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Ni-NTA XPure Agarose Resin

User's Guide

1. DESCRIPTION

Ni-NTA XPure Agarose Resin can be used to purify 6xHis-tagged proteins expressed in series of expression vectors, such as *E.coli*., yeast, insect cells and mammalian cells. **Ni-NTA XPure Agarose Resin** consists of 90μm beads of highly cross-linked 6% agarose, to which Nitilotriacetic acid (NTA) has been coupled. The chelating group has then been charged with nickel ions (Ni^{2+}). This form is very stable octahedral structure of nickel ions in the center, which can protect the nickel ions from attack of the competitive small molecule. The structure of Ni-NTA is compatible with a certain concentration of reducing agents, denaturing agents, detergents and other additives. **Ni-NTA XPure Agarose Resin** is highly stable and expand the range of suitable operating conditions.

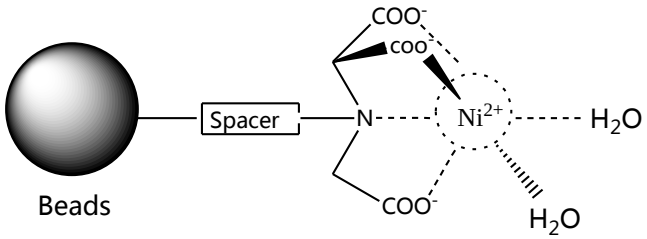

Fig.1. The chemical structures of Ni NTA Beads

Table 1. **Characteristics of Ni-NTA XPure Agarose Resin**

Item	Description
Matrix	Highly cross-linked 6% agarose
Static binding capacity	>40mg 6XHis-tagged protein/ml medium
Particle size（μm）	45–165
Maximum pressure	0.3 MPa, 3 bar
Storage solution	1X PBS containing 20% ethanol
Storage	2°C-8°C

Table 2. Chemical compatibilities for **Ni-NTA XPure Agarose Resin**

Reagent	Stability
Reductants	5 mM DTE 0.5-1mM DTT 20 mM β-mercaptoethanol

	5 mM TCEP 10 mM reduced glutathione
Denaturants	8 M urea 6 M Gua-HCl
Detergent	2% Triton™ X-100 (nonionic) 2% Tween™ 20 (nonionic) 2% NP-40 (nonionic) 2% cholate (anionic) 1% CHAPS (zwitterionic)
Other additives	500 mM imidazole 20% 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. PURIFICATION PROCEDUE

2.1 Buffer Preparation

The basic principle of the following recommended buffer and other buffer is low concentration of imidazole in Lysis and wash buffer and high in elution buffer. Water and chemicals used for buffer preparation should be of high purity. It is recommended filtering the buffers by passing them through a 0.22μm or 0.45 μm filter before use. **Ni-NTA XPure Agarose Resin** can be used for the his-tagged protein purification under native conditions and denaturing conditions, which need different buffer. See table 3 and table 4.

Table 3. Recommended buffer for his-tagged protein purification under native conditions

Name	Volume	Ingredient
Lysis Buffer	1L	50 mM NaH_2PO_4 （7.80 g $NaH_2PO_4 \cdot 2H_2O$）
		300 mM NaCl （17.54 g NaCl）
		10 mM imidazole （0.68 g imidazole）
		Adjust the buffer pH to 8.0 with NaOH solution
Wash Buffer	1L	50 mM NaH_2PO_4 （7.80 g $NaH_2PO_4 \cdot 2H_2O$）
		300 mM NaCl （17.54 g NaCl）
		20 mM imidazole（1.36 g imidazole）

		Adjust the buffer pH to 8.0 with NaOH solution
Elution Buffer	1L	50 mM NaH_2PO_4 （7.80 g $NaH_2PO_4 \cdot 2H_2O$）
		300 mM NaCl （17.54 g NaCl）
		250 mM imidazole （17.0 g imidazole）
		Adjust the buffer pH to 8.0 with NaOH solution

Table 4. Recommended buffer for his-tagged protein purification under denaturing conditions

