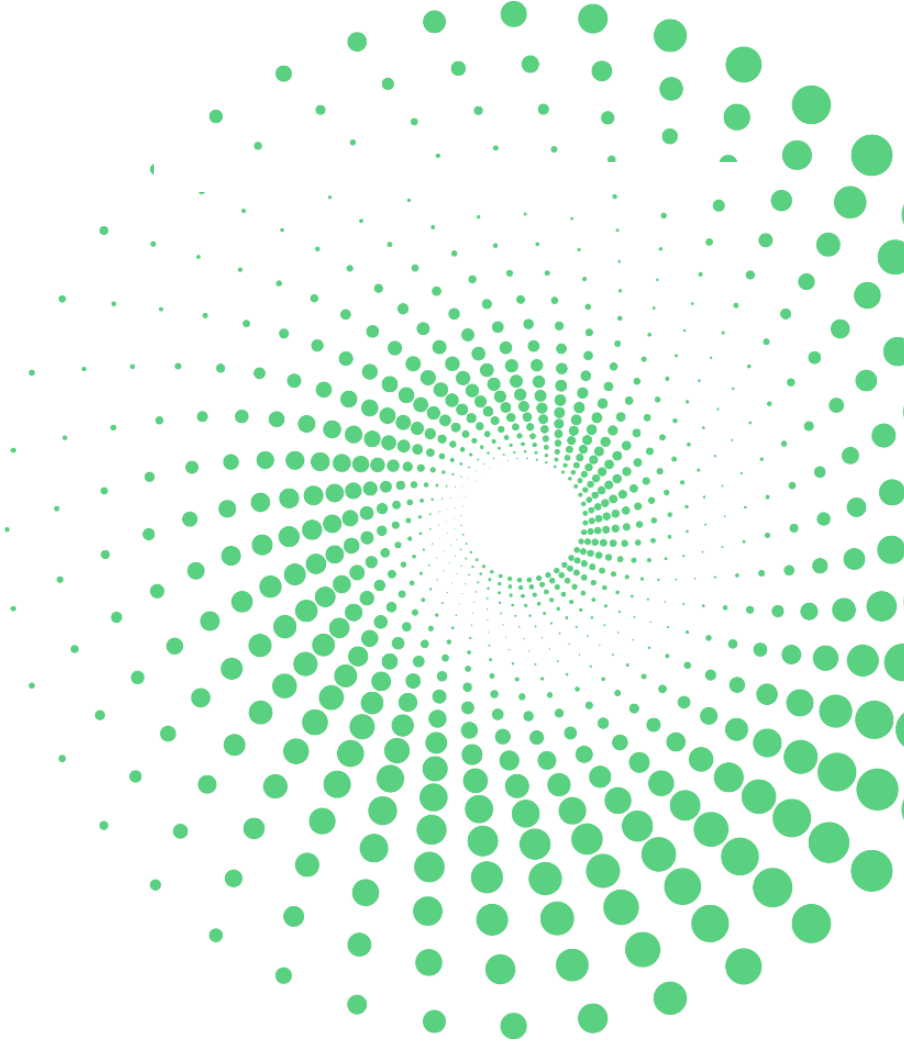

Instructions

His-tag Affinity Microsphere, Nickel ion, Ultra-flux

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GMExpression
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1. General information

Immobilised Metal Ion Affinity Chromatography (IMAC) leverages the specific affinity interactions between chelated transition metal ions and the side chains of specific amino acids, predominantly histidine, on proteins. Typically, Ni^{2+} is the preferred metal ion for purifying histidine-tagged proteins, although Cu^{2+}, Co^{2+}, and Zn^{2+} can also be used in IMAC resins for various applications, offering different selectivity and binding strengths.

1.1. GMExpression's Ultra-Flux Affinity Microsphere

Ultra-Flux Affinity Microsphere (UFAM) is an advanced protein purification matrix that provides high flow rate tolerance, high binding capacity and high resolution. This chromatography microsphere is prepared by using high-density polyacrylate microspheres as a core and various functional ligand groups or ligands cross-linked on its surface. UFAM is particularly suitable for purifying target proteins directly from supernatants (e.g. cell lysate supernatants) containing metal chelators such as EDTA and other components.

