WorkBeads 40 NTA
WorkBeads 40 IDA

WorkBeads™ 40 NTA and WorkBeads 40 IDA resins are based on nitrilotriacetic acid (NTA) and the iminodiacetic acid (IDA) chelating groups. The resins can be easily charged before use with a broad spectrum of divalent or trivalent transition metal ions, including Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ga^{3+} and Fe^{3+}. They can then be used for the Immobilized Metal Ion Affinity Chromatography (IMAC) purification of His-tagged proteins or other proteins with an affinity for metal ions. The selectivity of the metal-charged resin depends on both the choice of ligand (NTA or IDA) and the metal ion used. These resins can also be used for divalent metal ion removal.

- Easy charge of the resin with the metal ion of choice for optimal purity of the target protein
- High binding capacity and flow properties
- Reliable and reproducible results

Short protocol

This short protocol is for column packing, metal ion charging and IMAC purification of WorkBeads 40 NTA and WorkBeads 40 IDA resins. Detailed instructions and recommendations for optimization are given later in this instruction.

Packing

1. Make a slurry of the desired resin concentration in water.
2. Pour the slurry into the column.
3. Pack the resin with an appropriate flow rate.
4. Apply an axial compression of less than 2%.
5. Equilibrate the column with 20% ethanol for storage.

Charging the resin

1. Wash the column with 5 column volumes (CV) deionized water.
2. Charge the column by applying 2 CV 50 mM metal ion solution.
3. Wash the column with 10 CV deionized water.
4. Equilibrate the column with 20% ethanol for storage.

Purification

1. Equilibrate the column using 10 CV binding buffer.
2. Apply a clarified sample in the pH range 7 - 8.5. The sample should contain 10 mM imidazole.
3. Wash the column using 20 - 30 CV washing buffer.
4. Elute the target protein.

Alternative 1, step gradient: Desorb the target protein with 5 CV elution buffer.

Alternative 2, linear gradient: For increased purity, linear gradient elution is recommended. For example, use a gradient from 10 mM to 300 mM imidazole over 20 CV.

5. Wash the column with 5 CV deionized water to remove the buffer salts.
6. Equilibrate with 10 CV 20% ethanol for storage.

Principle

IMAC utilizes the affinity of histidine, cysteine and tryptophan amino acid side chains on the protein surface for binding to transition metal ions, such as Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}, immobilized via a metal chelating ligand on the chromatography resin. WorkBeads resins are available with immobilized nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) chelating ligands as illustrated in Figure 1.

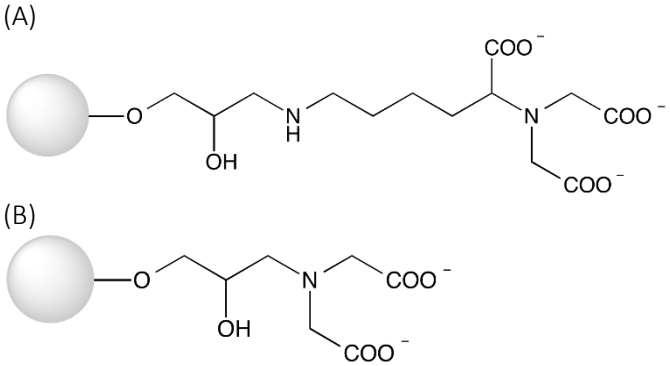

Figure 1. Structure of the chelating ligands used in WorkBeads 40 NTA (A) and WorkBeads 40 IDA (B) resins.

IMAC is most commonly used for purification of recombinant His-tagged proteins. A His-tag is usually composed of six to ten histidyl groups, and is typically placed at the N- or C-terminus of the target protein, although other positions are possible. His-tagged proteins will bind to the chelating ligand (through the metal ion) and unbound material will pass through the column. Bound proteins are desorbed by stepwise or gradient elution using a competing agent, or by applying a low pH buffer. WorkBeads 40 Ni-NTA is recommended as the primary choice for His-tagged protein purification and, in most cases, will give excellent results. For more difficult purifications, screening the sample is recommended with the eight different pre-charged WorkBeads IMAC resins available to find the optimal combination of ligand and metal ion, see *Related products*. Bio-Works also offer two different Screening kits with pre-charged WorkBeads IMAC resins pre-packed in BabyBio 1 ml and 5 ml columns.