Name	Volume	Ingredient
Lysis Buffer	1L	8 M Urea （480.50 g urea）
		100 mM NaH_2PO_4 （15.60 g $NaH_2PO_4 \cdot 2H_2O$）
		100 mM Tris·HCl （15.76 g Tris·HCl）
		Adjust the buffer pH to 8.0 with HCl solution
Wash Buffer	1L	8 M Urea （480.50 g urea ）
		100 mM NaH_2PO_4 （15.60 g $NaH_2PO_4 \cdot 2H_2O$）
		100 mM Tris·HCl （15.76 g Tris·HCl）
		Adjust the buffer pH to 6.3with HCl solution
Elution Buffer	1L	8 M Urea（480.50 g urea）
		100 mM NaH_2PO_4（15.60 g $NaH_2PO_4 \cdot 2H_2O$）
		100 mM Tris·HCl（15.76 g Tris·HCl）
		Adjust the buffer pH to 4.5 with HCl solution

2.2 Sample Preparation

2.2.1 Recombinant native protein expressed in *E.coli* or yeast

1. Single colonies were cultured in LB medium. According to the instruction, adding the inducers for a period of time.

2. Harvest cells from an appropriate volume of bacterial culture by centrifugation at 7,000rpm for 10-15min at 4℃. Discard supernatant and determine weight of pellet. Resuspend pellet in 1:10 ration (w/v) in lysis buffer and add lysozyme (0.2-0.4mg/ml cell paste, if the host cell containing pLysS or pLysE, it can be without lysozyme) and PMSF (1mM/ml cell paste).

3. If high concentration of cell suspension, it is consider to add 10μg/ml RNase A and 5μg/ml DNase I. Sonicate the cell suspension/lysate on ice.

4. Centrifuge the homogenized lysate at 10,000rpm for 20min at 4℃ to clarify sample. Save supernatant.

2.2.2 Native protein expressed in yeast, insect or mammalian cells

1. Harvest the cells from an appropriate volume of culture by centrifugation at 5,000rpm for 10-15min at 4℃. Save supernatant. If the supernatant without EDTA, histidine and reductant, it can be purified directly, otherwise it need dialysis to 1XPBS under 4℃ .

2. for a large volume of supernatant, it need precipitation by adding ammonium sulfate and dialysis to1XPBS under 4℃.

2.2.3 Inclusion bodies from *E.coli*

1. Harvest cells from an appropriate volume of bacterial culture by centrifugation at 7,000rpm for 10-15min at 4℃. Discard supernatant and determine weight of pellet.
2. Resuspend pellet in 1:10 ration (w/v) with Lysis Buffer. Sonicate the cell suspension/lysate on ice.
3. Centrifuge the homogenized sample at 10,000rpm for 20min at 4℃ to pellet the inclusion.
4. Resuspend pellet in 1:10 ration (w/v) with denaturing Lysis Buffer(containing 8M urea). Sonicate, as needed, to redissolve the pellet.
5. Analyze the concentration of the target protein and continue with purification protocols under denaturing conditions.

2.3 Column Purification

1. Mix the slurry by gently inverting the bottle several times to completely suspend the **Ni-NTA XPure Agarose Resin.** Close the column outlet leaving the net covered with packing buffer. Transfer the slurry to the column.
2. Allow the resin to settle down and the buffer to drain from the column. Add 5 column volumes Lysis Buffer to the column to equilibrate the beads.
3. Apply the sample to the column. Collect the flow-through for measuring the binding efficiency to the beads, i.e. by SDS-PAGE.
4. Wash the column with 10 column volumes Wash Buffer or until the absorbance of the effluent at 280 nm is stable.
5. Elute the target protein with Elution Buffer and collect the eluate.
6. Equilibrate the column with 5 column volumes of Lysis Buffer, distilled water and 1XPBS containing 20% ethanol. Finally store the beads with 1XPBS containing 20% ethanol at 4℃.