1.2. His-tag Affinity Microsphere

The microsphere resin consists of 50 µm beads with a highly cross-linked PA core, and the pore size is 80 nm. A chelating group is coupled to this core and charged with nickel (Ni^{2+}) ions.

The His-tag Affinity Microsphere is highly stable and compatible with a wide range of molecular biology reagents. This stability helps maintain the bioactivity of the protein of interest (POI) and increases yield, while also expanding the range of suitable operating conditions. The microsphere is rigid, tolerant to extreme temperatures, pressure, and pH environments, resistant to microbial contamination, and easy to pack and use.
These features make the His-tag Affinity Microsphere the preferred microsphere for high-performance purification of histidine-tagged proteins.

1.3. Key Features

- Low Ni^{2+} Leakage: Ensures minimal contamination.
- High Protein-Binding Capacity: Maximises purification efficiency.
- Porous Structure: Uniform distribution of pore and particle size with a specific surface area exceeding 500 m^2/g.
- High Functional Group Density: Extraordinary dynamic binding capacity, more than twice that of conventional agarose microsphere cores.
- Chemical Stability
  - pH Tolerance: Stable from pH 1 to 14 (recommended pH 3-13 for prolonged use).
  - Cleaning in Place (CIP): Can be cleaned with 0.5 M NaOH (recommended 0.1 M NaOH for prolonged use) for efficient removal of pyrogens and hetero-proteins.
  - Chelators & Reductive Compatibility: Works with samples containing metal chelators.
    Resistant to 30 mM EDTA for over 48 hours.
    Resistant to 20 mM DTT for over 48 hours.
    Resistant to 45 mM β-mercaptoethanol for over 48 hours.
- Mechanical Stability
  a. High-Pressure Tolerance: Withstands pressures up to 10 MPa.
  b. High Flow Rate: Supports flow rates up to 1800 cm/hour (recommended 150-700 cm/hour).
  c. Uniform Particle Size: Results in better chromatographic characteristics, excellent compression resistance, and reduced blockage risk.
- High Resolution
  Significantly higher resolution than agarose microspheres, with sharper elution peak shapes.

1.4. Intended Use

The products are intended for research use only and shall not be used in any clinical or in vitro procedures for diagnostic purposes.

1.5. Safety

Ensure all handling and usage of these products are conducted within a controlled laboratory environment, adhering to all relevant safety protocols and guidelines. Appropriate personal protective equipment (PPE) should be used for protection at all times, and waste disposal should comply with local regulations.

1.6. Table 1 - Resin characteristics

Matrix	Highly cross-linked high-density polyacrylate
Metal ion capacity	~ 30 µmol Ni^{2+}/ml resin
Average particle size	50 µm
Dynamic binding capacity	90 mg $(histidine)_6$-tagged protein/ml resin
Max. linear flow rate	1800 cm/h
Recommended flow rate	150~700 cm/h
Max. operating pressure	10 MPa
Chemical stability (for microsphere without metal ion)	
Stable in	0.01 M HCl, 0.1 M NaOH; tested for 1 week at 40°C. 1 M NaOH, 70 % acetic acid; tested for 12 hours. 2 % SDS; tested for 1 hour. 30 % 2-propanol; tested for 30 min.
PH stability (for microsphere without metal ion)	
Working range	3 ~ 13
Cleaning-in-place	1 ~ 14
Storage	
Storage conditions	20~25 % ethanol
Storage temperature	4°C to 30°C
Notice	**DO NOT freeze. DO NOT let the microsphere get dry for long.**
Buffers	
Native Binding Buffer	20 mM Na_3PO_4*, 0.5 M NaCl, 10 mM imidazole (can be 5~40 mM), pH 7.4~8
Native Wash Buffer	20 mM Na_3PO_4*, 0.5 M NaCl, 20 mM imidazole (can up to 40mM), pH 7.4~8
Native Elution Buffer	20 mM Na_3PO_4*, 0.5 M NaCl, 250 mM imidazole (can up to 500mM), pH 7.4~8
Denaturing Binding Buffer	20 mM Na_3PO_4, 0.5 M NaCl, 8 M Urea, pH 7.8
Denaturing Wash Buffer	20 mM Na_3PO_4, 0.5 M NaCl, 8 M Urea, pH 6
Denaturing Elution Buffer	20 mM NaH_2PO_4, 0.5 M NaCl, 8 M Urea, pH 4.0

