



INSTRUCTION

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 Ni²⁺ 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



Intended use

WorkBeads resins are developed and supported for both research and production-scale chromatography. WorkBeads resins are produced according to ISO 9001:2015, and Regulatory Support Files (RSF) are available to assist the process validation and submissions to regulatory authorities.

GoBio™ prepacked column family is developed for convenient, reproducible, and fast results and can be used from small scale purification through process development to full-scale manufacturing.

Safety

Please read the associated Safety Data Sheet (SDS) for WorkBeads NiMAC and the safety instructions for any equipment to be used.

Unpacking and inspection

Unpack the shipment as soon as it arrives and inspect it for damage. Promptly report any damage or discrepancies to complaints@bio-works.com

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 deionized water.
Before the first purification run do an initial wash with 0.5 M NaOH for 15 min and then 5 – 10 CV deionized water.
2. Equilibrate the column using 10 CV binding buffer.
3. Apply a clarified sample in the pH range 7.0 – 8.5. The sample should contain at least 10 mM imidazole.
4. Wash the column using 20 CV binding buffer.
5. 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.
6. Wash the column with 5 CV deionized water to remove the buffer salts.
7. 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 Ni²⁺, Co²⁺, Cu²⁺ and Zn²⁺, 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. GoBio Mini NiMAC columns are recommended for His-tagged protein purification when minimal Ni²⁺ leakage is necessary and when higher concentrations of DTT (20 mM) and EDTA (20 mM) need 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 GoBio Mini 1 mL and 5 mL columns.

Imidazole is recommended for elution. This is the most common 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.

Resin characteristics

WorkBeads NiMAC is an agarose-based chromatographic resin immobilized with chelating groups that bind nickel ions very tightly. The Ni²⁺-charged resin binds His-tagged proteins but has unusually low affinity for other host proteins that tend to bind other resins made for Immobilized Metal Ion Affinity Chromatography (IMAC).

The very tightly bound nickel ions result in extremely low nickel ion leakage. This property makes WorkBeads NiMAC excellent for purification of His-tagged proteins from large feed volumes containing chelating substances. Typical sources of this problem are extracts from eukaryotic cells, e.g., insect cells, that commonly contain reducing agents added during the extraction. WorkBeads NiMAC resin cannot be stripped of the Ni²⁺-ions and recharged.

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:

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

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.

Evaluation of the packed column

Test the column efficiency to check the quality of packing. Testing should be done after packing, at regular intervals during the usage of the column or when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_s). These values are easily determined by applying a sample such as 1% acetone solution to the column.

For optimal results, the sample volume should be 2.5% of the column volume (CV) and the flow rate 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Note: The calculated number of plates will vary according to the test conditions and should only be used as a reference value. Keep test conditions and equipment constant so that results are comparable. Changes of for example solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.

Measuring HETP and A_s

Calculate HETP and A_s from the UV curve (or conductivity curve).

$$\text{HETP} = \frac{L}{N}$$

$$N = 5.54 \times \left(\frac{V_R}{W_h} \right)^2$$

L = bed height (cm)

N = number of theoretical plates

V_R = volume eluted from the start of sample application to the peak maximum

W_h = peak width measured as the width of the recorded peak at half of the peak height

V_R and W_h are in the same units

The concept of reduced plate height is often used for comparing column performance. The reduced plate height, h , is calculated:

$$h = \frac{\text{HETP}}{d_{50v}}$$

d_{50v} = Median particle size of the cumulative volume distribution (cm)

As a guideline, a value of < 3 is very good.

The peak must be symmetrical, and the asymmetry factor as close to 1 as possible. (A typical acceptable range is $0.7 < A_s < 1.3$). A change in the shape of the peak is usually the first indication of bed deterioration.

Peak asymmetry factor calculation:

$$A_s = \frac{a}{b}$$

a = ascending part of the peak width at 10% of peak height

b = descending part of the peak width at 10% of peak height

Figure 2 on next page shows a UV trace for acetone in a typical test chromatogram from which the HETP and A_s values are calculated.

