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This MACS® product is for *in vitro* research use only and not for diagnostic or therapeutic procedures.

ProCatch His Resin User Manual

Order no. 130-092-184
130-092-183
130-092-182

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1. Description

Components	ProCatch His Resin 10 mL (# 130-092-184) 25 mL (# 130-092-183) 100 mL (# 130-092-182)
Intended use	Purification of His-tagged proteins
Product format	ProCatch His Resin contains agarose supplied as 50% slurry in 20% ethanol storage buffer (pH 7.0).
Storage	Store resin as slurry in 20% ethanol at 4 °C.

ProCatch His Resin characteristics

Capacity	5–10 mg His-tagged protein/mL resin
Matrix	Sepharose™ 6B (GE Healthcare)
Bead size	45–165 µm
Max. linear flow rate*	80 cm/h
Max. operating pressure	14 psi = 0.1 MPa
pH stability	3–14

$$* \text{volumetric flow} \left(\frac{\text{mL}}{\text{min}} \right) = \frac{\text{volumetric flow} \left(\frac{\text{cm}^3}{\text{h}} \right) \times \text{column cross-sectional area} (\text{cm}^2)}{60 \left(\frac{\text{min}}{\text{h}} \right)}$$

The cover photo shows a replica of the DNA model built in 1953 by James D. Watson and Francis Crick at the Cavendish Laboratory in Cambridge. This model is located at Heureka, the Finnish Science Centre. Photography by Alexander Budde; © Miltenyi Biotec GmbH, Germany. Detailed information on the history of the Watson-Crick model can be found in: de Chadarevian, S. (2003) Relics, replicas and commemorations. Endeavour 27: 75–79.

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1.1 Background and product applications

The major goal in protein purification is to reach high protein purities quickly with a method that can be easily applied to diverse proteins. Purifying proteins with a combination of “classical” methods such as ion-exchange chromatography and gel filtration is problematic as each protein will require development of a customized purification protocol.

In contrast, the use of protein tags allows a one-step affinity chromatography purification independent of the protein itself. To influence the structure and function of the protein as little as possible, the tag should be short, and it should be placed either at the N- or C-terminus. The polyhistidine tag or His-tag consisting of 6–10 histidines is an established protein tag that fulfills these requirements, enabling a rapid and standardized purification of recombinant proteins.

Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) was first introduced methodically as a specific affinity technique for protein separation by Porath *et al.*¹ IMAC makes use of the reversible interaction between various amino acid side chains and immobilized metal ions. The binding of histidine side chains depends on the immobilized transition metal ions such as Ni²⁺, Co²⁺, Cu²⁺, Fe³⁺ or Zn²⁺.²⁻³ Exposed cysteine and tryptophan side chains may also be involved in binding to metal ions, but the adsorption efficiency is lower than with histidine. Several variants of IMAC have been developed for protein separation, matrix-assisted protein refolding and protein surface topography studies.⁴

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ProCatch His Resin

ProCatch His Resin is a high performance nickel-chelator agarose for the fast purification of recombinant proteins containing a histidine tag. The separation principle depends on the affinity between neighboring histidines (6–10) of the histidine tag and an immobilized metal ion (Ni²⁺). ProCatch His Resin technology is based on a novel tetradentate metal chelator for optimal binding of polyhistidine-tagged proteins.

The versatile chemistry developed by Miltenyi Biotec prevents leaching of metal ions during the purification process and is compatible with strong denaturants such as 8 M urea and reducing agents such as 20 mM β-mercaptoethanol. ProCatch His Resin allows stringent wash steps to remove unspecifically bound proteins and to elute pure target protein. The Resin is optimized for protein purification under native and denaturing conditions.

ProCatch His Resin shows high stability to a wide range of additives and is compatible with a variety of compounds in buffers (see 7. Appendix). ProCatch His Resin can be reused (see 3.6).

Product applications

ProCatch His Resin was developed for large scale, fast purification and high recovery of recombinant proteins containing a His-tag sequence. The ProCatch His Resin offers exceptional performance and is compatible with all prokaryotic and also eukaryotic expression systems. It was designed for multiple preparation formats such as batch - gravity flow purification, gravity flow purifications and low pressure column purifications.



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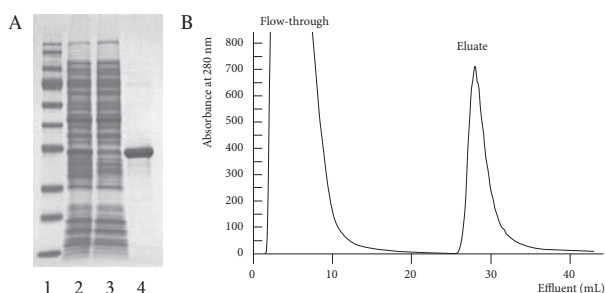


Figure 1: Purification of a 30 kDa histidine-tagged protein with ProCatch His Resin.