* Or 50 mM NaH_2PO_4

1.7. Table 2 - Working Concentrations Compatible with His-tag Affinity Microsphere

Caustic sanitiser (for CIP)	0.1 M NaOH (0.5M for fast CIP)
Reducing agents	1 5 mM DTE 5 mM DTT 20 mM β-mercaptoethanol 5 mM TCEP 10 mM reduced glutathione
Denaturing agents	8 M urea 6 M Guanidine-HCl
Detergents	2% Triton™ X-100 (nonionic) 2% Tween™ 20 (nonionic) 2% NP-40 (nonionic) 2% Chlorate (anionic) 1% CHAPS (zwitterionic)
Other additives	500 mM imidazole 25% ethanol 50% glycerol 100 mM Na_2SO_4 1.5 M NaCl 1 mM EDTA 60 mM citrate
Buffer	50 mM sodium phosphate, pH 7.4 100 mM Tris-HCl, pH 7.4 100 mM Tris-acetate, pH 7.4 100 mM HEPES, pH 7.4 100 mM MOPS, pH 7.4 100 mM sodium acetate, pH 4

2. General Considerations for Using His-tag Affinity Microsphere

2.1. Binding Conditions

2.1.1. pH and Salt Concentration

- Recommended binding at neutral to slightly alkaline system (pH 7~ 8).
- Use 0.5 to 1.0 M NaCl to eliminate ion-exchange effects.
- Sodium phosphate buffers are commonly used.
- Tris-HCl can be used, but may reduce binding strength in cases of weak metal-protein affinity.

2.1.2. Chelating Agents

Best to avoid using chelating agents such as EDTA or citrate in buffers.

2.1.3. Imidazole Concentration

- Recommended Binding Buffer:
  - 20 mM sodium phosphate
  - 500 mM NaCl
  - 5~40 mM imidazole
  - pH 7.4
- Adjusting Imidazole Concentration:
  - Higher Final Purity: Increase imidazole concentration in the sample and binding buffer.
  - Higher Yield: Decrease imidazole concentration (may result in lower final purity).

2.1.4. Inclusion Bodies

- For recombinant histidine-tagged proteins expressed as inclusion bodies, include up to 6 M Guanidine-HCl or 8 M urea in all buffers.
- Protein unfolding generally occurs within high concentrations of urea or Guanidine-HCl; refolding is protein-dependent.

2.2. Elution Conditions

2.2.1. Imidazole Elution:

- The purity of histidine-tagged proteins can be increased by washing with a binding buffer containing high imidazole concentrations.
- Determine the optimal imidazole concentration for sample loading, washing, and elution using a gradient from 5 to 500 mM.

2.2.2. Alternative Elution Methods:

- Lowering pH within the range of 7.5 to 2.5 can be used for elution.
- Chelating agents like EGTA and EDTA can strip metal ions from the microsphere resin, causing protein elution.
- Elution with ammonium chloride or histidine has also been reported.

2.3. Buffer Preparation

2.3.1. High Purity:

- Use high-purity water and chemicals for buffer preparation.
- Filter buffers through a 0.22 μm filter before use.
- Use high-purity imidazole to ensure low or no absorbance at 280 nm.

2.3.2. Recommended Buffers:

- Binding Buffer: 20 mM sodium phosphate, 0.5 M NaCl, 5~40 mM imidazole, pH 7.4.
- Elution Buffer: 20 mM sodium phosphate, 0.5 M NaCl, 250~500 mM imidazole, pH 7.4.

