BabyBio NiMAC

The ready-to-use BabyBio™ NiMAC comprises prepacked WorkBeads™ NiMAC resin which is pre-charged with strongly bound nickel ions, providing very high resistance to reducing agents such as DTT and also EDTA. The strong nickel ion binding of this resin is key to its suitability for purification of large volumes of His-tagged proteins from various sources and it requires minimal sample pre-treatment that would normally cause metal ion stripping.

BabyBio NiMAC are available in two column sizes: 1 ml and 5 ml for quick and convenient purification of His-tagged proteins by using Immobilized Metal Ion Affinity Chromatography (IMAC).

BabyBio NiMAC columns can be used to purify up to at least 40 mg or 200 mg protein using a 1 ml or a 5 ml column.

- Ready-to-use columns for fast results
- 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 general and short protocol is for the use of metal ion charged BabyBio NiMAC. Detailed instructions and recommendations for optimization are provided later in this instruction. Recommended buffers are listed in Table 2.

1. Connect the column to the chromatography system, syringe or pump.
2. Equilibrate the column using 10 column volumes (CV) binding buffer.
3. Apply a clarified sample in the pH range 7.0 - 8.5. To the sample add imidazole to a concentration of 10 mM.
4. Wash the column using 20 - 30 CV binding buffer, or a more stringent washing buffer.
5. Elute the target protein.
   Alternative 1: Desorb the target protein with 5 CV elution buffer.
   Alternative 2: For increased purity, gradient elution is recommended. For example, a gradient from 10 mM to 300 mM imidazole over 20 CV.
6. After the elution re-equilibrate the column with 10 CV binding buffer.
7. Wash the column with 5 CV deionized water to remove the elution buffer.
8. Equilibrate the column with 5 CV 20% ethanol for storage. Close the column using the provided cap and plug.

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, we recommend screening using the eight available pre-charged WorkBeads IMAC resins to determine 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 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 loss of affinity for the metal ion and thus to release of the protein. It is important to consider the target protein stability at low pH.

Instructions

Purification can be carried out at room temperature or at temperatures down to 4°C. Operation at a low temperature may require a reduced flow rate due to the increased viscosity of the buffer. All steps can be carried out with a syringe, a peristaltic pump or a chromatography system. If the chromatography system has a pressure limit functionality, set the maximum pressure over the column to 3 bar (remember to take the pressure contribution of the system fluidics into account).

1. Prepare the sample

After cell disruption or extraction, clarify the sample by centrifugation at 10 000 – 20 000 × *g* for 15 - 30 minutes. It is generally recommended that you also pass the sample through a 0.22 - 0.45 µm filter (e.g. a syringe filter) to avoid introducing any remaining particles onto the column. If the sample only contains small amounts of particles, centrifugation may be omitted and filtration alone is sufficient. Application of a sample that has not been properly clarified may reduce the performance and lifetime of the column. The sample should be applied under conditions similar to those of the binding buffer. Add imidazole to the sample to the same concentration as in binding buffer, generally 10 mM imidazole.

2. Connect the column

Cut off or twist off the end at the outlet of the column, see Figure 1. **Note:** It is of great importance to cut off the tip at the very end of the cone, preferably using a scalpel. The function of the cone is to create a tight seal when the column is connected. Incorrect removal of the end piece will affect the performance of the column.

Connect the column to your equipment using the recommended connectors shown in Table 1. Fill the equipment with deionized water or buffer and make drop-to-drop connection with the column to avoid introducing air into the column. Carry out all steps, except for sample application, at 1 ml/min (BabyBio 1 ml column) or 5 ml/min (BabyBio 5 ml column).

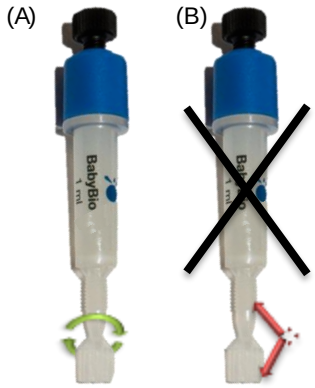

Figure 1. Removal of the cut-off end at the column outlet should be done by cutting or by twisting (A), not bending (B).

Table 1. Recommended connectors for coupling BabyBio columns to the equipment of choice.

Equipment	Accessories for connection
Syringe	Female luer or male coned 10-32 threads
Chromatography system	Fingertight connectors (coned 10-32 threads) for 1/16” o.d. tubing

3. Remove the storage solution

The column contains 20% ethanol on delivery. This storage solution should be washed out before use. Wash the column with 5 CV deionized water or buffer. Avoid flow rates higher than 2 ml/min for BabyBio 1 ml columns or 10 ml/min for BabyBio 5 ml columns before the storage solution has been removed to avoid overpressure due to the high viscosity of the 20% ethanol solution.