A: Marker (lane 1), lysate from cell extract (lane 2), the flow-through (lane 3) and the eluate (lane 4) were loaded onto a Coomassie-stained SDS gel.

B: Corresponding chromatogram. Proteins leaving the column were monitored by measuring absorbance at 280 nm.

1.2 Reagent and instrument requirements

Required buffers

Stock solutions

- 100 mM sodium phosphate buffer (preparation at 25 °C, see table 1)
- 3 M NaCl
- 0.5 M imidazole

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Buffers for native purification

- 1× Native Binding / Wash Buffer (also used as Lysis Buffer):
50 mM sodium phosphate, 300 mM NaCl (pH 8.0)
- 1× Native Imidazole Buffer:
50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole (pH 8.0)
- 1× Native pH Elution Buffer:
50 mM sodium acetate, 300 mM NaCl (pH 5.0)

Buffers for denaturing purification

▲ Do not autoclave denaturing buffers. The pH of denaturing buffers should be adjusted prior to use due to dissociation of urea.

- 1× Denaturing Binding / Wash Buffer (also used as Lysis Buffer):
50 mM sodium phosphate, 8 M urea, 300 mM NaCl (pH 8.0)
- 1× Denaturing Imidazole Elution Buffer:
50 mM sodium phosphate, 8 M urea, 300 mM NaCl, 150 mM imidazole (pH 8.0)
- 1× Denaturing pH Elution Buffer (prepare fresh prior to use):
50 mM sodium acetate, 8 M urea, 300 mM NaCl (pH 5.0)



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Table 1: Dilution scheme for 0.1 M sodium phosphate buffer

pH	Volume of 0.5 M Na ₂ HPO ₄ (mL)	Volume of 1 M NaH ₂ PO ₄ (mL)
7.0	115.4	42.3
7.2	136.8	31.6
7.4	154.8	22.6
7.6	169.0	15.5
7.8	179.2	10.4
8.0	186.4	6.8
Data from ISCO (1982)		

Dilute the combined stock solutions to 1000 mL final volume with distilled H₂O. The pH is calculated according to the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK}' + \log\left[\frac{(\text{proton acceptor})}{(\text{proton donor})}\right] \quad \text{where } \text{pK}' = 6.86 \text{ at } 25^\circ\text{C}.$$

Required reagents

- Imidazole
- (Optional) Nuclease, e.g. Benzonase® Nuclease (Novagen, # 71205), or DNase I
- (Optional) β-Mercaptoethanol (β-ME)
- (Optional) 5 mg/mL NiCl₂ (for regeneration of resin)
- (Optional) Triton X-100® (for lysis of eukaryotic cells)

Required disposables

- Gravity flow column with end caps (for gravity flow purification)
- Chromatography column (for low pressure column purification)

Required instruments

- Chromatography system (for automated low pressure column protein purification)
- Ultrasonic homogenizer (sonicator)
- MACSmix™ Tube Rotator (# 130-090-753)

1.3 Related products

For highly pure small scale isolation of His-tagged proteins, the μMACS His Protein Isolation Kit (# 130-091-124) is recommended. This kit allows the magnetic affinity purification based on monoclonal antibodies.

2. Purification strategy

▲ To prevent protein degradation of native proteins, perform all work, especially the lysis, on ice (4–8 °C).

▲ Proteinase inhibitors can be added to the Binding/Wash Buffer. For details, refer to the appendix where appropriate inhibitors and effective concentrations are listed.

▲ To obtain the desired bed volume, use twice the volume of 50% slurry (e.g. 2 mL of 50% resin slurry will result in a resin bed volume of 1 ml).

▲ Reducing agents, such as 10 mM β-Mercaptoethanol (β-ME), can be included in the Binding/Wash Buffer to prevent protein aggregation during preparation. Use up to 25 mM β-ME as maximum final concentration.

▲ **Do not use DTT or DTE**, since these reducing agents are not compatible with this protocol.

▲ If β-ME cannot be used, up to 10 mM reduced glutathione may be used in combination with ProCatch His Resin.

Protein purification using ProCatch His Resin

Generally, ProCatch His Resin is optimized for working with transformed *E. coli* cultures or insect cells. Additionally, ProCatch His Resin can be used for large scale protein isolations from eukaryotic cells.

The following table gives an overview of possible protein purification methods and applications.

Table 2: Protein purification with ProCatch His Resin

Method	Recommended use
Batch - Gravity flow (see 3.2)	Purification with more than 1 mL resin bed volume (i.e. more than 2 mL resin slurry)
Gravity flow (see 3.3)	Rapid purification with up to 1 mL resin bed volume
Automated low pressure (see 3.4)	Flow-regulated column purification in chromatography system
Test scale (see 3.5)	Test of expression level of target protein

A first experimental trial can be started with 2 mL of ProCatch His Resin suspension (which equals 1 mL resin bed volume) per 6 mg of anticipated polyhistidine-tagged protein.

