Ni-NTA Spin Kit Handbook

Ni-NTA Spin Kit

Ni-NTA Spin Columns

For manual or automated purification of

His-tagged proteins

- 可在天然和变性条件下纯化
- 一步法，均一性高达95%
- 即用型离心柱，可用于快速自动化或手工流程

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www.tthbio.com

www.galaxybio.cn

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产品简介

Ni-NTA Spin Kits提供一种简单快速的提取6xHistagged 基因工程蛋白的方法。非常适合从成批诱导培养物提取蛋白，整个提取过程仅需15分钟，可以纯化多达300μg的6xHis-tagged蛋白。为小量离心纯化His-tagged重组蛋白，高通量筛选重组蛋白首选。

Ni-NTA column 内含有Ni-NTA 硅胶基质可以抑制非特异性疏水作用。Ni-NTA Spin Kit 和Columns以微离心柱形式提供Ni-NTA 硅胶基质用于平行进行多组样品制备。每个离心柱可纯化高达300ug 6xHis-tagged 蛋白。Ni-NTA column可以在30分钟内处理多达24个样本，可用于筛选功能蛋白，选择全长翻译产物的克隆表达，比较表达水平的差异。与所有Ni-NTA基质类似，Ni-NTA离心柱可以在天然或变性条件下一步纯化蛋白。Ni-NTA Spin Kit 可以以离心形式纯化6xHis-tagged 蛋白。

包装

	Cat:DC110
Ni-NTA Spin Columns	50套
Guanidine HCl盐酸胍	40g
Urea	100g
1 M Imidazole, pH 8.0	50ml
10x Tris-HCl缓冲液 (500 mM Tris-HCl, 3 M NaCl, pH 8.0贮存液)	100ml
质控菌株（甘油菌 ）	100ul

贮存：常温保存.