Imidazole is recommended for elution. This is the most commonly used competing agent but histidine, ammonium chloride or histamine can also be used. Before sample application the column should be equilibrated with a low concentration of the competing agent in order to prevent non-specific binding of endogenous proteins that may bind via histidine clusters for example. This is easily done by using the recommended binding buffer.

Elution with a continuously decreasing pH gradient is an alternative to imidazole and after optimization, a pH step gradient could be more appropriate for scale-up. At pH 3 - 5, the histidine residues (pK_a approx. 6) are protonated which leads to the loss of affinity for the metal ion and to a release of the protein. It is important to consider the target protein stability at low pH.

Column packing

WorkBeads resins are cross-linked using a proprietary method that results in a very rigid resin, which tolerate pressures of several bars and consequently can run at high flow rates. Follow both this general advice when packing a column and the column manufacturer's specific instructions. Preferably, use a column with an adjustable adaptor. In some instances, a packing reservoir or column extension may be needed.

Note: Always make sure that the maximum pressure of the column hardware is not exceeded. Backpressure caused by the chromatography system components connected downstream of the column may reduce the maximum flow that can be used. Wear eye protection.

1. Wash the resin

The resin is provided in 20% ethanol. To avoid undue backpressure when packing, wash the desired amount of resin with several column volumes of deionized water before packing.

2. Make a slurry

Add deionized water to the washed resin to obtain a 40% to 60% slurry concentration. Make approximately 15% extra slurry to compensate for the compression during packing. The amount of slurry to be prepared can be calculated according to:

$$\text{Slurry volume} = \frac{\text{bed volume x } 100}{\text{\% slurry}} \text{ x } 1.15$$

3. Pour the slurry into the column

Pour the slurry slowly down the side of the column to avoid formation of air bubbles. Preferably, use a packing reservoir or an extra column tube to extend the column volume to accommodate the entire slurry volume during packing. If no packing adaptor is available packing can be done by stepwise additions and packing. Although not recommended this will give acceptable results for most applications.

4. Pack the bed

Pack the resin with a downward flow higher than the intended operational flow. We recommend 700 cm/h for columns up to 25 mm i.d. and with 200 mm bed height. Make sure the packing flow rate does not exceed the maximum pressure of the column hardware or the resin. The operational flow should not be more than 75% of the packing flow rate.

5. Close the column

When the bed height is constant mark the bed height on the column. Stop the flow and remove the packing reservoir or extra tube. Note that the bed height will increase temporarily when the flow is stopped. If needed, adjust the bed height by removing excess resin. Be careful not to remove too much resin. Gently fill the column with packing solution to its rim without disrupting the packed bed. Insert the adjustable adapter on top of the packed bed. Apply a small axial compression of less than 2% of the final bed height by lowering the adapter into the packed bed.

6. Apply a flow

Apply a flow of 450 cm/h (taking account of section 4) and check for any gap formation above the surface of the resin bed. If a gap is observed, stop the flow and adjust the adaptor to eliminate the gap.

Metal ion charging

1. Equilibrate the column

Wash the column with 5 CV deionized water.

2. Metal ion charging

Charge the column with the metal ion of choice by applying approximately 2 CV 50 mM metal solution in deionized water. See recommended salts in Table 1.

3. Remove unbound metal ion

Remove the excess metal solution by washing the column with 10 CV deionized water.

Table 1. Recommended metal ions salts for charging. Other metal salts can possibly be used.