Buffers

Optimal buffer conditions and elution strategy depend on the recombinant protein of interest.

Increasing the ionic strength of the Binding/Wash Buffer, e.g. by increasing the concentration of NaCl up to 0.5 M, can reduce non-specific binding of proteins.

If the protein of interest is unstable at pH 8, the purification can be carried out using buffers with pH 7.

Choosing buffers for sample preparation

The solubility of the protein of interest and the planned downstream application are important factors to decide between native or denaturing buffers.

Buffers for native purification

Native buffers provide conditions to preserve the native surface charge of the protein and its functions. They are recommended if the protein is soluble and functional active protein should be eluted for downstream assays such as enzymatic tests.

Buffers for denaturing purification

Proteins overexpressed in prokaryotes are sometimes insoluble and aggregated in so-called inclusion bodies. To increase the solubility of a protein, a purification under denaturing conditions using a denaturing agent such as 8 M urea, can be performed.

▲ **Note:** Using high concentrated guanidinium solutions (6 M) as a denaturing agent is not compatible with SDS-PAGE: a precipitate is formed when loading samples containing guanidinium on an SDS polyacrylamid gel. To prevent precipitation, dialyze such samples in a buffered solution containing 8 M urea overnight before gel-loading.

Use either buffers for native purification or buffers for denaturing purification throughout the entire process (cell lysis, protein purification). After purification yield and purity of the protein can be checked by SDS-PAGE analysis.

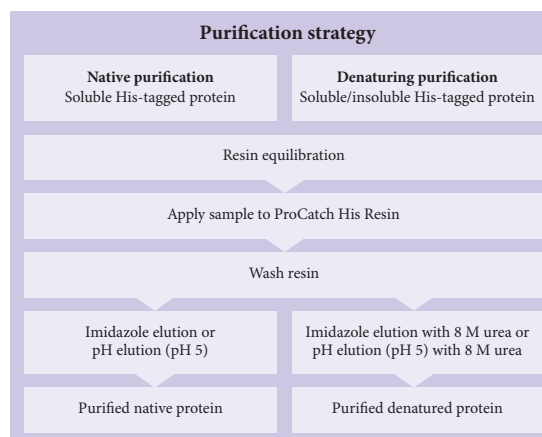


Figure 2: Strategy scheme for the purification of His-tagged proteins.

Choosing buffers for protein purification and elution

Elution of recombinant His-tagged proteins from ProCatch His Resin can be performed by addition of imidazole or a pH shift.

Imidazole in Binding/Wash Buffer reduces non-specific binding of proteins to the resin. A concentration of up to 5–10 mM imidazole in Binding/Wash Buffer generally improves protein purity; however, compatibility with the target protein has to be checked for each individual

protein. If Binding/Wash Buffer with imidazole is also used for cell lysis, the yield of protein in the eluate may be lower.

▲ Storage (especially freezing) of the sample in Elution Buffer containing imidazole can promote precipitation of the purified native protein. To prevent precipitation, dialyze the fractions overnight with Binding/Wash Buffer before freezing or use ultrafiltration or gel filtration with sephadex columns.

Alternatively, polyhistidine-tagged proteins that are stable in the pH range of 5.0–7.0 can be eluted without imidazole using a pH shift to pH 5 using the pH Elution Buffer.

▲ Imidazole like proteins absorbs at 280 nm in a spectrophotometer. This can cause problems when calculating the protein concentration. Protein concentration of eluted protein should be determined by Bradford assay⁵ and SDS-PAGE.

▲ The purity of His-tagged proteins may be optimized by using step-gradients for elution, e.g. 20 mM, 40 mM, 75 mM and 150 mM imidazole. This has to be determined for each protein.

Volumes

The anticipated yield and expression rate of the His-tagged protein depends on the protein itself and the expression system used. Therefore buffer volumes have to be adjusted empirically.

Concerning the buffer volumes in this manual, it is advisable to start with 2 mL of Binding/Wash Buffer for 20–25 mL of *E.coli* culture for purification of a polyhistidine-tagged protein.

Lysis

The lysis is one of the most crucial steps during protein purification. For special applications the Binding/Wash Buffer has to be adapted. Factors such as ionic strength, pH, the concentration and type of detergent, the presence of divalent cations, cofactors, and stabilizing ligands influence the effectiveness of the Binding/Wash Buffer. Generally, the buffer should not contain SDS as it denatures proteins.

▲ Do **not** use lysozyme for lysis of the cells because it may interfere with the functionality of the target protein.