2.4. Nickel-Ion Leakage

- General Conditions: Leakage of Ni^{2+} from His-tag Affinity Microsphere is low.
- Critical Applications: Perform a Blank Flush before loading the sample to further decrease leakage.
- Buffer Selection: Use binding and elution buffers **without** reducing agents to minimise leakage.

2.5. Reducing Agents

- Compatibility: His-tag Affinity Microsphere is compatible with reducing agents.

- Discolouration: High concentrations of reducing agents can cause discolouration, which typically does not affect resin performance.
- Minimising Discolouration: Perform a Blank Flush using buffers without reducing agents before purification.

3. Packing a Column: Step-by-Step Instructions

Step 1. Assemble the Column

- Set up the column and packing reservoir if needed.

Step 2. Remove Air

- Flush the end piece and adapter with distilled or Milli-Q water to remove air.
- Ensure no air is trapped under the column bed support.
- Close the column outlet, keeping the bed support covered with water.

Step 3. Prepare the Slurry

- Resuspend the microsphere resin and pour the slurry into the column in one continuous motion.
- Pour the slurry down, holding it against the column wall with a glass rod to minimise air bubbles.

Step 4. Fill in the Column

- If using a packing reservoir, fill the rest of the column and reservoir with distilled or Milli-Q water.
- Mount the adapter or lid of the packing reservoir and connect the column to a pump.
- Avoid trapping air bubbles under the adapter or in the inlet tube.

Step 5. Set the Flow Rate

- Open the bottom outlet of the column and set the pump to the desired flow rate.
- If the recommended pressure or flow rate cannot be achieved, use the maximum flow rate your pump can deliver to obtain a well-packed bed.

  Note: For subsequent chromatography procedures, do not exceed 75% of the packing flow rate.

Step 6. Maintain Flow Rate

- Maintain the packing flow rate for at least 3 slurry bed volumes (or 3 column volumes) after a constant bed height is reached.
- Mark the bed height on the column wall.

Step 7. Stop the Pump

- Stop the pump and close the column outlet.

Step 8. Fit the Adapter

- If using a packing reservoir, disconnect it and fit the adapter to the column.

Step 9. Adjust the Adapter

- With the adapter inlet disconnected, push the adapter down into the column until it reaches the marked bed height.
- Allow the packing solution to flush the adapter inlet.
- Lock the adapter in position.

Step 10. Start Equilibration

- Connect the column to a pump or chromatography system and start equilibration.
- Re-adjust the adapter if necessary.

Following these steps will ensure a well-packed column, ready for efficient chromatography.

4. Sample Preparation

For optimal growth, induction, and cell lysis conditions, refer to established protocols. Ensure the sample is fully dissolved to avoid column clogging. Follow these steps:

Step 1. Centrifugation and Filtration:

Centrifuge the lysate and filter the supernatant through a 0.45 µm or/and 0.22 µm filter to remove cell debris or other particulate material.

Step 2. Buffer Adjustment:

- If the sample is dissolved in a buffer other than 20 mM phosphate buffer with 0.5 M NaCl pH 7.4, adjust its NaCl concentration to 0.5 M and pH to 7 ~ 8.
- Note: Avoid using strong bases or acids for pH adjustments to prevent precipitation.

Step 3. Imidazole Addition:

To prevent the binding of host cell proteins with exposed histidine, add the same concentration of imidazole to the sample as to the binding buffer.

5. Purification Procedure

Note: Before performing the purification, please read Chapter 2 (General Considerations) and Chapter 4 (Sample Preparation) in the manual.

5.1. [OPTIONAL] First-Time Use Resin Activation

5.1.1. Ethanol Removal

If the column contains 20~25% ethanol, wash it with 5 column volumes (CV) of distilled or Milli-Q water at a flow rate of 50 to 100 cm/h.

5.1.2. [Optional] NaOH Incubation:

Incubate the microsphere resin with 1 CV of 0.1 M NaOH at room temperature for 10~30 minutes.

5.1.3. NaOH Wash:

Wash with 5 CV of 0.1 M NaOH at a flow rate of 50 to 100 cm/h.