蛋白纯化一般常识

操作流程（见figure1）

- 准备细胞裂解液，结合Ni-NTA硅胶膜
- 洗涤至少3次
- 洗脱 6xHis-tagged蛋白

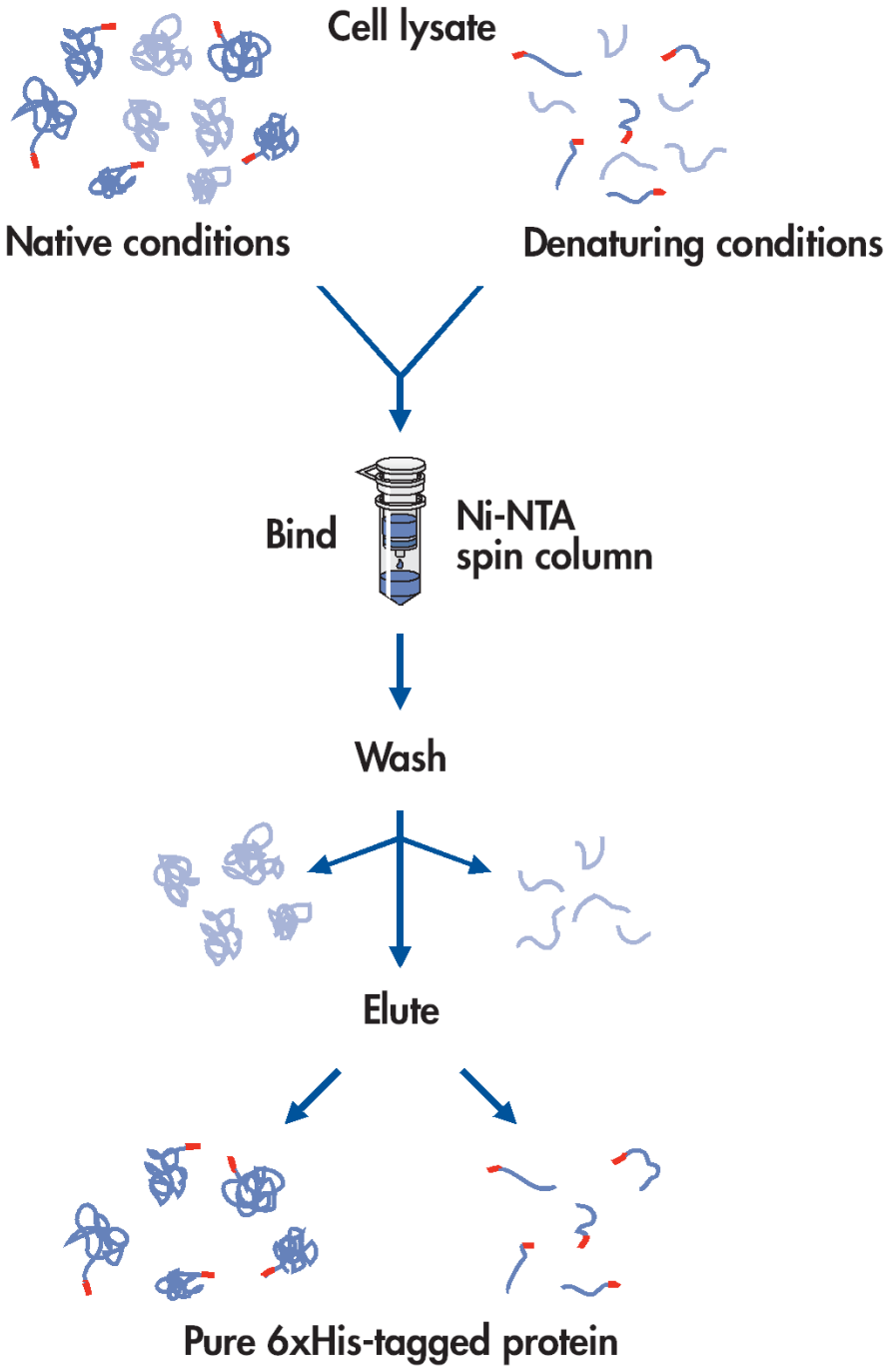

Figure 1. Ni-NTA Spin purification procedure.

注意事项

- 为确保柱子结合效率，蛋白结合一步离心速度最好不要超出 270 x g (approx. 1600 rpm) 高速离心会导致蛋白不能有效结合
- 避免高浓度的溶液（见Table 1）.
- 细胞裂解液避免高浓度的螯合剂和离子活性剂（见Table 1）
- 细胞在上柱子前，要高速离心除掉不容物。溶液粘稠裂解液，上柱后，适当延长离心时间
- spin columns 离心时最好是打开盖子，以便能够离心彻底。
- 有些蛋白不稳定可以添加蛋白酶抑制剂，防止降解

变性条件下准备细胞裂解液

可以使用6 M盐酸胍或者8 M尿素。优先使用尿素，因为样品中高浓度盐酸胍会影响下一步的SDS-Page电泳。

估算一下细菌的表达量很重要，表达量>10 mg/L,占细胞总蛋白12%多，裂解上清需要10倍的浓缩。600 µl细胞裂解液含His-tagged蛋白60µg左右表达量低于1 mg/L的蛋白，需要50倍体积的浓缩

自然条件下准备细胞裂解液

自然条件下纯化蛋白，需要估算一下可溶蛋白和包含蛋白的比例，可溶蛋白需要一定浓度和量。

- 自然条件下纯化蛋白，常常会有不含6xHis-tagged非特异性的蛋白吸附，因此需要在裂解液和洗涤液加入一定浓度的咪唑。
- 大多数蛋白使用10－20 mM咪唑；如果目的蛋白此条件不能结合，把咪唑浓度降到1–5 mM.
- 加入β-mercaptoethanol (up to 20 mM) 或者 DTT (up to 10 mM) 可以打开任何二硫键，防止杂蛋白结合6xHis-tagged蛋白，从而造成纯化的蛋白不纯。
- 收集的菌体至少在－20℃冷冻30min， 才能被含有溶菌酶(1 mg/ml)和DNAase的裂解液裂解。新鲜收集的菌体需要超声破碎。
- 所有的缓冲液都需要足够的离子强度阻止非特异吸附。 All buffers should have sufficient ionic strength 结合洗涤可以300 mM NaCl. 最大可以用到2 M NaCl.