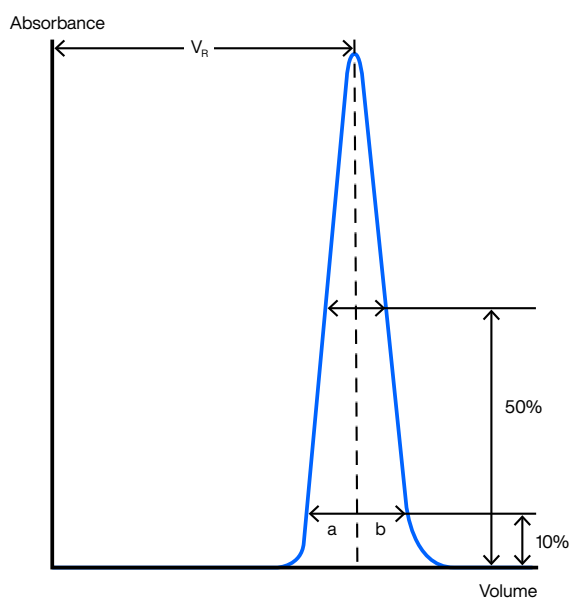


Fig 1. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

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 and/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 the concentration when the target protein 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

When using affinity chromatography it is recommended to do an initial wash with a Cleaning-in-Place (CIP) solution before the first purification to wash out any loosely bound ligands and/or Ni²⁺-ions to stabilize the binding capacity over time.

First wash out the storage solution (20% ethanol) with approx. 10 CV deionized water and then do an initial wash with 3 – 5 CV 0.5 M NaOH for 15 minutes followed by 5 – 10 CV deionized water.

After this wash with 5 – 10 CV binding buffer to get the pH back to the same as the binding buffer before loading the sample.

After sample application, remove unbound and loosely bound impurities by washing the column with 15 – 30 CV binding buffer or if needed a more stringent washing buffer, or until desired A₂₈₀ 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.

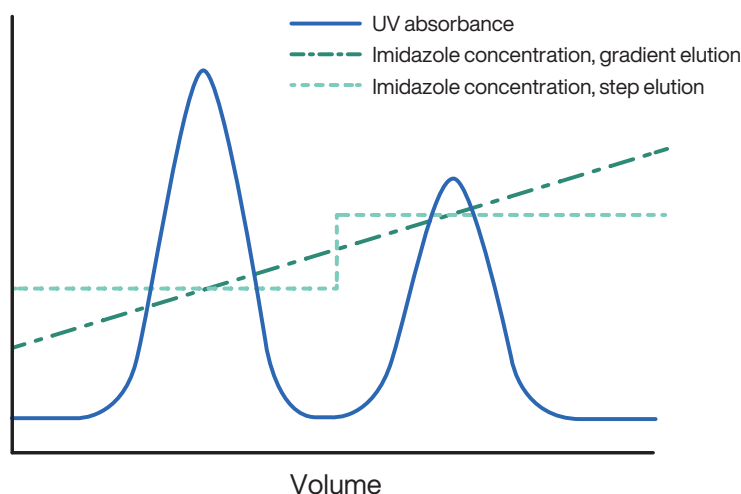


Fig 2. Optimization of step elution with imidazole. A test run with linear gradient elution gives information about suitable imidazole concentrations to be used in step elution.

Scale-up

After developing a chromatographic procedure in a small-scale column, e.g., 7 (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:

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

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., 7 × 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)
16x100	4	150	5.0
26x100	4	150	13.3
80x200	8	150	126
130x200	8	150	332
200x200	8	150	785
240x200	8	150	1131
330x250	10	150	2138

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 40/100 SEC, WorkBeads 40/1000 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, WorkBeads 40 DEAE and WorkBeads 40 TREN resins provide different selectivities for ion exchange chromatography.

These resins are also available as prepacked columns in different sizes in the GoBio column family.