5.1.4. Renatural Wash:

Wash with 5 CV of distilled or Milli-Q water at a flow rate of 50 to 100 cm/h.

5.2. [OPTIONAL] Blank Flush

To minimise nickel leakage and discolouration, perform a Blank Flush using binding and elution buffers without reducing agents.

5.2.1. Wash the Column:

Use 5 CV (column volumes) of distilled or Milli-Q water.

5.2.2. Elution Buffer Wash:

Wash with 5 CV of elution buffer.

5.2.3. Equilibrate:

Equilibrate with 10 CV of binding buffer.

5.3. Purification:

5.3.1. Initial Wash:

- If the column contains 20~25% ethanol, wash it with 5 CV (column volumes) of distilled or Milli-Q water.
- Use a linear flow rate of 50 to 100 cm/h.

5.3.2. Equilibration:

- Equilibrate the column with 5 to 10 CV of binding buffer (recipe see table 1).
- Recommended linear flow rate: 150~700 cm/h.
- In some cases, perform a Blank Flush before final equilibration/sample application.

5.3.3. Sample Application:

- Add/Inject the pretreated sample (see Sample Preparation) to the column.

5.3.4. Washing:

- Wash with a binding or washing buffer (recipe see Table 1) until the optical absorbance reaches the baseline.

5.3.5. Elution:

- Elute with elution buffer (recipe see Table 1) using a constant concentration or step/ linear gradient.
  - Step Elution: 5 column volumes of elution buffer are usually sufficient.
  - Linear Gradient: A shallow gradient over 20 column volumes may separate proteins with similar binding strengths.
- Note: Use the elution buffer as a blank when measuring absorbance manually. If imidazole needs to be removed from the protein, use a desalting column.

5.4. Optimisation:

5.4.1. Imidazole concentration of the buffers:

- Imidazole at low concentrations minimises the binding of unwanted host cell proteins.
- Include imidazole in the sample at the same concentration as in the banding buffer.
- Optimise imidazole concentration to balance high purity and high yield.
- Start with 5 to 40 mM in the binding and 250 to 500 mM in the Elution buffer for most of the His-tagged proteins.

5.4.2. Choice of affinity Ion:

- Ni^{2+} is usually the first choice for purifying most $(histidine)_6$-tagged recombinant proteins.
- The strength of binding is affected by several factors, including the affinity tag's length, position, and exposure, the type of ion used, and the pH of buffers.
- Test different metal ions (e.g., Cu^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+}, Fe^{3+}) based His-tag Affinity Microsphere.

6. Regenerating the microsphere resin

6.1. Stripping and Recharging

The microsphere resin **DOES NOT** need to be stripped and recharged (affinity ions) between each purification if the same protein is being purified. For different proteins of interest (POI), if the microsphere's binding capacity is not significantly decreased and the purity of the POI is not strictly required, the microsphere resin **DOES NOT** need to be stripped and recharged.

⚠ Note: The waste liquid containing divalent nickel is classified as toxic. Dispose of it following hazardous waste regulations—do not discharge into the sewer system.

6.1.1. Recharging Frequency:

It is sufficient to recharge the microsphere resin after 7 to 10 purifications, or even more rounds, depending on factors such as:

- Cell lysate
- Lysate volume
- Target protein

6.1.2. Ni^{2+} Stripping - Remove Residual Ni^{2+}:

Step 1. Wash with 5 column volumes of 20 mM sodium phosphate, 0.5 M NaCl, 50~100 mM EDTA, pH 7.4.

Step 2. Remove residual EDTA by washing with 5~10 column volumes of binding buffer, followed by 5 column volumes of distilled or Milli-Q water before recharging the column.

6.1.3. Ni^{2+} Recharging - Recharge the resin with affinity ions:

Step 1. Load 0.5 column volumes of 0.1~0.2 M $NiSO_4$ and incubate for 30 min. Chlorides, or sulphate salts of other metals, may also be used.

