WorkBeads NiMAC

WorkBeads™ NiMAC resin is precharged with very strongly bound nickel ions which provides very high resistance to reducing agents such as DTT and chelating substances such as EDTA. The high stability allows purification of proteins from sources, such as eukaryotic cell extracts, that normally would cause significant nickel ion stripping from the resin. This reduces the need for sample pre-treatment. The resin provides high purity and binding capacity and the possibility to use high flow rates for minimized process time.

- Resin with extra strongly bound Ni^{2+} resulting in extremely low nickel ion leakage
- Highly resistant to reducing agents up to 20 mM DTT
- Highly resistant to chelating substances present in eukaryotic extracts or up to 20 mM EDTA
- High purity and reproducible results

Short protocol

This short protocol is for column packing and purification using WorkBeads NiMAC. 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 ca 2%.
5. 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 7.0 - 8.5. The sample should contain 10 mM imidazole.
3. Wash the column using 20 CV binding 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.

IMAC is commonly used for the 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. BabyBio™ NiMAC columns are recommended for His-tagged protein purification when minimal Ni^{2+} leakage is necessary and also when higher concentrations of DTT (20 mM) and EDTA (20 mM) have to be included in the sample/buffers. For optimization of for example purity, a screening is recommended using the eight available different pre-charged WorkBeads IMAC resins are recommended 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 prepacked 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 binding buffer containing a low concentration of the competing ligand to prevent non-specific binding of host cell proteins that may bind via, e.g., histidine clusters on their surface.

Elution with a continuously decreasing pH gradient is an alternative to imidazole and after optimization a pH step gradient may 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 thus to the 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 without the risk of destroying the column hardware. Wear eye protection and other personal protection equipment required for the work.

1. Wash the resin

The resin is provided in 20% ethanol. To avoid overpressure when packing, wash the desired amount of resin with several column volumes of deionized water before packing. If packing is done in 20% ethanol the flow rate should be reduced to half to compensate for the high viscosity of 20% ethanol causing high backpressure.

2. Make a slurry

Add deionized water to the washed resin to obtain a 50% to 70% 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 300 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 ca 2-5% of the final bed height by lowering the adapter into the packed bed.

6. Apply a flow

Apply a flow of 200 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.

Purification

WorkBeads NiMAC resin is excellent for purification of His-tagged proteins with minimal Ni^{2+} leakage also when high concentrations of reducing agents such as 20 mM DTT or chelating substances such as 20 mM EDTA have to be included in the sample/buffers. Recommended buffers for purification of His-tagged proteins are shown in Table 1.

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 1. Recommended buffers for purification of His-tagged proteins.

Buffer	Composition
Binding buffer[1]	50 mM sodium phosphate buffer,300 mM NaCl, 10 mM imidazole, pH 8.0
Washing buffer[1]	50 mM sodium phosphate buffer, 300 mM NaCl, 20 - 100 mM imidazole, pH 8.0
Elution buffer	50 mM sodium 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 binding buffer or if needed a more stringent washing buffer, or until desired $A_{280\ nm}$ absorbance of the wash fractions (e.g., 0.01 - 0.02) is obtained. When gradient elution is used most host cell protein impurities will elute earlier than the His-Tagged protein. This reduces the need for a stringent washing solution. 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, including DTT, EDTA, 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.

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 7.0 - 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 this treatment. In some case subsequent renaturation is desired.

Addition of reducing agents

It is possible to include up to 20 mM DTT or other similar reducing agents, and 20 mM EDTA or other chelators both in the sample and buffers without losing any binding capacity.

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 be 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 often gives increased purity compared to step elution, but step elution may be desired to obtain the highest possible concentration of the target protein and is most common in large-scale purifications. The imidazole concentration can be optimized for step elution by carry out an initial linear gradient test run to find required concentration for elution, see Figure 2.

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

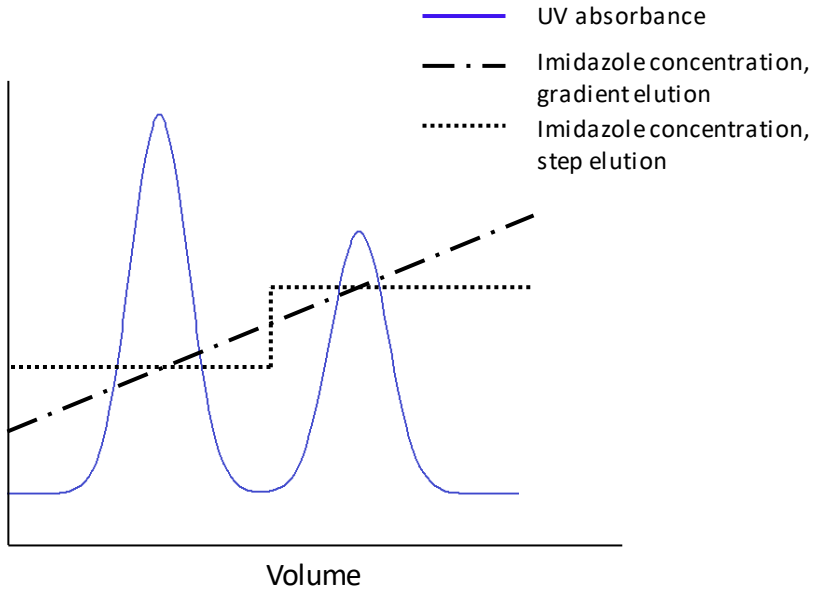

Figure 2. Optimization of step elution with imidazole. A test run with linear gradient elution gives information about suitable imidazole concentrations to be used in step elution.

