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Protocol

Cat. No. PC2002

HyperChrom Ni-NTA HP Agarose

Product description

HyperChrom Ni-NTA HP Agarose is an affinity chromatography medium made by chemically coupling the transition metal ion Ni2+ with tetracoordination to a ligand of Nitrilotriacetic acid (NTA) using a highly crosslinked agarose gel as a matrix. It has a very stable octahedral structure, nickel ions are at the center of the octahedron, which protects nickel ions from attack by small molecules, is more stable, and can withstand harsh conditions such as reducing agents, denaturants or couplants in a certain concentration. In addition, due to the pressure resistance of the matrix, which can withstand pressures up to 0.3 MPa, the product can be used for industrial large-scale protein purification, enabling purification of the protein of interest at relatively high flow rates. This chromatography medium uses the interaction between Ni^{2+} and the side chains of certain amino acids (mainly histidine) on the protein to achieve the separation and purification of proteins containing and not containing these amino acids and containing a large and low number of these amino acids.

HyperChrom Ni-NTA HP Agarose is the first choice for purifying histidine-tagged proteins, with the advantages of high load, easy regeneration, and low cost. Compared to HyperChrom Ni-NTA FF Agarose, HyperChrom Ni-NTA HP Agarose has a finer particle size and provides higher resolution.

Components and storage conditions

Components	PC2002-25 mL	PC2002-100 mL
HyperChrom Ni-NTA HP Agarose	25 mL	100 mL
Store the components at 4°C for 5 years.		

Product parameters

HyperChrom Ni-NTA HP Agarose chromatography media parameters

Name	Description
Chromatography media type	Affinity chromatography media
Ligation	NTA-Ni^{2+}
Scaffolding	Highly cross-linked agarose
Average particle size	34 μm
Ligand density	~15 μmol Ni^{2+}/mL chromatography medium

Dynamic load	≥40 mg histidine-tagged protein/mL chromatography medium*
Flow rates are recommended	90-150 cm/h
Maximum flow rate	200 cm/h
Withstand pressure	0.3 MPa
Use temperature	4 - 30°C
pH stability **	2-14
Chemical resistance	Common aqueous solution, 0.01 M HCl, 0.1 M NaOH, 8 M urea, 6 M guanidine hydrochloride, 30% isopropanol ***

* Dynamic load measurement conditions: column loading height: 10 cm, test flow rate 150 cm/h; Test buffer: 0.02 M PB, 0.5 M NaCl, 5 mM imidazole, pH 7.4; test sample: tagged protein (1 mg/ml, 6*His); Index: When the protein penetration reaches 10%, the amount of protein loaded per unit volume of media (mL) (mg).

**After 7 days in the environment of chromatography at 40 °C and pH 2-14, the physical and chemical properties and functions of the chromatography medium did not change significantly.

*** 30% is v/v, volume ratio.

Experimental manipulation

1. **Preparation of buffers**

Buffer type	His-tagged protein buffer components	Inclusion body His-tagged protein buffer components
Lysis Buffer	50 mM NaH_2PO_4 300 mM NaCl 10 mM imidazole NaOH pH adjusted to 8.0, 0.22 µm or 0.45 µm filtration sterilization	8 M Urea 100 mM NaH_2PO_4 100 mM Tris· HCl The hydrochloric acid solution was filtered and sterilized at pH to 8.0, 0.22 µm or 0.45 µm
Balance/Bind/Wash Buffer	50 mM NaH_2PO_4 300 mM NaCl 20 mM imidazole NaOH pH adjusted to 8.0, 0.22 µm or 0.45 µm filtration sterilization	8 M Urea 100 mM NaH_2PO_4 100 mM Tris· HCl The hydrochloric acid solution was filtered and sterilized at pH to 6.3, 0.22 µm or 0.45 µm
Elution Buffer	50 mM NaH_2PO_4 300 mM NaCl 250 mM imidazole NaOH pH adjusted to 8.0, 0.22 µm or 0.45 µm filtration sterilization	8 M Urea 100 mM NaH_2PO_4 100 mM Tris· HCl The hydrochloric acid solution was adjusted to pH to 4.5, 0.22 µm or 0.45 µm and filtered for sterilization
Store Buffer	20% ethanol, stored at 4-30°C	20% ethanol, stored at 4-30°C