2.4 Analysis

Identify the fractions containing the His-tagged protein. Use UV absorbance, SDS-PAGE, or western blot.

3. CLEANING-IN-PLACE

A column used to purify protein from cell extract usually contains some soluble substances and cell debris that are nonspecifically absorbed onto the matrix. Cleaning-in-Place eliminates material not removed by regeneration and prevents progressive buildup of contaminants. If the column is to be reused, these

contaminants should be cleaned from the column, as they were not completely removed during the sample clarification steps.

Remove the strong hydrophobic binding protein, lipoprotein and lipid

Wash the column using 5-10 column volumes 30% isopropanol contacting for 15-20min. Or you can choose the 2CV acidic or alkaline solution containing detergents, for example, 0.1 M acetic acid solution contains 0.1-0.5% non-ionic detergent, contacting for 1-2 hours .

Finally wash the column with 10CV distilled water

Remove the proteins combined with ion interacting

Wash the column with 1.5M NaCl solution contacting for 10-15min.

Finally wash the column with 10 column volumes distilled water

4. REGENERATING THE MEDIUM

In general, **Ni-NTA XPure Agarose Resin** may be used a number of times before it becomes necessary to recharge them with metal ions. When the back pressure is too high or the capacity significantly lower, it need to strip the metal ions and recharge the **Ni-NTA XPure Agarose Resin** as the following procedure.

Wash the column with one of the following solutions.

1) 0.2M acetic acid with 6 M Guanidine Hydrochloride, 2 column volumes ;
2) Rinse with 5 column volumes of distilled water ;
3) 2% SDS, 3 column volumes ;
4) Rinse with 5 column volumes of distilled water ;
5) 70% ethanol ,5 column volumes ;
6) Rinse with 5 column volumes of distilled water ;
7) 100 mM EDTA (pH 8.0), 5 column volumes ;
8) Rinse with 5 column volumes of distilled water ;
9) 100mM $NiSO_4$, 5 column volumes ;
10) Rinse with 5 column volumes of distilled water ;

After regeneration, the medium can be used immediately, otherwise, it need to be suspended in an equal volume of 1X PBS containing 20% ethanol at 4°C.

5. TROUBLESHOOTING

Problem	Probable cause	Solution
Back pressure exceeds 1 bar	Column is clogged	Cleaning in place (Part 3).
		Increase the centrifugation speed or filtering the sample.
	Sample is too viscous	Increase sonication or add DNase I (5 µg/ml with 1mM Mg^{2+}). Incubate on ice for 15min.
	Buffer is too viscous	Dilute sample by adding more homogenization buffer.
No protein is eluted	Expression of target protein in extract is very low	Check expression level of protein by estimating the amount in the extract, flow through, elute fraction and pellet upon centrifugation. Or apply large sample volume.
	Target protein is found in the flow through	Reduce imidazole concentration in lysis buffer sample and wash buffer. Increase buffer pH.
	Elution conditions are too mild.	Increase imidazole concentration in Elution buffer. Or decrease buffer pH.
		Strip nickel ion by using 10-100mM EDTA solution, at the same time you can obtain target protein.
	Protein degradation or purification cause the his-tag to be removed.	Operate at 4°C. Add protease inhibitors.
		Make a new construct with his-tag attached to other terminus.
His-tagged protein is not pure	Wash is not enough	Increase the volume of Wash Buffer.
	Association between the his-tagged protein and protein contaminant.	Optimize the wash condition by adjusting the pH and imidazole concentration.
		Add an additional chromatography step, that is ion exchange, hydrophobic interaction or size exclusion.
The color of medium becomes shallow.	The nickel ions was stripped.	Chelate nickel ions according to the part 4.
The medium are brown.	The buffer contains DTT.	Reduce the concentration of DTT under 2mM.
Protein precipitates during purification	Temperature is too low	Perform the purification at room temperature.
	Aggregate formation	Add solubilization agents to the samples and buffers, for example 0.1% Triton X-100 , Tween-20 and ≤20% glycerol to maintain protein solubility.

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