Metal ion immobilized	Metal salt recommended
Ni^{2+}	50 mM Nickel(II) sulfate
Co^{2+}	50 mM Cobalt(II) sulfate
Cu^{2+}	50 mM Copper(II) sulfate
Zn^{2+}	50 mM Zinc sulfate
Ga^{3+}	50 mM Gallium(III) nitrate
Fe^{3+}	50 mM Iron(III) sulfate

Purification

The charged WorkBeads 40 NTA and WorkBeads 40 IDA resins are excellent for purification of His-tagged proteins. Recommended buffers for purification of His-tagged proteins are shown in Table 2.

Purification can be carried out at room temperature or at temperatures down to 4°C. Operation at a low temperature may require reduced flow rate due to increased viscosity. Prepare the sample according to sample preparation below before starting. Equilibrate the column with 5 - 10 CV equilibration buffer before use.

Note: To avoid bacterial growth and poor column performance, use only freshly prepared and filtered buffers.

Table 2. Recommended buffers for purification of His-tagged proteins.

Buffer	Composition
Binding buffer[1]	50 mM Na-phosphate buffer,300 mM NaCl, 10 mM imidazole, pH 8.0
Washing buffer[1]	50 mM Na-phosphate buffer, 300 mM NaCl, 20 - 100 mM imidazole, pH 8.0
Elution buffer	50 mM Na-phosphate buffer, 300 mM NaCl, 300 mM imidazole, pH 8.0

1. The imidazole concentration may have to be optimized. A too high concentration may elute the target during washing. An imidazole concentration just below where the target proteins is still bound will prevent impurities to bind. This is an ideal washing buffer.

Sample preparation

After cell disruption or extraction, clarify the sample by centrifugation at 10 000 - 20 000 × *g* for 15 - 30 minutes. It is generally also recommended to pass the sample through a 0.22 - 0.45 µm filter, e.g., a syringe filter, to avoid transferring any remaining contaminating particles onto the column. Large sample volumes may be clarified by filtration through depth filters or by tangential flow filtration, which may be cheaper and more efficient than investing in a large-scale centrifuge. Application of a sample that has not been properly clarified may reduce the performance and lifetime of the packed column.

Note: Add imidazole to the sample in the same concentration as in the binding buffer.

Purification

After sample application, remove unbound and loosely bound impurities by washing the column with 20 - 30 CV washing buffer or until desired $A_{280\,nm}$ absorbance of the wash fractions (e.g., 0.01 - 0.02) is obtained. Binding buffer can be used instead of washing buffer if the target protein binding is weak. However, this may decrease the final purity. Binding buffer can also be an option when using gradient elution since most of the impurities will elute earlier than the His-tagged target protein. Elute the target protein by either desorption of the target protein with 5 CV elution buffer or for high purity, gradient elution is recommended. For example, use a gradient from 10 mM imidazole to 300 mM imidazole over 20 CV.

Purification additives

A multitude of additives can be used in IMAC, including various buffer substances, salts, detergents and stabilizers. Integral membrane proteins can be purified in the presence of detergents. Denaturing agents such as guanidine-HCl or urea can be used, although they may denature the target protein. Proteins expressed as inclusion bodies often have an incomplete folding. Dissolution of the inclusion body followed by IMAC purification in the presence of a denaturing agent, and finally renaturation can be done, although significant further development may be required to obtain native protein structure.

Note: The metal pre-charged WorkBeads NTA and WorkBeads IDA are compatible with normal use of reducing agents, such as 5 mM dithiothreitol (DTT), 5 mM dithioerythritol (DTE) and 20 mM β-mercaptoethanol. However, the use of chelating substances and reducing agents may under some conditions affect the performance. If needed, Tris(2-carboxyethyl)phosphine (TCEP) is recommended as reducing agent.

Optimization

The following section will give tips on some parameters that can be tuned to get the optimal conditions for increased purity.

Optimization of binding

Low imidazole concentration

The sample and the binding buffer should contain a low concentration of imidazole (not below 10 mM) to reduce unwanted binding of host cell proteins, and to avoid pH affects that may interfere with protein binding. Keep in mind that if the imidazole concentration is too high the His-tagged protein will not bind at all. Use high quality imidazole which has little or no absorbance at 280 nm.