#Buffer suitability recommendations:

- **Buffer**: Phosphate buffer is preferred, pH neutral to weak alkaline (7~8), avoid the use of EDTA and citrate, Tris-HC can also be used, but should be avoided in the case of weak affinity between metal ions and proteins. In order to reduce the non-specific binding of host proteins to media, low concentrations of imidazole (20~40 mM) are usually added to the equilibrium buffer and sample. 0.15~0.5 M NaCl must be added to the buffer to eliminate ion adsorption.
- **pH**: The pH of the buffer needs to be flexibly adjusted considering factors such as the isoelectric point of the sample.

2. Sample preparation

2.1 Bacteria express proteins

a) Pick single colonies into LB medium containing suitable resistance and add the corresponding inducer according to the vehicle instructions to induce the corresponding time.

b) After expression, the culture was transferred to a centrifuge bottle, centrifuged at 7,000 rpm for 15 min, the bacteria were collected, and then 1/10 volume of lysis buffer and PMSF (PMSF was added before fragmentation at a final concentration of 1 mM), and other protease inhibitors could also be added, but could not affect the binding of the protein of interest to the resin.

c) Lysozyme is then added to a working concentration of 1 mg/mL. *Note: If the expressed host cell contains pLysS or pLysE, lysozyme can be omitted.*

d) Suspend the bacterial pellet (if the concentration of the bacterial solution is high, consider adding 10 μg/mL RNase A and 5 μg/mL DNase I), mix well, and place it on ice to sonicate the cells until the bacterial solution is basically clear.

e) Collect the above clarified protein solution and centrifuge at 10,000 rpm at 4 °C for 20-30 min. The supernatant was filtered, sterilized by 0.22 μm/0.45 μm membrane, placed on ice for later use or stored at -20°C.

2.2 Yeast, insects, and mammalian cells secrete expressed soluble proteins.

Transfer the cell culture medium to a centrifuge bottle, centrifuge at 5,000 rpm for 10 min, and collect the supernatant. If the supernatant does not contain EDTA, histidine and reducing agent, it can be directly purified on the column; if it contains EDTA, histidine and reducing agent, it needs to be dialyzed at 1×PBS at 4 °C before going to the column. *Note: For a large volume of supernatant, ammonium sulfate needs to be added for precipitation concentration, and then dialyzed at 1×PBS at 4 °C before going to the column.*

2.3 Inclusion body proteins (in the case of bacteria)

a) Transfer the culture to a centrifuge flask at 7,000 rpm for 15 min and collect the bacteria to de-supernatant.

b) According to the ratio of bacteria: lysate = 1:10 (w/v), the bacteria are fully suspended, mixed well,

and broken in an ice bath ultrasonically.

c) Transfer the crushed solution to a centrifuge tube, centrifuge at 10,000 rpm, centrifuge at 4 °C for 20-30 min, and remove the supernatant. Steps 2) and 3) can be repeated once.

d) The inclusion bodies were fully suspended according to the ratio of bacteria: lysate (containing 8 M urea) = 1:10 (w/v). *Note: Different inclusion bodies have different solubility, and 6M guanidine hydrochloride can also be used instead of 8M urea, because guanidine hydrochloride dissolves inclusion bodies more completely.*

e) Purification of His-tagged proteins under denaturing conditions.