▲ Do **not** use EDTA and EGTA because they strip Ni²⁺ from the resin.

▲ Harvested eukaryotic cells should be lysed in an appropriate buffer containing a mild detergent⁶, e.g. Triton X-100.

▲ When sonicating cells for lysis, check viscosity of the sample. Genomic DNA in the solution, which is not completely sheared, increases sample viscosity and can affect the recovery of the protein. Decrease the sample's viscosity by degradation of DNA and RNA with nuclease (as recommended by the manufacturer; be aware that nucleases require Mg²⁺). This should improve the yield of protein. Alternatively, dilute the sample five-fold with Binding/Wash Buffer before applying it to the resin.

For lysis of yeast cells or bacteria we refer to any of the common protocols found in the literature⁶.

3. Protocols

3.1 Sample preparation

3.1.1 Lysate from prokaryotic cells: native or denatured purification

The expression rate of proteins is highly variable and must be determined empirically, e.g. by SDS-PAGE. Typical amounts of expressed proteins are in the range from 5–50 mg per liter culture volume. One mL of ProCatch His Resin bed volume is equal to 2 mL of the 50% slurry used and can bind up to 10 mg polyhistidine-tagged protein. The binding capacity of the resin depends on the specific protein. Calculate the bed volume of ProCatch His Resin for the amount of cells used for purification and for the anticipated yield of protein.

Native purification:

Preparation: Pre-cool 1× Native Binding / Wash Buffer (4 °C).

1. Centrifuge the cell culture at 3,000–5,000×g for 15 min at 4 °C to harvest the cells. Decant the supernatant.
 2. Resuspend the cell pellet by vortexing in 2 mL pre-cooled 1× Native Binding / Wash Buffer (4 °C) per 25 mL culture.
(Optional) Add protease inhibitors such as PMSF and leupeptin (see 7.3).
 3.
 - For sample volume up to 200 mL: sonicate the sample 3×10 sec, interrupted by a 30-sec pause on ice between each sonication step.
 - For sample volume ≥200 mL: sonicate the sample 3×30 sec, interrupted by a 2-min pause on ice between each sonication step.
- ▲ **Note:** Excessive sonication can destroy protein functionality.

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4. (Optional) If the viscosity of the lysate is high, add nuclease, e.g. 1 µL (25 units) Benzonase Nuclease per mL of buffer and incubate for 15 min on ice.
5. Centrifuge the lysate at 10,000–15,000×g for 30 min at 4 °C to pellet the debris.
6. Transfer the supernatant carefully to a fresh tube. Do not disturb the pellet. The clarified sample contains the protein which can be analyzed by taking a small part (1–10 µL) for SDS-PAGE analysis.
7. Continue with protein purification (see 3.2–3.5).

Denaturing purification:

1. Centrifuge the cell culture at 3,000–5,000×g for 15 min at 4 °C to harvest the cells. Decant the supernatant.
2. Resuspend the cell pellet by vortexing in 2 mL of (optional) pre-cooled 1× Denaturing Binding / Wash Buffer (4 °C, containing 8 M urea) per 25 mL of culture.
3. Gentle agitate the sample until it becomes translucent.
4. (Optional) If the viscosity of the lysate is high, add 1 µL (25 units) Benzonase Nuclease per mL of buffer and incubate for 15 min on ice.
▲ **Note:** Benzonase Nuclease works in urea.
5. Centrifuge the lysate at 10,000–15,000×g for 30 min at 4 °C to pellet the cell debris.
6. Transfer the supernatant carefully to a fresh tube. Do not disturb the pellet. The clarified sample contains the protein which can be analyzed by taking a small part (1–10 µL) for SDS-PAGE analysis.



3.1.2 Lysate from eukaryotic cells

The expression rate of proteins is highly variable and must be determined empirically. For insect cells, 2–20 mg fusion protein from 10⁸ cells can be expected. One mL of ProCatch His Resin bed volume is equal to 2 mL of the 50% slurry used and can bind up to 10 mg polyhistidine-tagged protein. Calculate the bed volume of ProCatch His Resin for the amount of cells used for purification and for the anticipated yield of protein.

1. Prepare Native Binding / Wash Buffer for lysis: Add Triton X-100 to Native Binding / Wash Buffer to a final concentration of 1% and use 1 mL of the buffer per 10⁷ cells. Pre-cool at 4 °C
(Optional) Add protease inhibitors such as PMSF and leupeptin (see 7.3).
2. Transfer cells to a centrifugation tube and centrifuge for 5 min at 300×g at 4 °C.
3. Remove supernatant. Place the tube containing the cell pellet on ice and add 1 mL of Native Binding / Wash Buffer containing 1% Triton X-100. Mix well by vortexing.
4. Incubate on ice for 15 min with occasional vortexing.
5. Centrifuge at 10,000–15,000×g for 30 min at 4 °C to sediment the cell debris.
6. Transfer the supernatant to a fresh tube and proceed with protein purification (see 3.2–3.5).