Slightly basic pH

Binding of His-tagged proteins is preferably carried out at pH 7 - 8.5. A lower pH protonates the histidine residues (pK_a approx. 6), and causes desorption of bound proteins.

Tuning the flow rate

Binding of His-tagged proteins to a metal chelating column is a rather fast mechanism, and a high flow rate will usually not affect the yield when moderate loadings are applied. It may be useful to lower the flow rate under some circumstances (for some proteins or sample compositions, or at low temperature).

Addition of a denaturing agent

If the target protein is insoluble or present as inclusion bodies, a denaturing agent (e.g., 8 M urea or 6 M guanidine-HCl) can be used to dissolve the target protein. The denaturing agent should be included in all buffers during purification. The protein is usually denatured by the treatment. In some cases, subsequent renaturation is desired.

Optimization of washing and elution

Washing

A continuously decreasing UV signal is an indication of unbound material being washed out. The amount of washing buffer applied should be continued until the UV signal is stable and is the same as for the washing buffer. The binding affinity for some His-tagged proteins may be very strong due to extra His-residues on the protein surface or to multimeric properties. Those cases allow more stringent washing conditions (higher concentration of imidazole), which can give higher purity. The washing step can also be optimized by increasing the imidazole concentration in an additional washing step. Note that if the imidazole concentration is too high it may cause elution of the target protein.

300 - 500 mM NaCl is usually included in the elution buffer to reduce electrostatic interactions. In rare cases it may we worthwhile to optimize the ionic strength. Other parameters such as pH and additives can be considered for optimization of the purity and stability of the purified target protein.

Elution

Elution can be carried out using a high imidazole concentration, 300 mM imidazole is usually sufficient. A stronger binding may require higher imidazole concentrations for elution. Aggregates of His-tagged proteins can bind via multiple tags thus increasing the affinity. Optimization of the imidazole concentration may allow elution of the His-tagged protein without the aggregates.

The optimal imidazole concentration is dependent on purity and recovery requirements as well as properties of the target protein and the sample. Applying gradient elution gives often increased purity than step elution, but step elution may be desired to obtain the highest possible concentration of the target protein and also when scaling-up. In order to optimize the imidazole concentration for step elution an initial linear gradient test run should be carried out to obtain suitable step elution conditions for purification of the sample, see Figure 2.

Note: Remember to take the system dead volume into account when comparing the print out of the gradient and the trace.

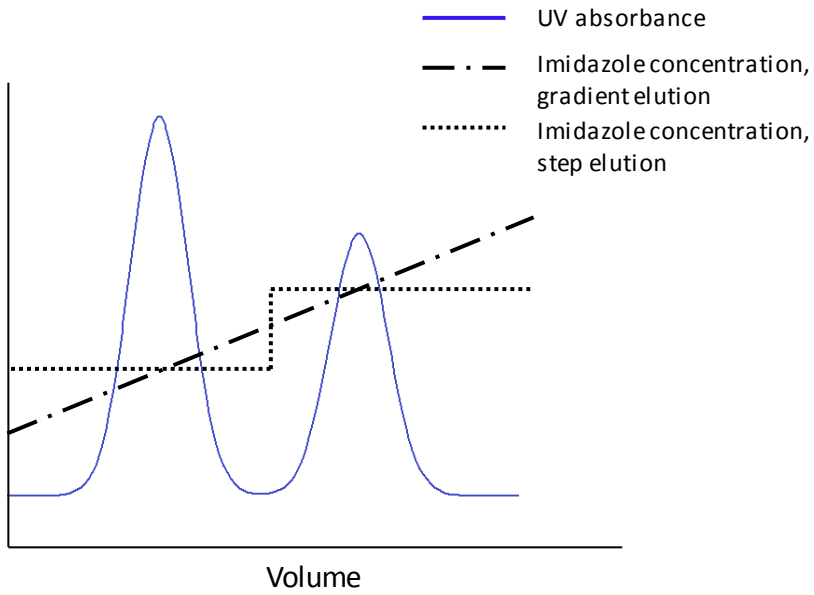

Figure 2. Optimization of step elution with imidazole. A test run with linear gradient elution gives information about suitable imidazole concentrations to be used in step elution.