3. Chromatographic conditions

- Flow rate selection: Linear flow rate of 90-300 cm/h is generally selected according to the height of the column bed.
- Sample preparation: To prevent the sample from clogging the column, the sample needs to be filtered with a 0.2/0.45 μm (after inclusion body disruption) microporous membrane before loading, and it is recommended that the pH and conductivity of the sample be adjusted to be consistent with the equilibrium buffer (the pH and conductivity of the sample can be adjusted by dilution, ultrafiltration, and desalting).

4. Loading columns

The following column loading methods are suitable for filling laboratory-scale chromatography columns:

4.1 Supplies required for column mounting.

a) Chromatography medium: HyperChrom Ni-NTA HP Agarose

b) Chromatographic empty column: laboratory-scale chromatography empty column and column loader

c) Solution required:

- Column loading solution: purified water.
- Exhaust solution: purified water.

d) Column loading tools: sand core funnel, stirring rod, measuring cylinder, etc

4.2 Preparation before column loading

a) Calculate the volume of chromatography medium Vm required for column loading (volume of the chromatography medium part after sufficient sedimentation), and calculate the formula:

Vm = cross-sectional area of the chromatography column X height of the column bed where the column is planned to be loaded x compression ratio of the chromatography medium.

(Note: The compression ratio of HyperChrom Ni-NTA HP Agarose is 1.15).

b) The chromatography medium was transferred to the sand core funnel, and the column loading solution was cleaned and filtered with about 3 times the volume of the chromatography medium, and the column chromatography medium to be loaded was replaced with the column loading solution.

c) To prepare the glue suspension of the column chromatography medium to be loaded, the suitable column loading glue suspension ratio of HyperChrom Ni-NTA HP Agarose chromatography medium is 50%-70%. In order to obtain an accurate chromatography medium volume, the chromatography medium can be placed in a graduated cylinder and settled overnight or centrifuged at low speed (3000 rpm, 5 min) to simulate the natural sedimentation effect of the chromatography medium, and then measured.

d) Check the empty column to be used to ensure it is clean and leak-free.

4.3 **Column mounting**

a) Exhaust the column bottom membrane (screen) with purified water.

b) After sufficient exhaustion, screw the plug or close the column bottom valve at the bottom of the column interface, and continue to inject a small amount of purified water until the bottom of the column is covered.

c) Adjust the chromatography column to vertical.

d) The column head is connected to the chromatography system, which provides a low flow rate of 5 m L/min through the chromatography system and exhausts the column head membrane (screen) with purified water.

e) Thoroughly stir the prepared chromatography medium suspension with a stir bar, and then slowly pour into the prepared chromatography empty column at one time.

Note: If the volume of the glue suspension exceeds the empty column volume, it should be extended by using a column loader or connecting another empty column tube with a connector.

f) Place the gaseated stigma into the chromatography column, fully fit the glue suspension level, and remove all air bubbles. Then tighten the column head seal.

g) Start the system pump, adjust the flow rate to 300 cm/h, and use the liquid flow to press the column bed. During this period, the pressure should not exceed 0.3 MPa. If the pressure is over, the flow rate needs to be reduced (see the table below for flow rate conversion).

CID / V/FR / LFR	10 mm	16 mm	26 mm	50 mm
60 cm/h	0.8 mL/min	2.0 mL/min	5.3 mL/ min	19.6 mL/min
100 cm/h	1.3 mL/min	3.3 mL/min	8.8 mL/min	32.7 mL/min
1 50 cm/h	2.0 mL/min	5.0 mL/min	13.3 mL/ min	49.1 mL/min
200 cm/h	2.6 mL/min	6.7 mL/min	17.7 mL/ min	65.4 mL/min

300 cm/h	3.9 mL/min	10.0 mL/min	26.5 mL/min	98.1 mL/min
600 cm/h	7.9 mL/min	20.1 mL/ min	53.1 mL/min	196.3 mL/ min

Note：
CID: Chromatographic inner diameter
V/FR: Volumetric flow rate
LFR: Linear flow rate

Table 1 Flow rate conversion table of different specifications of chromatography column

h) After the column bed is stabilized (the glue surface no longer falls), mark the position of the glue surface at this time. Stop the pump and press the column head down to 2-3 mm below the marked position.

i) Re-apply the flow rate of 300 cm/h, if the glue surface no longer drops, that is, the column loading is completed. If the glue surface falls, repeat steps h-j.