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3.2 Batch – gravity flow column protein purification

This protocol is for protein purification with more than 1 mL resin bed volume. One bed volume of ProCatch Resin (e.g. 1 mL) is obtained by using double the volume (e.g. 2 mL) of resuspended resin.

▲ Depending on your lysate, use either buffers for native or denaturing purification.

3.2.1 Preparation of the ProCatch His Resin

1. Resuspend the ProCatch His Resin thoroughly.
2. Transfer the required amount of resin suspension to a sterile tube that has enough volume for 10–20 times the resin bed volume.
3. Pellet the resin by centrifugation at 700×g for 2 min.
4. Discard the supernatant.
5. Pre-equilibrate the resin by adding 10 volumes of 1× Binding / Wash Buffer and briefly mixing.
6. Pellet the resin by centrifugation at 700×g for 2 min and discard the supernatant.
7. Repeat steps 5 and 6.

3.2.2 Batch - gravity flow column His-tagged protein purification

1. The cell lysate can now be added to the ProCatch His Resin.



2. Put the sample into the MACSmix Tube Rotator and rotate for 20 min to bind the target protein to the resin.
3. Pellet the resin by centrifugation at 700×g for 5 min.
4. Carefully remove as much supernatant as possible. Do not touch the pellet.
5. Resuspend the resin by adding 10–20 volumes of 1× Binding/Wash Buffer. Rotate the sample for 10 min by using the MACSmix Tube Rotator.
6. Pellet the resin by centrifugation at 700×g for 5 min.
7. Discard the supernatant.
8. Repeat steps 5–7.
9. Add one volume of the 1× Binding/Wash Buffer to the resin and resuspend by vortexing.
10. Fill a 2 mL gravity flow column (with end-cap) with the resin and remove air bubbles. Let the resin settle down.
11. Allow the buffer to drain by removing the end-cap of the column until the buffer surface reaches the top of the resin bed.
12. (Optional) For more stringent wash conditions, wash the column with 5 volumes of 1× Binding/Wash Buffer containing 5–10 mM imidazole.
13. Add 5 volumes of 1× Elution Buffer to the column to elute the polyhistidine-tagged protein. Collect the eluate in 0.5–1 mL fractions.

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14. To determine which fraction contains the highest amount of the polyhistidine-tagged protein, a Bradford protein assay and SDS-PAGE analysis can be performed. In most cases, the majority of the protein will be found in the fractions containing the first two bed volumes.

3.3 Gravity flow column protein purification

This protocol is for protein purification with less than 1 mL resin bed volume. One bed volume of ProCatch Resin (e.g. 1 mL) is obtained by using double the volume (e.g. 2 mL) of resuspended resin.

▲ Depending on your lysate, use either buffers for native or denaturing purification.

3.3.1 Preparation of ProCatch His Resin in the column

1. Resuspend the ProCatch His Resin thoroughly.
2. Transfer appropriate volume of the resuspended resin to the disposable column sealed with an end cap.
3. Remove air bubbles. Let the resin settle down completely in the column and let the liquid pass through.
4. Apply 10 resin bed volumes of 1× Binding/Wash Buffer to equilibrate the column. Avoid air bubbles and do not disturb the resin.
5. Continue with the purification.



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3.3.2 Gravity flow column protein purification

1. Apply your sample to the column.
2. Wash the column with 10 bed volumes of 1× Binding/Wash Buffer.
 - ▲ Note: (Optional) Use 5–10 mM imidazole in 1× Binding/Wash Buffer for more stringent wash conditions.
3. Add 5 resin bed volumes of 1× Elution Buffer to the column to elute the polyhistidine-tagged protein. Collect the eluate in 0.5–1 mL fractions.
4. To determine which fraction contains the highest amount of the polyhistidine-tagged protein, a Bradford protein assay and SDS-PAGE analysis can be performed. In most cases, the majority of the protein will be found in the fractions containing the first two bed volumes.

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3.4 Automated low pressure column protein purification

This protocol is for flow-regulated column protein purification in chromatography systems.

▲ Depending on your lysate, use either buffers for native or denaturing purification.

▲ Use a flow rate of 75 cm/h for column packing and a flow rate of 30–60 cm/h for all other steps. A flow rate >80 cm/h will damage the resin.

▲ It is recommended to load the sample at a flow rate of 30–60 cm/h. If the protein does not bind, reduce the flow rate. Do not change the flow rate between equilibration and sample loading.

▲ If the target protein is unstable at room temperature, perform the chromatography at 4 °C.