4. Equilibrate the column

Equilibrate the column with 5 - 10 CV of binding buffer (see Table 2 for recommended buffers). Other neutral buffers, with at least 10 mM of imidazole, can also be used.

Table 2. Recommended buffers for purification.

Buffer	Composition
Binding buffer	50 mM sodium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 8.0
Washing buffer	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

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

5. Apply the sample

Apply the sample at 0.5 - 1 ml/min for the BabyBio 1 ml or 2 - 5 ml/min for the BabyBio 5 ml columns. An excessive flow rate will reduce the binding capacity and may reduce the yield.

6. Wash
After sample application, remove unbound impurities by washing the column with 20 - 30 CV of washing buffer or until the desired $A_{280\ nm}$ absorbance of the wash fractions (e.g. 0.01 - 0.02) is obtained. The binding buffer is the first choice for washing, but a more stringent washing solution can occasionally be used if the binding of the target protein is sufficiently strong. Excessive washing may reduce the yield. If a gradient elution is used, most of the host cell protein impurities will bind less strongly and elute earlier than the His-tagged target protein. In this situation, stringent washing is of less interest.

7. Elute
Alternative 1: Elute the target protein with 5 CV elution buffer.
Alternative 2: For increased purity, gradient elution is recommended. For example, a gradient from 10 mM to 300 mM of imidazole over 20 CV can be applied.

8. Re-equilibrate
Before the next purification, re-equilibrate the column with 10 CV binding buffer.

9. Remove the elution buffer
Wash the column with 5 CV deionized water to remove the salts of the elution buffer.

10. Column storage
Equilibrate the column with 5 CV 20% ethanol for storage. Close the column using the cap and plug (included).

Purification additives
BabyBio NiMAC columns are compatible with a multitude of additives, including EDTA, DTT, 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 are often incompletely folded. Dissolution of the inclusion body followed by IMAC purification in the presence of a denaturing agent, and finally renaturation, is sometimes performed, although significant further development may be required to obtain native protein structure.

Scale-up

BabyBio NiMAC 1 ml columns are commonly used for purification of up to at least 40 mg of protein sample, but a higher capacity is often possible. However, this depends on the properties (mainly size) of the target protein and the flow rate applied. The capacity is also dependent on the sample composition and conditions used for the purification. Scale-up from a BabyBio 1 ml column can easily be done using a BabyBio 5 ml column and applying a sample volume five times larger. BabyBio columns can be connected in series with a maximum of five columns (column stacking). This will increase the capacity accordingly. By connecting BabyBio columns in series, column volumes from 1 ml to 25 ml can be obtained. This means that a binding capacity of at least 1000 mg of His-tagged protein can be achieved.

Note: Due to the very tight binding of the nickel ions it is possible to load large volumes of samples, where other charged IMAC resins would be stripped of the nickel ions in the same situation. It is important to clarify the sample to prevent fouling of the column and increased backpressure.

BabyBio columns can be connected together easily without accessories. The pressure drop across each column bed will be the same as for a single column, but the upstream columns will be exposed to a higher internal pressure since this is affected by the added pressure drops across the downstream columns. It may therefore be necessary to decrease the flow rate accordingly to avoid reaching the maximum pressure limit in the first column. If possible, the maximum pressure of the chromatography system should be set according to Table 3. Remember to take the system fluidics contribution to the pressure into account. In addition, downstream system components may produce backpressure which will add to the pressure inside the upstream columns. This will not affect the resin inside the column but may cause overpressure of the column hardware.

Table 3. Recommended maximum pressure settings for BabyBio columns connected in series. Note that the maximum pressure over each column is always 3 bar.

Number of columns in series	Max pressure BabyBio 1 ml (bar)	Max pressure BabyBio 5 ml (bar)
1	3.0	3.0
2	6.0	6.0
3	9.0	9.0
4	12	10[1]
5	15	10[1]

1 The maximum pressure is defined by the column hardware maximum pressure.

The column size should be selected based on the estimated amount of protein to be purified. A test run with a defined small volume of sample on a BabyBio 1 ml column should be used to estimate the concentration of the target protein in the sample. A general recommendation is to use 70 - 80% of the column binding capacity to avoid loss due to overload of the column. For large sample volumes with low concentrations of the target protein, it may be suitable to use a larger column than calculated in order to allow higher sample flow rates, and consequently shorter application time. For example, using a 5 ml column instead of a 1 ml column allows a flow rate five times higher due to the larger cross-section of the column. Bear in mind that excessive flow rates may reduce the binding capacity.