Note: The recommended workflow speed does not exceed 75% of the flow rate of the loading column.

5. Column efficiency determination (optional).

Select one of the two test methods shown in the table below for column effectiveness testing. Use the mobile phase equilibrium chromatography column to the baseline to be stable, load the sample into the chromatography column, continue to use the mobile phase for rinsing, and after the chromatographic peak is completed to return to the baseline, end the run, integrate the chromatographic peak, and evaluate the loading effect.

Table 2 Statistical table of two column efficiency measurement methods

	Acetone method	**NaCl method**
Sample	1% (v/v) acetone in water	2 M NaCl in water
Sample volume	1% column volume	1% column volume
Mobile phase	water	0.2 M NaCl in water
velocity of flow	30 cm/h	30 cm/h
Detector	UV 280 nm	electrical conductivity

The main evaluation criteria for the effect of column loading are N/m (number of plates per meter) and as (symmetry factor), which are calculated as follows:

$$\frac{N}{m} = 5.54x(\frac{V_R}{W_h})^2 \times \frac{1}{L}$$

$$As = {}^{b}/_{a}$$

Column efficiency qualification standards: $N/m > 3000$, $0.8 < As < 1.5$

*Parameter Notes:

L = column height, VR = reserved volume, Wh = half-peak width, a = left half-peak width at 10% peak height, b = right half-peak width at 10% peak height

6. Chromatographic steps

a) Equilibrium: Adequately equilibrate the column to pH and conductivity stable using equilibration buffer and is essentially the same as the equilibration buffer, which typically requires 3-5 times the column volume.

b) Sample loading: Determine the sample loading volume and loading amount on the HyperChrom Ni-NTA HP Agarose based on the binding load measured in the small test experiment. [Note 1] In order to prevent the sample from blocking the column, it is recommended that the sample needs to be filtered with a 0.22/0.45μm microporous filter membrane before loading; [Note 2] If the viscosity of the sample increases, even if the loading volume is small, it will cause a large back pressure of the chromatography column; [Note 3] The loading amount should not exceed the binding capacity of the column; [Note 4] Large sample volumes can also cause large backpressures, making the injector more difficult to use.

c) Washing: With equilibration buffer or another suitable buffer, rinse the column until UV stable and return to baseline.

d) Elution: Elution is achieved by increasing the concentration of imidazole, which can gradually increase the concentration of imidazole in the eluate through a linear gradient or a step gradient to elute molecules with different binding strengths.

e) Re-equilibration: Re-equilibrate the chromatography column with equilibration buffer.

7. Cleaning and recycling

As the number of uses of the chromatography medium increases, contaminants (e.g., lipids, endotoxins, proteins, etc.) accumulate on the chromatography column. Regular in-place cleaning is essential to keep the column in stable working condition. Determine the frequency of in-place cleaning according to the degree of contamination of the chromatography medium (if the contamination is serious, it is recommended that in-place cleaning should be carried out after each use to ensure repeatable results and extend the working life of the chromatography medium).

a) Strip buffer with 2-5 column volumes of metal ions (0.05 M PB, 0.5 M NaCl, 0.1-0.2 M EDTA, pH 7.0).

b) Rinse the chromatography column with 2-3 column volume 0.5 M NaCl solution to remove residual EDTA.

c) For different types of impurities and contaminants, cleaning can be carried out under the following conditions:

- Removal of bound proteins due to strong ion adsorption: wash with 2 M NaCl solution in 5x column volume.

- Denature, removal of precipitated proteins: Wash first with 1 M NaOH solution in 5x column volumes, then wash the lye with 5-10 column volumes of purified water.
- Removal of hydrophobic and lipids: Wash the chromatography column first with 2x column volume of 70% ethanol or 30% isopropanol, then rinse with 5-10x column volume of purified water.