Assemble the column according to the manufacturer's instructions.

1. Resuspend the ProCatch His Resin thoroughly and pour the slurry into the column. Avoid air bubbles in the column.
2. Let the resin settle down. The process can be accelerated by applying a pump attached to the column output which allows the buffer to flow through. For packing, use a linear flow rate of 75 cm/h. Do not allow the resin to dry out.
3. Adjust the top adaptor to the column and connect it to the chromatography system according to manufacturer's instructions.



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4. Equilibrate the column with 5 column volumes 1× Binding/Wash Buffer. Check the optical density of the eluate at 280 nm. Wash with 1× Binding/Wash Buffer until the absorbance reaches the baseline.
▲ **Note:** Do not exceed a flow rate of 60 cm/h for equilibration.
5. Centrifuge the sample at $\geq 15,000\times g$ for 20–30 min at 4 °C and/or filter the sample through a 0.45 μm filter and apply the pretreated sample to the column. Start collecting the fractions.
6. Wash the column with 1× Binding/Wash Buffer, until the baseline (280 nm) is stable (approx. 5–10 column volumes).
7. Elute the target protein by applying 5 column volumes of 1× Elution Buffer. Usually the polyhistidine-tagged protein elutes in the fractions containing the second and third column volumes. Collect the eluate in appropriate fraction volumes.
8. Check which fraction contains the majority of the polyhistidine-tagged protein by Bradford protein assay and SDS-PAGE analysis.

3.5 Test scale protein purification

This quick protocol for bacterial lysates is intended to:

- check for the presence of the polyhistidine-tagged protein.
- examine the expression level of the protein.

In this protocol denaturing conditions with 8 M urea are used.

Please note that the test scale purification is not optimized for highest purity.

▲ It is recommended to take a small sample (10–50 μL) after each critical step and to analyze these samples by SDS-PAGE to monitor the experimental procedure.

1. Transfer 1 mL bacterial cell culture to a microcentrifuge tube.
2. Harvest the cells by centrifugation at $14,000\times g$ for 2 min at 4 °C.
3. Discard the supernatant.
4. Add 500 μL of 1× Denaturing Binding/Wash Buffer.
5. Lyse cells by vortexing until the pellet is completely dissolved.
6. Centrifuge the lysate at $14,000\times g$ for 5 min.
7. Save a 50 μL aliquot of the supernatant for analysis. Transfer the supernatant to a clean microcentrifuge tube containing 50 μL of pre-washed ProCatch His Resin (prepare the resin according to 3.2.1 by using a starting volume of 100 μL slurry).

8. Add lysate to resin. Gently mix the sample at room temperature for 10 min using the MACSmix Tube Rotator.
9. To pellet the resin with bound protein, centrifuge at $14,000\times g$ for 1 min.
10. Carefully remove the supernatant and save a 10 μL aliquot for analysis.
11. Add 1 mL of 1× Denaturing Binding/Wash Buffer.
12. Vortex briefly until the solution is resuspended.
13. To pellet the resin, centrifuge at $14,000\times g$ for 1 min.
14. Discard the supernatant and save a 10 μL aliquot for analysis.
15. Repeat steps 11–14.
16. Add 50 μL of 1× Denaturing Elution Buffer to the pellet and vortex briefly to elute the bound polyhistidine-tagged protein.
17. Centrifuge at $14,000\times g$ for 1 min.
18. Carefully transfer the supernatant to a fresh tube. This fraction contains the polyhistidine-tagged protein.
19. Repeat steps 16–18.
20. Analyze fractions of interest by performing SDS-PAGE analysis.

3.6 Regeneration of ProCatch His Resin

ProCatch His Resin can be regenerated for reuse. Two protocols are available for regeneration with acetic acid or EDTA, respectively. EDTA regeneration strips off Ni^{2+} from the resin. To avoid cross-contamination it is only recommended with identical samples.

3.6.1 Stripping off protein contaminations with acetic acid

Required: 200 mM acetic acid and ddH_2O . The regeneration can be done in a Falcon tube or in a column.

Protocol for Falcon tubes

1. Transfer the resin into a fresh Falcon tube.
2. Add 5 mL of 200 mM acetic acid per 1 mL bed volume resin and rotate in the MACSmix Tube Rotator for 15 min.
3. Centrifuge at $1,000\times g$ for 5 min and discard the supernatant.
4. Wash with 5 mL ddH_2O and centrifuge at $1,000\times g$ for 5 min.
5. Measure the pH of the supernatant and discard it.
6. Repeat steps 4 and 5 at least two times until the pH of the supernatant is between pH 5.5 and 7.0.

Protocol for columns

1. Wash column with 5 mL of 200 mM acetic acid per 1 mL bed volume resin.

- Wash at least three times with 5 mL ddH₂O until the pH of the flow-through is between 5.5 and 7.0.