Scale-up

After developing a chromatographic procedure in a small scale column, e.g., 10 (i.d.) × 100 mm (bed height), WorkBeads resins can be packed into larger columns for scale-up. Large scale purification is often carried out in columns with bed heights of 200 - 300 mm.

Scale-up principles

During scale-up the ratio between sample volume and column volume should be kept constant. The column volume is scaled up by increasing the column diameter while keeping the bed height the same (e.g., 200 mm). The linear flow rate should remain the same while the volumetric flow rate increases. The volumetric flow rate for each column can be calculated according to:

$$\text{Volumetric flow rate (ml/min)} = \frac{\text{Linear flow rate (cm/h)} \times \text{Column cross sectional area (cm}^2\text{)}}{60}$$

Flow

The concepts of volumetric flow, linear flow rate and residence time is important when doing scale-up in chromatography. Volumetric flow is measured in ml/min or l/min, linear flow in cm/h and residence time in minutes. The relationship between these metrics are:

$$\text{Linear flow rate (cm/h)} = \frac{\text{Volumetric flow (ml/min)} \times 60}{\text{Column cross sectional area (cm}^2\text{)}}$$

$$\text{Residence time (minutes)} = \frac{\text{Column bed height (cm)} \times 60}{\text{Linear flow rate(cm/h)}}$$

In the initial process development phase it is common to use a small column, e.g., 10 × 100 mm, to save sample, buffers and time. This column has a shorter bed height than the final column, which may have a bed height of 200 mm or more. The flow rate for the larger column can be calculated from the flow established on the small column, using the equation above by keeping the residence time of the small column the same for the larger column. This will allow an increase of the linear flow in proportion to the increase in bed height between the columns see Table 3 for examples. If the column bed heights are constant during scale-up the linear flow rate should be also constant (as well as the residence time).

Table 3. Example of scale-up parameters.

Column dimension	Residence time (minutes)	Linear flow rate (cm/h)	Volumetric flow rate (ml/min)
10 × 100	4	150	1.96
25 × 200	4	300	24.5
50 × 200	4	300	98.2

Additional purification

His-tagged protein purification on WorkBeads IMAC resins gives high purity in a single purification step. For very high purity requirements, it can be necessary to add a second purification step. The additional purification step is used to remove remaining proteins and/or impurities from the sample. In research-scale purification, size exclusion chromatography (SEC/gel filtration) is often a good polishing step since it removes impurities, the imidazole used for elution and potential aggregates of the target protein. Size exclusion chromatography can be done using WorkBeads 40/1000 SEC, WorkBeads 40/100 SEC and WorkBeads 40/10 000 SEC resins having different separation ranges. Ion exchange chromatography is suitable for both research scale purification and process scale. WorkBeads 40S, WorkBeads 40Q and WorkBeads 40 DEAE resins provide different selectivities for ion exchange chromatography. These resins are also available as ready-to-use 1 ml and 5 ml BabyBio S, BabyBio Q and BabyBio DEAE columns.

To find out more about Bio-Works chromatography resins for additional purification, please visit www.bio-works.com

Desalting and buffer exchange

Target protein eluted after purification by IMAC using the conditions describe above will contain imidazole and a relatively high concentration of salt which usually needs to be removed. Buffer exchange or desalting of the sample can be done using BabyBio Dsalt 1 ml or BabyBio Dsalt 5 ml (see *Related products*). Chromatographic desalting is especially useful when the sample needs to be processed rapidly to avoid degradation. For larger sample volumes BabyBio Dsalt columns can be connected in series and for process-scale, diafiltration should be considered.

Maintenance of the resin

Unpacking and inspection

Unpack the shipment as soon as it arrives and inspect it for damage. Promptly report any damage or discrepancies to your local supplier.