For columns larger than 20 ml, we recommended packing a single column using bulk resin, as the limitations of column stacking will then impact chromatographic performance. To find out more about Bio-Works bulk chromatography resins, please visit www.bio-works.com and read about our WorkBeads resin packages.

Optimization

The following paragraphs will give indications as to which parameters can be fine-tuned to optimize conditions for purification.

Optimization of the binding

Low imidazole concentration

The sample and the binding buffer should contain a low concentration of imidazole, but not below 10 mM, to reduce unwanted binding of host cell proteins and to avoid pH effects that may interfere with the protein binding. Keep in mind that the His-tagged protein will not bind at all if the imidazole concentration is too high. 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. At lower pH the histidine residues will be protonated (pK_a approx. 6) and will not bind to the column.

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. At low temperature, or for some target proteins or sample compositions, it may be useful to lower the flow rate to obtain optimal yield.

Addition of a denaturing agent

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

Addition of reducing and chelating 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

Prolonged or more stringent wash

A continuously decreasing UV signal is an indication of unbound material being washed out. The washing should be continued until the UV signal is stable and is the same as or similar to 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 for 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.

Increased imidazole concentration

An additional washing step with a higher imidazole concentration in the washing buffer can be tested. Note that if the imidazole concentration is too high it may cause premature elution of the target protein.

Additives

In IMAC, 300 - 500 mM NaCl is usually included in the eluents 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 to increase the purity and stability of the target protein.

Addition of reducing and chelating agents

It is possible to include up to 20 mM DTT and 20 mM EDTA in both in the binding and elution buffers without losing any binding capacity.

Optimizing elution conditions

Elution can be performed using a high imidazole concentration (but rarely higher than 300 mM). A stronger binding may require higher imidazole concentrations for elution. Aggregates of His-tagged protein bind via multiple tags, thus increasing the affinity. By optimizing the imidazole concentration, it is possible to elute the His-tagged protein separately from the aggregates.

Step elution

The optimal imidazole concentration is dependent on the purity and recovery requirements as well as the properties of the target protein and sample. Applying gradient elution often gives increased purity over 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 performed to obtain suitable step elution conditions, see Figure 2.

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

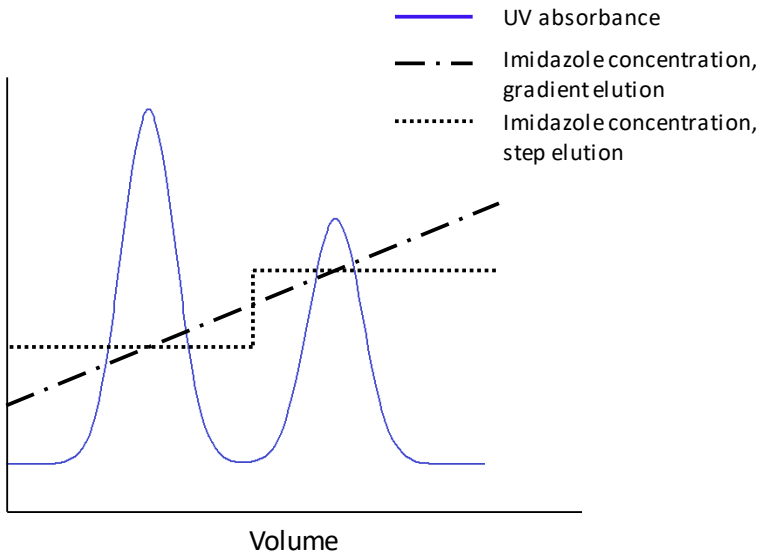

Figure 2. Optimization of step elution with imidazole. A test run with linear gradient elution provides information on suitable imidazole concentrations to be used in step elution.

Extra purification step

Optimization of the overall purification process by tuning the binding, washing and/or elution steps, is a possibility. However, an extra purification step based on another chromatography technique is recommended (see *Additional purification*).

Desalting and buffer exchange

After the purification on BabyBio NiMAC the eluted target protein preparation will contain imidazole from the elution buffer. In most cases it needs to be removed. Removal of low M_r substances such as imidazole, salts or buffer substances can be easily and quickly carried out by desalting using BabyBio Dsalt 1 ml or 5 ml columns, or by packing a larger column with WorkBeads Dsalt resin (see *Related products*). This takes a couple of minutes instead of many hours, as for dialysis. BabyBio Dsalt columns are also useful when samples need to be processed rapidly, to avoid degradation.