Note: The flow rate can be selected from 30-60 cm/h during the cleaning process; reverse cleaning can be used when the blockage is serious.

d) After re-attaching nickel with 0.1 M $NiSO_4$ solution with 0.5 column volume, the chromatography medium can be used normally.

8. Destruction and recycling

- Since HyperChrom Ni-NTA HP Agarose chromatography media is difficult to degrade in nature, incineration of discarded chromatography media is recommended to protect the environment.
- For chromatography media exposed to bioactive samples such as viruses and blood, please follow local biosafety requirements before destroying or disposing of them.

9. Storage recommendations

The preloaded column is stored at the factory with 20% ethanol and closed with upper and lower plugs to keep the chromatographic packing wetted. After opening and using the preloaded column, the user can first rinse the preloaded column with pure water, then rinse 2-3 times the column volume with 20% ethanol, and then seal it with a plug (solution volatilization will cause the chromatographic packing to dry out and shrink, affecting its separation performance). For unopened chromatography media, keep in the original container.

10. FAQs

issue	Possible causes	Recommend a solution
The column backpressure is too high	The packing is blocked	The lysate may contain tiny solid particles, and it is recommended to filter with a membrane (0.22 μm or 0.45 μm) before loading the column, or centrifuge for removal
		Samples contain high concentrations of nucleic acids, extend the disruption time until the viscosity decreases, or add DNase I (final concentration 5 μg/mL), Mg^{2+} (final concentration 1 mM), and incubate on ice for 10-15 min
	The sample is too viscous	Organic reagents or protein-stabilizing agents (e.g., glycerol, etc.) may cause increased back pressure and

		decrease the operating flow rate.
There is no protein of interest in the eluting component	Proteins may be inclusion bodies	The lysate can be detected by electrophoresis to analyze whether there is a protein of interest in the supernatant, and the inclusion body protein needs to be purified according to the inclusion body protein
	The expression is too low	Optimize the expression conditions and use the inclusion body purification buffer system
	The target protein binding is relatively weak and has been washed down during the washing step	Increase the pH of the Wash Buffer, or decrease the concentration of imidazole
	The protein of interest binds too strongly and is not easy to elute off	Lower the pH of Elution Buffer, or increase the concentration of imidazole in Elution Buffer
		Peel off nickel ions using 10-100 mM EDTA solution while obtaining proteins
	Protein degradation	When the bacteria are broken, some protease inhibitors need to be added
		Perform the purification operation at 4°C
Impure elution components (containing multiple proteins)	Incomplete washing	Increase the Wash Buffer volume
	The sample contains other His-tagged proteins	Optimize washing conditions by adjusting pH or imidazole concentration. The eluting components were further purified using other purification methods (e.g., deionization exchange, hydrophobicity, etc.).
The filler color becomes lighter or turns white	Nickel ions shed or peeled off	Re-hang nickel ions according to the operation of filler regeneration
The filler is brown	The buffer contains reducing agents such as DTT	Appropriately reduce the concentration of reducing agent DTT, or switch to thioethanol
Protein precipitation occurs during sample loading	The operating temperature is too low	Load samples at room temperature
	Proteins aggregate	Add stabilizer to the sample and all buffers, such as

		0.1% Triton X-100 or Tween-20

Notes

1. It is recommended that the buffer and protein solution used for purification be filtered through a 0.22 μm or 0.45 μm membrane and then used on the column.
2. Sterilization: Since the 20% ethanol preservation solution does not have the effect of sterilization and pyrogenization, it is recommended that HyperChrom Ni-NTA HP Agarose media can be treated with 70% ethanol for more than 12 h before and during use, or the denickelized medium can be treated with 1 M NaOH for 0.5~1 h to reduce the risk of microbial contamination.
3. This product is for scientific purposes only.

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