Cleaning and recharging with metal ions

Small amounts of impurities can be found in some samples, which tend to adsorb to the resin by unspecific interactions. This may reduce the resin performance. It is therefore common to strip off the metal ions, followed by cleaning and then recharge the resin with fresh metal ions. If the resin is packed in a column; stripping, cleaning and recharging the resin can be carried out as followed:

Wash with:

1. 5 CV deionized water
2. 10 CV 50 mM Na_2-EDTA, pH 8.0
3. 10 CV 100 mM NaOH
4. 10 CV deionized water
5. 2 CV 50 mM metal salt solution in deionized water
6. 10 CV deionized water
7. 10 CV 20% ethanol (for storage)

Storage

Store at 2 to 25°C in 20% ethanol.

Additional information

Intended use

WorkBeads 40 NTA and WorkBeads 40 IDA resins are intended for research, process development and industrial use only. The resins shall not be used for preparation of material for clinical or diagnostic purposes.

Safety

Please read the associated Material Safety Data Sheets (MSDS) and the safety instructions for any equipment to be used. Nickel and cobalt salts are considered to be allergenic and potentially carcinogenic. Use recommended safety equipment.

Product description

	WorkBeads 40 NTA	WorkBeads 40 IDA
Target substance	His-tagged proteins, proteins containing histidine cysteine and/or tryptophan amino acid side chains	His-tagged proteins, proteins containing histidine cysteine and/or tryptophan amino acid side chains
Matrix	Highly cross-linked agarose	Highly cross-linked agarose
Average particle size[1] (D_{V50})	45 µm	45 µm
Chelating ligand	Nitrilotriacetic acid (NTA)	Iminodiacetic acid (IDA)
Metal ion capacity[2]	50 - 60 µmol Cu^{2+}/ml resin	50 - 60 µmol Cu^{2+}/ml resin
Max flow rate (20 cm bed height and 5 bar)	600 cm/h	600 cm/h
Chemical stability	Compatible with all standard aqueous buffers used for protein purification, 8 M urea, 6 M guanidine-HCl, non-ionic detergents, 20% ethanol. Chelating substances (e.g. EDTA) will strip off the metal ions. Stripped column 10 mM HCl (pH 2), 10 mM NaOH (pH 12), 10 mM sodium citrate-HCl (pH 3).	
pH stability	2 - 12	2 - 12
Storage	2 to 25 °C in 20% ethanol	2 to 25 °C in 20% ethanol

1. The median particle size of the cumulative volume distribution.
2. Metal ion capacity is determined by frontal analysis at 50% breakthrough using cupper solution.

Related products

Product name	Pack size[1]	Article number
Prepacked columns		
BabyBio IDA His-tag Screening kit 1 ml[2]	4 x 1 ml	45 700 001
BabyBio NTA His-tag Screening kit 1 ml[2]	4 x 1 ml	45 700 101
Bulk resins		
WorkBeads 40 Ni-NTA	25 ml	40 651 001
WorkBeads 40 Co-NTA	25 ml	40 651 401
WorkBeads 40 Cu-NTA	25 ml	40 651 301
WorkBeads 40 Zn-NTA	25 ml	40 651 501
WorkBeads 40 Ni-IDA	25 ml	40 650 001
WorkBeads 40 Co-IDA	25 ml	40 650 401
WorkBeads 40 Cu-IDA	25 ml	40 650 301
WorkBeads 40 Zn-IDA	25 ml	40 650 501

1. Other pack sizes can be found in the complete product list on www.bio-works.com
2. Includes one column each charged with Ni^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}

Ordering information

Product name	Pack size	Article number
WorkBeads 40 NTA	25 ml	40 602 001
	150 ml	40 602 003
	1 L	40 602 010
WorkBeads 40 IDA	25 ml	40 601 001
	150 ml	40 601 003
	1 L	40 601 010

Orders: sales@bio-works.com or contact your local distributor.

For more information about local distributor and products please visit www.bio-works.com or contact us at info@bio-works.com

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