Step 2. Wash with 5 column volumes of distilled or Milli-Q water, followed by 5 column volumes of binding buffer (to adjust pH) before storage in 20~25% ethanol.

Note: In some applications, substances such as denatured proteins or lipids cannot be eluted in the regeneration. These substances can be removed by the following step: Cleaning-in-Place (CIP).

6.2. Cleaning-in-Place (CIP)

When a decrease in performance or an increase in back pressure is noted, the column should be cleaned. Before cleaning, strip off the Ni^{2+} ions using the above-mentioned procedure (6.1.2). Recommend using a reversed flow direction for cleaning. After cleaning, recharge the resin with Ni^{2+} before storage in 20~25% ethanol.

CIP Protocols

The Ni^{2+} stripped microsphere resin can be cleaned using specific protocols to ensure optimal performance.

6.2.1. Ionic Bound Proteins

- Wash: Use several column volumes of 1.5 M NaCl.
- Rinse: Follow with several column volumes of distilled or Milli-Q water.

6.2.2. Precipitated Proteins, Bound Proteins, and Lipoproteins

- Wash: Use 0.5 M NaOH with a contact time of 1 hour (or more to remove endotoxins).
- Rinse: Follow with approximately 10 column volumes of binding buffer, then 10 column volumes of distilled or Milli-Q water.

6.2.3. Hydrophobically Bound Proteins, Lipoproteins, and Lipids

- Option 1:
  - Wash: Use 5 to 10 column volumes of 30% isopropanol and incubate with 30% IPA for 15~20 minutes.
  - Rinse: Follow with approximately 10 column volumes of distilled or Milli-Q water.
- Option 2:
  - Wash: Use 2 column volumes of detergent in a basic or acidic solution (e.g., 0.1 to 0.5% nonionic detergent in 0.1 M acetic acid) with a contact time of 1 to 2 hours.
  - Rinse: Remove residual detergent by washing with 5 to 10 column volumes of 70% ethanol.
  - Final Rinse: Follow with approximately 10 column volumes of distilled or Milli-Q water.

7. Storage of Microsphere Resin

The microsphere resin can be stored at 4~30°C in a solution of 20~25 % v/v ethanol. For optimal storage, use a mixture of 25% ethanol and 75% resin slurry by volume. This ensures the resin remains stable and ready for future use.

DO NOT freeze. DO NOT let the microsphere get dry for long.

8. Troubleshooting

If you encounter issues with the His-tag Affinity Microsphere, the following tips may help. For further assistance, please email GMExpression for technical support.

8.1. Column Has Clogged

Cell Debris:

- Cell debris in the sample may clog the column. Clean the column according to Cleaning-in-Place (CIP).
- Centrifuge and/or filter the sample through a 0.22 µm or a 0.45 µm filter (refer to Sample Preparation).

8.2. Sample Is Too Viscous

High Nucleic Acid Concentration:

- If the lysate is very viscous due to a high concentration of host nucleic acid, continue sonication until the viscosity is reduced.
- Alternatively, add DNase I to 5 µg/ml, Mg^{2+} to 1 mM, and incubate on ice for 10 to 15 minutes.
- Another option is to draw & flush the lysate through a syringe needle several times.

8.3. Protein Is Difficult to Dissolve or Precipitates During Purification

Solubilisation:

- Refer to Table 2 for reducing agents, detergents, glycerol, and denaturing agents that may be used.

- Mix with the suitable reagent and stir gently for 30 minutes to aid the solubilisation of the tagged protein (inclusion bodies may require longer mixing).
- Note that Triton X-100 and NP-40 (but not Tween) have high absorbance at 280 nm and cannot be easily removed by buffer exchange.

8.4. Histidine-Tagged Protein Found in the Pellet

8.4.1. Insufficient Sonication:

- Check cell disruption by microscopy or check A260 to see the content of nucleic acids.
- Adding lysozyme (up to 0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) before sonication may improve results.
- Avoid frothing and overheating to prevent denaturing the target protein. Over-sonication can lead to co-purification of host proteins with the target protein.