3.6.2 Stripping off protein contaminations and Ni²⁺ with EDTA

Required: 100 mM EDTA solution pH 11 (use EDTA disodium or tetrasodium salt), NiCl₂ solution (5.0 mg/mL) and ddH₂O. The regeneration can be done in a Falcon tube or in a column.

Protocol for Falcon tubes

- Transfer the resin into a fresh Falcon tube.
- Add 5 mL of 100 mM EDTA solution per 500 µL bed volume resin and rotate in the MACSmix Tube Rotator for 15 min.
- Centrifuge at 1,000×g for 5 min and discard the supernatant.
- Wash with 5 mL H₂O bidest. and centrifuge at 1,000×g for 5 min.
- Measure the pH of the supernatant and discard it.
- Repeat steps 4 and 5 at least two times until the pH of the supernatant is between pH 5.5 and 7.0.
- Add 5 mL of NiCl₂ solution per 500 µL bed volume resin.
- Rotate for 5–10 min in the MACSmix Tube Rotator.
- Centrifuge at 1,000×g for 5 min and discard the supernatant.
- Wash with 5 mL H₂O bidest. and centrifuge at 1,000×g for 5 min.
- Measure the pH of the supernatant and discard it.

- Repeat steps 10 and 11 at least two times until the pH of the supernatant is between pH 5.5 and 7.0.

Protocol for columns

- Add 5 mL of 100 mM EDTA solution per 500 µL bed volume resin.
- Wash at least three times with 5 mL H₂O bidest. until the pH of the flow-through is between 5.5 and 7.0.
- Add 5 mL of NiCl₂ solution per 500 µL bed volume resin.
- Wash at least three times with 5 mL H₂O bidest. until the pH of the flow-through is between 5.5 and 7.0.

4. Tips & hints

Detergents—these are partially hydrophobic and partially hydrophilic and can solubilize membranes and membrane proteins. They can be used to increase protein solubility and to decrease aggregation. Non-ionic detergents such as Triton X-100 and Tween 20 tend to be more gentle in their actions than ionic detergents and are more suitable for protein-protein interaction studies.

Salts—increasing the salt concentration in the buffer will decrease ionic interactions between proteins in a cell lysate. Up to 500 mM NaCl may be helpful to reduce binding of non-specific proteins.

pH—increasing or decreasing the pH of the buffer will change the net charge of the proteins depending on their pI and therefore influence the extent of non-specific ionic interactions. For an effective protein binding, the Binding/Wash Buffer should have a pH of 7.0–8.0. Lowering the pH below 7.0 results in protonation of His residues and elution of the protein.

DTT and β-ME—these reducing agents are often used to prevent loss of enzyme function via oxidation and to prevent protein aggregation due to malformed disulfide bridges during protein isolation. β-ME and reduced glutathione are compatible with ProCatch His Resin whereas DTT cannot be used.

EDTA—this chelation agent binds divalent cations and can be used to prevent the action of certain enzymes that require ions such as Mg²⁺ or Ca²⁺. It can also prevent protein-protein interactions that are dependent on the presence of divalent cations. EDTA strips off Ni²⁺ from the resin.

Phosphatase inhibitors—when active kinases or phosphorylated proteins are to be isolated, we recommend the addition of 1 mM activated sodium orthovanadate (not compatible with reducing agents) and 1–10 mM sodium fluoride to inhibit phosphatase activity.

5. Troubleshooting

Viscosity of the sample is too high

High concentration of nucleic acid in the lysate:

- continue sonication until the viscosity is reduced, add nuclease and incubate on ice for 10–15 min.
- dilute the sample 1:5 with additional Binding / Wash buffer.

Protein of interest is found in the flow-through fraction

His-tag is not present:

- make sure that the tag is in frame at a correct position (usually at the N- or the C-terminus). Check the sequence of the reading frame for internal start or termination sites.

His-tag is not accessible:

- if the protein fails to bind under native conditions, purification under denaturing conditions can provide the necessary accessibility. If a purification under denaturing conditions is successful, cloning the tag to the other terminus of the target protein may increase tag exposure, which is necessary for a successful native purification.

Inadequate binding conditions:

- check pH and the composition of all buffers. Make sure that no chelating agents such as EDTA are present in the buffers. Check compatibility of reagents (see 7. Appendix).
- decrease the concentration of imidazole in the Binding / Wash Buffer.

Protein of interest is found in the wash fraction

Wash stringency is too high:

- lower the imidazole concentration in the Wash Buffer.

Inadequate wash buffer:

- check pH and composition of the Wash Buffer. If chelating agents (e.g. EDTA) are present the protein may elute during the wash.

No polyhistidine-tagged protein is found in any fraction

No protein is expressed:

- check cell lysate for presence of the His-tagged protein by SDS-PAGE and Western blot against His-tag.

