ❑ NTA-based resins are robust against reducing and chelating agents

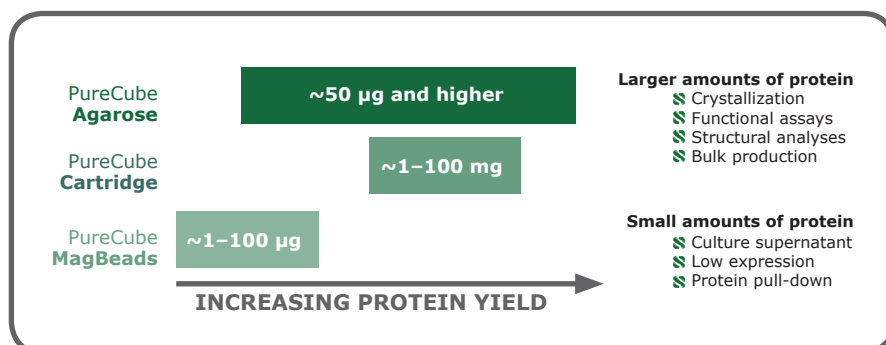


Fig. 5: Protein yields and applications for which different IMAC formats are used. Generally, magnetic beads are best implemented with low concentration samples and agarose-based resins are used for larger yields. Indicated protein yields are only guidelines.

Featured products:

- PureCube Ni-NTA Agarose (Cat. No. 31103)
- PureCube Ni-NTA MagBeads (Cat. No. 31201)
- PureCube Ni-NTA Cartridge (Cat. No. 31305)
- PureCube Ni-IDA Agarose (Cat. No. 30103)
- PureCube Ni-IDA MagBeads (Cat. No. 30201)
- PureCube Ni-IDA Cartridge (Cat. No. 30305)
- PureCube Co-NTA Agarose (Cat. No. 31403)

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