8.4.2. Insoluble Protein (Inclusion bodies):

- Solubilise (and unfold) the protein using denaturants such as 4 to 6 M Guanidine-HCl, 4 to 8 M urea, or strong detergents.
- Prepare buffers containing 20 mM sodium phosphate, 8 M urea, or 6 M Guanidine-HCl, and suitable imidazole concentrations, pH 7.4 to 7.6.
- Use the above formula, combined with 10 mM imidazole or the concentration selected during optimisation trials, as binding buffer and as elution buffer for sample preparation. To minimise the sample dilution time, solid urea or Guanidine-HCl can be added.

8.5. Histidine-Tagged Protein Found in the Flowthrough and Purified Fractions

Capacity Exceeded:

- Increase the volume of His-tag Affinity Microsphere used for your purification.

8.6. No Histidine-Tagged Protein in the Purified Fractions

8.6.1. Mild Elution Conditions:

- Elute with an increasing imidazole gradient or decreasing pH to determine the optimal elution conditions.
- Consider using EDTA or EGTA (10–100 mM) to strip the nickel ions from the resin and facilitate protein elution.

8.6.2. Protein Precipitation in the Column:

- Try detergents or change NaCl concentration, or elute under denaturing (unfolding) conditions (use 4 ~ 8 M urea or 4 ~ 6 M Guanidine-HCl) to remove precipitated proteins.
- For the next experiment, decrease the amount of sample or decrease protein concentration by eluting with a linear imidazole gradient instead of imidazole steps.

8.6.3. Nonspecific Hydrophobic or Other Interaction:

- Add a nonionic detergent to the elution buffer (e.g., 0.2% Triton X-100) or increase the NaCl concentration.

8.7. Eluted Protein Is Not Pure (Multiple Bands on SDS-PAGE)

8.7.1. Partial Degradation by Proteases:

- Add protease inhibitors (use EDTA with caution).

8.7.2. Contaminants with High Nickel Ions:

- Optimise imidazole concentration for the binding buffer. If optimised conditions do not remove contaminants, further purification by ion exchange chromatography may be necessary.

8.7.3. Contaminants Associated with Tagged Proteins:

- Add detergent and/or reducing agents before sonication of the cells. Increase detergent levels (e.g., up to 2 % Triton X-100 or 2 % Tween 20), or add glycerol (up to 50 %) to the wash buffer to disrupt nonspecific interactions.

8.8. Removal of Dust or Inorganic Impurities from Microsphere Resin

If the microsphere resin is contaminated with dust or inorganic particulates, the following cleaning protocols may be applied:

8.8.1. Option 1 - Alkaline Cleaning

Base Incubation: Incubate the resin with 1 CV of 0.1~0.5% nonionic detergent prepared in 0.1 M NaOH at room temperature for 30 minutes.

NaOH Wash: Rinse with 5 CV of 0.1 M NaOH at a flow rate of 50~100 cm/h.

Renatural Wash: Rinse with 5 CV of distilled or Milli Q water at a flow rate of 50~100 cm/h.

8.8.2. Option 2 - Acidic Cleaning

Acid Incubation: Incubate the resin with 1 CV of 0.1~0.5% nonionic detergent prepared in 0.1 M acetic acid at room temperature for 30 minutes.

Acid Wash: Rinse with 5 CV of 0.1 M acetic acid at a flow rate of 50~100 cm/h.

Renatural Wash: Rinse with 5 CV of distilled or Milli Q water at a flow rate of 50~100 cm/h.

9. Other types of microsphere resin

We also have affinity gels of different microsphere sizes and ligands with various ions for sale.

Such as gels with Cu/Co/Ni/Zn ions, NTA/IDA/Amine/Amalose/Heparin/Thiopropyl/HisTalon/Alkali-tolerant protein A-derived ligand.

We also have corresponding high-pressure peristaltic pumps, standard affinity/elution columns/chambers of various volumes and materials (stainless steel, PEEK or high-density polypropylene) compatible with FPLC from GE (Cytiva), Agilent, etc.

Please contact us via email for a quotation and technical support.