目的蛋白洗脱

目的蛋白的洗脱通过低PH或者咪唑竞争洗脱。一般单体蛋白在PH5.9或者咪唑浓度大于100 mM 可以洗脱下来。多聚体一般在pH4.5 or或200 mM咪唑洗托。推荐使用Buffer E pH4.5或者250–500 mM 咪唑 pH 8.0。 100 mM EDTA 可以通过剥夺镍离子，洗脱所以的结合蛋白。

使用Ni-NTA spin column, 可以纯化300 µg的f 6xHis-tagged蛋白。实际提取的量与蛋白表达的量、上样量、溶液粘稠度有关。

一般洗脱总体积在100–300 µl，单次洗脱最好不要低于50ul。为获得高浓度的蛋白，建议每次50ul洗脱，洗脱两次。

培养与诱导表达

准备工作

- LB液体培养基配方: 胰蛋白胨(Tryptone) 10g/L 、酵母提取物(Yeast extract) 5g/L 、氯化钠(NaCl) 10g/L 。根据经验值用NaOH调节该培养基的pH,使其达到7.4(该pH适合目前使用最广的原核表达菌种

E.coli的生长)

- 卡那霉素贮存液： 25 mg/ml水溶液，无菌过滤，保存在－20℃。使用终浓度 25 μg/ml (也就是 1000稀释).
- 氨苄青霉素贮存液: 100 mg/ml 25 mg/ml水溶液，无菌过滤，保存在－20℃。使用终浓度 25 μg/ml (也就是 1000稀释).
- IPTG 贮存液: 1 M水溶液，无菌过滤，保存在－20℃。使用终浓度 1mM， (也就是 1000稀释).

Protocol

1. 第一天，挑去一个单克隆细菌，放入10mlLB培养基，加入相应抗生素， 37℃摇床振荡培养过夜。

2. 第二天，过夜培养菌液 1:60稀释，加入相应抗生素. 37℃摇床振荡培养OD_{600} 为 0.6.

3.加入 IPTG终浓度 1 mM ， 37℃摇床振荡，诱导4小时

视情况可以加入0.1–1 mM PMSF，防止蛋白酶降解蛋白

4. 4000 x g 15 min，小心倒掉培养基，收集菌体，-20℃保存。

方案一：自然条件下，从细菌裂解液种纯化蛋白

试剂准备

- **裂解液Lysis Buffer (NPI-10):**
  1XTris-HCl缓冲液, 10 mM imidazole; 1%Nonidet P40或者Tritonx-100 , pH 8.0
- **洗涤液WashBuffer (NPI-20):**
  1XTris-HCl缓冲液, 10 mM imidazole; 1%Nonidet P40或者Tritonx-100 , pH 8.0
- **洗脱液Elution Buffer (NPI-500):**
  1XTris-HCl缓冲液, 10 mM imidazole; 1%Nonidet P40或者Tritonx-100 , pH 8.0
- DNAase
- Lysozyme.

操作：

1. 5ml-10ml诱导菌,4000 x g 15 min,小心倒掉培养基,收集菌体,-20℃保存

2. 用700 µl Lysis Buffer (NPI-10)重悬细菌，加入. DNAase, Lysozyme.

3. 冰浴30分钟.

4. 4°C离心12,000 x g ,10分钟. 收集上清，留出20ul做SDS-PAGE蛋白电泳

5. 加600ul Lysis Buffer (NPI-10) 于Ni-NTA spin column.亲和柱中，平衡5分钟。然后 890 x g (approx. 2900 rpm)离心除去液体备用.