Sonication is insufficient:

- check cell disruption by measuring the release of nucleic acids at 260 nm. (A solution containing 50 µg per mL of double stranded DNA has an absorbance (optical density) of 1.0 at a wave length of 260 nm). Use longer sonication times.

Protein is insoluble in inclusion bodies:

- use protocol for denaturing purification with buffers containing 8 M urea. If the protein of interest in inclusion bodies is not solubilized by urea, try using 6 M guanidinium-Cl instead.

Inadequate elution conditions:

- if elution with 150 mM imidazole or pH 5 is not successful, try eluting with 250 mM imidazole. If protein is still bound, use 100 mM EDTA, pH 11, to strip off the resin. If this procedure still does not help, the protein may be precipitated on the resin. In this case, add 1–5 mM β-ME to all buffers before loading.

Protein degraded during extraction:

- add protease inhibitors (see 7. Appendix).
- work on ice when preparing cell lysates.
- use less stringent sonication conditions.

Eluted protein contains contaminations

Not enough Wash / Binding Buffer used:

- use larger volumes of wash buffer.

Wrong column size:

- if the bed volume is too large, reduce the amount of ProCatch His Resin. For highest purity, all metal chelate sites should be occupied by His-tagged proteins. Therefore, slightly overloading the resin with His-tagged protein is advisable.

Binding and wash conditions are not stringent enough:

- use 5–10 mM imidazole in Wash / Binding Buffer. In some cases, step-gradients for elution may be helpful, e.g. 20 mM/ 40 mM/ 75 mM/ 150 mM imidazole.

Excessive non-specific interactions:

- increase the final concentration of NaCl up to 0.5 M.
- add non-ionic detergent (e.g. up to 2% Triton X-100 or 2% Tween 20) to Wash / Binding Buffer.

Contaminants are bound to the target protein via disulfide bridges:

- add up to 20 mM β-Mercaptoethanol to the Binding / Wash Buffer to reduce the disulfide bonds.

Histidine-rich proteins are co-purified with the polyhistidine-tagged protein:

- use a second purification method, e.g. purification with a monoclonal antibody using the µMACS His Isolation Kit.
- eukaryotic cells contain histidine-rich proteins. Therefore, use prokaryotic expression systems if possible.

Column matrix is blocked

Cellular debris clogged the column frit / filter:

- change the frit / filter.
- completely remove the resin from the clogged column and resuspend it. Transfer the resin into a fresh column.
- centrifuge the sample at 12,000×g for 20–30 min at 4 °C and / or filter the sample through a 0.45 µm filter before applying it to the column.

Protein has precipitated in the column:

- try regeneration procedure (see 3.6).

Color of the resin changes

Resin changes from blue to white:

- the Ni²⁺ ions are removed due to the presence of chelators (e.g. EDTA) in the sample. Remove chelators by gel filtration and regenerate resin with NiCl₂ (see 3.6).

Resin turns to grey or brown:

- the resin is exposed to reducing agents such as DTE or DTT. Replace the reducing agents completely by gel filtration and regenerate resin with NiCl₂ (see 3.6). Use β-ME or reduced glutathione instead.

6. References

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3. Sulkowski, E. (1985) Purification of proteins by IMAC. *Trends Biotechnol.* 3: 1–7.
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7. Appendix

7.1 Compatible reagents

Reagent	Acceptable concentration
β-Mercaptoethanol (β-ME)	25 mM
Glutathione (reduced)	10 mM
Triton X-100	2%
Tween 20	2%
Urea	8 M
Guanidinium-Cl	6 M
NaCl	2 M
Imidazole	250 mM
MES	20 mM
MOPS	50 mM
HEPES*	50 mM
Tris*	50 mM
Acetic acid	200 mM (regeneration)

* Tris and HEPES are not the buffers of choice for metal chelate chromatography because they contain amino groups that interact with metal chelate resins.

7.2 Incompatible reagents

Reagent	Concentration
DTT (dithiothreitol)	any
DTE (dithioerythritol)	any
EDTA (ethylenediaminetetraacetic acid)	any
EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid)	any

7.3 Protease inhibitors

Inhibitor	Final concentration	Stock solution*
Aprotinin	0.3 μM	1 mg/mL (= 500×) in H ₂ O (pH 7)
E-64	10 μM	0.36 mg/mL (= 100×) in 1:1 mixture H ₂ O:EtOH (pH 7)
Leupeptin	10 μM	5 mg/mL (= 1000×) in H ₂ O (pH 7)
PMSF	1 mM	17 mg/mL (= 100×) in EtOH, isopropanol or methanol; inactivated by DTT

* Store stock solutions at -20 °C (except for PMSF: store at room temperature).

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