Additional purification

His-tagged protein purification on BabyBio NiMAC gives high purity in a single 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. WorkBeads 40/100 SEC, WorkBeads 40/1000 SEC and WorkBeads 40/10 000 SEC resins facilitate the purification of target proteins of different sizes. WorkBeads 40S, WorkBeads 40Q and WorkBeads 40 DEAE resins are excellent for ion exchange chromatographic purification. These resins are also available as ready-to-use BabyBio Q, BabyBio S and BabyBio DEAE columns, as well as prepacked OptioBio™ 40S 10x100 and OptioBio 40Q 10x100 columns with 7.9 ml column volume.

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

Maintenance of the column

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 BabyBio NiMAC due to the very tight binding of the Ni^{2+} ions to the chelating groups immobilized on the matrix.

Storage

Equilibrate the column in 20% ethanol and close it securely using the included plug and cap. Store the column at 2 to 25°C.

Additional information

Intended use

BabyBio columns are intended for research and process development only. BabyBio columns shall not be used for preparation of material for clinical or diagnostic purposes.

Safety

Please read the associated Safety Data Safety (SDS) for BabyBio columns, and the safety instructions for any equipment to be used. Nickel salts are considered to be allergenic and potentially carcinogenic. Use recommended safety equipment. Note that the maximum backpressure of BabyBio columns are 0.3 MPa (3 bar, 43 psi).

Product information

	BabyBio NiMAC
Target substance	His-tagged proteins
Resin	WorkBeads NiMAC
Matrix	Rigid, highly cross-linked agarose
Average particle size[1] (D_{V50})	45 µm
Precharged metal ion	Nickel (II) ions, Ni^{2+}
Dynamic binding capacity[2]	> 40 mg His-tagged protein/ml resin
Column volume	1 ml 5 ml
Column dimensions	7 x 28 mm (1 ml) 13 x 38 mm (5 ml)
Recommended flow rate BabyBio 1 ml BabyBio 5 ml	 1 ml/min (150 cm/h) 5 ml/min (225 cm/h)
Maximum flow rate[3] BabyBio 1 ml BabyBio 5 ml	 5 ml/min (780 cm/h) 20 ml/min (900 cm/h)
Maximum back pressure	0.3 MPa, 3 bar, 43 psi
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), 8 M urea, 6 M guanidine-HCl, non-ionic detergents, 20% ethanol and 1 M NaOH
pH stability	2 – 14
Storage	2 to 25°C in 20% ethanol

1. The median particle size of the cumulative volume distribution.
2. The binding capacity is dependent on the size of the target protein, and on the competition with impurities.
3. Aqueous buffers at 20°C. Decrease the maximum flow rate if the liquid has a higher viscosity. Higher viscosities can be caused by low temperature (use half of the maximum flow rate at 4°C), or by additives (e.g. use half of the maximum flow rate for 20% ethanol).

Related products

Product name	Pack size[1]	Article number
Prepacked columns		
BabyBio NTA His-tag Screening kit 1 ml[2]	1 ml × 4	45 700 101
BabyBio NTA His-tag Screening kit 5 ml[2]	5 ml × 4	45 700 102
BabyBio IDA His-tag Screening kit 1 ml[2]	1 ml × 4	45 700 001
BabyBio IDA His-tag Screening kit 5 ml[2]	5 ml × 4	45 700 002
BabyBio Dsalt 5 ml	5 ml × 5	45 360 107
BabyBio S 5 ml	5 ml × 5	45 200 107
BabyBio Q 5 ml	5 ml × 5	45 100 107
BabyBio DEAE 5 ml	5 ml × 5	45 150 107
OptioBio 40S 10x100	7.9 ml × 1	55 420 011
OptioBio 40Q 10x100	7.9 ml × 1	55 410 011
Bulk resins		
WorkBeads NiMAC	25 ml	40 653 001
WorkBeads NiMAC	300 ml	40 653 003
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
WorkBeads 40S	25 ml	40 200 001
WorkBeads 40Q	25 ml	40 100 001
WorkBeads 40 DEAE	25 ml	40 150 001
WorkBeads 40/1000 SEC	25 ml	40 300 001
WorkBeads 40/1000 SEC	300 ml	40 300 003
Accessories		
Column plug male 1/16''	10	70 100 010
Column cap female 1/16''	10	70 100 020

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
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

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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