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 is:

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

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

In the initial process development 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 2 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 2. 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 NiMAC resin 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 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*) or by packing a larger column with WorkBeads Dsalt resin. 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 WorkBeads Dsalt allow scale-up and should be considered a fast alternative to diafiltration.

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.

Recharging with metal ions

It is not possible to strip and recharge WorkBeads NiMAC due to the very tight binding of the Ni^{2+} ions to the chelating group of the resin.

Cleaning-in-place (CIP)

When running complex feeds, small amounts of impurities tend to adsorb to the resin by unspecific interactions. Cleaning of resin with up to 0.5 M NaOH for 15 minutes followed by 15 minutes distilled H_2O in repeated cycles are recommended if resin gets fouled.

Storage

Store at 2 to 25°C in 20% ethanol.

Additional information

Intended use

WorkBeads resins are developed and supported for both research and production-scale chromatography. WorkBeads resins are produced with validated methods and Regulatory Support Files (RSF) are available to assist the process validation and submissions to regulatory authorities.

Safety

Please read the associated Safety Data Sheets (SDS) and the safety instructions for any equipment to be used. Nickel salt is considered to be allergenic and potentially carcinogenic. Use recommended safety equipment.

Product description

	WorkBeads NiMAC
Target substance	His-tagged proteins
Matrix	Highly cross-linked agarose
Average particle size[1] (D_{V50})	45 µm
Precharged ions	Nickel (II) ions, Ni^{2+}
Static binding capacity	> 80 mg/ml resin
Dynamic binding capacity[2]	> 40 mg/ml resin
Metal ion capacity[3]	> 60 µmol Cu^{2+}/ml resin
Max flow rate (20 cm bed height and 5 bar)[4]	600 cm/h
Chemical stability	Compatible with all standard aqueous buffers used for protein purification, and additives such as 20 mM Na_2-EDTA, 20 mM dithiothreitol (DTT), 20 mM TCEP, 20 mM β-mercaptoethanol, 8 M urea, 6 M guanidine-HCl, non-ionic detergents, 500 mM imidazole, 30% isopropanol, 0.5 M NaOH
pH stability	3 – 9 (working range) 2 – 14 (cleaning-in-place)
Storage	2 to 25 °C in 20% ethanol

1. The median particle size of the cumulative volume distribution.
2. Binding capacity may vary depending on protein characteristics and on flow rate used. A lower flow rate usually increases the dynamic binding capacity.
3. Metal ion capacity is determined by frontal analysis at 50% breakthrough using copper solution.
4. Optimal flow rate during binding is depending on the sample.

Related products

Product name	Pack size[1]	Article number
Prepacked columns		
BabyBio NiMAC 1 ml	1 ml × 1	45 655 311
	1 ml × 2	45 655 312
	1 ml × 5	45 655 313
	1 ml × 10	45 655 314
BabyBio NiMAC 5 ml	5 ml × 1	45 655 315
	5 ml × 2	45 655 316
	5 ml × 5	45 655 317
	5 ml × 10	45 655 318
BabyBio NTA His-tag Screening Kit 1 ml[2]	1 ml x 4	45 700 101
BabyBio NTA His-tag Screening Kit 5 ml[2]	5 ml x 4	45 700 102
BabyBio IDA His-tag Screening Kit 1 ml[2]	1 ml x 4	45 700 001
BabyBio IDA His-tag Screening Kit 5 ml[2]	5 ml x 4	45 700 002
Bulk resins		
WorkBeads 40 Ni-NTA	25 ml	40 651 001
	150 ml	40 651 003
WorkBeads 40 Co-NTA	25 ml	40 651 401
	150 ml	40 651 403
WorkBeads 40 Cu-NTA	25 ml	40 651 301
	150 ml	40 651 303
WorkBeads 40 Zn-NTA	25 ml	40 651 501
	150 ml	40 651 503
WorkBeads 40 Ni-IDA	25 ml	40 650 001
	150 ml	40 650 003
WorkBeads 40 Co-IDA	25 ml	40 650 401
	150 ml	40 650 403
WorkBeads 40 Cu-IDA	25 ml	40 650 301
	150 ml	40 650 303
WorkBeads 40 Zn-IDA	25 ml	40 650 501
	150 ml	40 650 503

1. All different pack sizes are available 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 NiMAC	25 ml	40 653 001
	150 ml	40 653 003
	1 L	40 653 010

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

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

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