Desalting and buffer exchange

Buffer exchange or desalting of a sample can be used before analysis and/or after purification. This can be carried out quickly and easily in lab-scale using GoBio Mini Dsalt 1 mL, GoBio Mini Dsalt 5 mL, GoBio Prep 16x100 Dsalt (20 mL) and GoBio Prep 26x100 Dsalt (53 mL) prepacked columns depending on sample volumes. GoBio Prod prepacked columns starting from 1 L are available for larger sample volumes, see "Related products".

These columns are very useful alternatives to dialysis for larger sample volumes or when samples need to be processed rapidly to avoid degradation.

Pre-swollen WorkBeads Dsalt is also available in bulk for packing column format of choice.

To find out more about Bio-Works' chromatography products visit www.bio-works.com

Maintenance 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 5 – 10 CV deionized H₂O in repeated cycles are recommended if resin gets fouled.

Storage

Store at 2 to 25°C in 20% ethanol.

Product description

WorkBeads NiMAC	
Target substance	His-tagged proteins
Matrix	Highly cross-linked agarose
Average particle size (D_{v50}) ¹	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 $\mu\text{mol Cu}^{2+}$ /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 $\text{Na}_2\text{-EDTA}$, 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.

GoBio preppacked column family

GoBio preppacked column family is developed for convenient, reproducible and fast results and includes columns with different sizes and formats.

GoBio Mini 1 mL and GoBio Mini 5 mL for small scale purification and screening using a shorter packed bed.

GoBio Screen 7x100 (3.8 mL) for reproducible process development including fast and easy optimization of methods and parameters.

GoBio Prep 16x100 (20 mL) and GoBio Prep 26x100 (53 mL) for lab-scale purifications and scaling up.

GoBio Prep 16x600 (120 mL) and GoBio Prep 26x600 (320 mL) for preparative lab-scale size exclusion chromatography.

GoBio Prod 80x200 (1 L), GoBio Prod 130x200 (2.7 L), GoBio Prod 200x200 (6 L), GoBio Prod 240x200 (9 L) and GoBio Prod 330x250 (21.4 L) for production-scale purifications.

Related products

Product name	Pack size ¹	Article number
Prepacked columns		
GoBio Mini NiMAC 1 mL	1 mL × 1	45 655 311
	1 mL × 5	45 655 313
	1 mL × 10	45 655 314
GoBio Mini NiMAC 5 mL	5 mL × 1	45 655 315
	5 mL × 5	45 655 317
	5 mL × 10	45 655 318
GoBio Mini Dsalt 1 mL	1 mL × 5	45 360 103
GoBio Mini Dsalt 5 mL	5 mL × 5	45 360 107
GoBio Screen 7x100 NiMAC ²	3.8 mL × 1	55 653 001
GoBio Prep 16x100 NiMAC ²	20 mL × 1	55 653 021
GoBio Prep 26x100 NiMAC ²	53 mL × 1	55 653 031
GoBio Prep 16x100 Dsalt ²	20 mL × 1	55 700 021
GoBio Prep 26x100 Dsalt	53 mL × 1	55 700 031
GoBio Prod 80x200 NiMAC ²	1 L	55 653 042
GoBio Prod 130x200 NiMAC ²	2.7 L	55 653 062
GoBio Prod 200x200 NiMAC ²	6 L	55 653 072
GoBio Prod 240x200 NiMAC ²	9 L	55 653 082
GoBio Prod 330x250 NiMAC ²	21.4 L	55 653 093
GoBio Prod 80x200 Dsalt ²	1 L	55 700 042
GoBio Prod 130x200 Dsalt ²	2.7 L	55 700 062
GoBio Prod 200x200 Dsalt ²	6 L	55 700 072
GoBio Prod 240x200 Dsalt ²	9 L	55 700 082
GoBio Prod 330x250 Dsalt ²	21.4 L	55 700 093
Bulk resins		
WorkBeads Dsalt	300 mL	40 360 003
	1 L	40 360 010

¹ All different pack sizes are available on www.bio-works.com

² Packed on request.

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
	5 L	40 653 050

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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