6. 加入600 µl 上清液体于平衡过的Ni-NTA spin column亲和柱中， 270 x g (approx. 1600 rpm)离心5分钟，如果没有完全离心掉液体，可以适当延长离心时间或提高转速。 确保转速不要太高，在 270 x g (approx. 1600 rpm) 左右，速度过高，蛋白不能有效结合。

7. 以600 µl Buffer NPI-20洗涤Ni-NTA spin column亲和柱两次，890 x g (approx. 2900 rpm)离心2分钟，除去液体. 如果没有完全离心掉液体，可以适当延长离心时间或提高转速。洗涤次数和蛋白表达量决定了纯化蛋白纯度。如果蛋白纯度不够可以增加两次洗涤。

8. 以300 µl 的Buffer NPI-500洗脱两次，890 x g (approx. 2900 rpm)离心2分钟

大于>80%6xHis-tagged蛋白洗脱在第一次300 µl 洗脱液里，剩余的在第二次300ul洗脱液里

方案二：变性条件下，从细菌裂解液种纯化蛋白

试剂准备

- Buffer A: 6 M GuHCl; 0.1 M NaH2PO4; pH 8.0
- Buffer B 7 M urea; 0.1 M NaH2PO4;pH 8.0
- Buffer C: 8 M urea; 0.1 M NaH2PO4; pH 6.3
- Buffer D: 8 M urea; 0.1 M NaH2PO4; pH 5.9
- Buffer E: 8 M urea; 0.1 M NaH2PO4; pH 4.5
- DNAase
- lysozyme.

由于加入尿素，需要校正溶液PH。本实验方法适合冻存过的菌体。没有冻存过的，新鲜菌体需要超声破碎。

操作

1. 把冻存的菌体解冻，重悬于 700 µl Buffer B，加入适量DNAase和lysozyme.

2. 室温 15 min，溶液应该变清亮。

优先使用Buffer B，不影响下一步SDS-PAGE电泳。如果在. Buffer B不能够溶解，需要使用Buffer A，后者需要1:6稀释后才能跑SDS-PAGE电泳，或者通过，TCA沉淀、或加入乙醇至50后沉淀，在做SDS-PAGE电泳。

注意GuHCl存在下，DNAase 是没有活性的，基因组必须通过离心除去。不溶性蛋白和基因组很容易堵死柱子，使得实验无法进行。

3. 12,000 x g ，室温离心 15–30 min ，取上清。

可以留出20 µl 做 SDS-PAGE 分析。

4. 以600 µl Buffer B平衡 Ni-NTA spin column ， 890 x g (approx. 2900 rpm). 离心2 min

spin columns 打开盖子，以便完全离心

5. 加入 600 µl 上清， 270 x g(approx. 1600 rpm) ，离心5 min，收集穿透液 collect the flow-through.

高表达的菌液600 µl上清保证含有150–180 µg的6xHis-tagged蛋白，低表达需要50ml诱导菌液浓缩到600ul上清，保证含有30–150 µg的 6xHis-tagged 蛋白。

由于上清粘稠度不同，需要充分考虑一下离心时间，尽量在700 x g (approx. 2000 rpm)，.低速离心3-4min以确保结合效率。 个别粘稠度高的适当延长时间

6. 加入600 µl Buffer C， 890 x g (approx. 2900 rpm)，离心2分钟。

洗涤这步可以用Buffer C甚至Buffer A 。大多数蛋白可以溶解于Buffer C，否则，Buffer C and Buffer E，以6 M 盐酸胍代替8M的尿素。

7. Repeat step 6.

蛋白表达量高时，洗涤两次一班都可以得到较纯的蛋白；表达量低时，需要增加洗涤次数。

8. 加入50-200 µl Buffer E，890 x g (approx. 2900 rpm)，离心2min，洗脱蛋白. >80% 蛋白在第一次洗脱液里，可以分别收集做SDS-PAGE电泳。如果想得到高浓度的蛋白，尽量减少洗脱体积。

如果 6xHis-tagged 单体需要从多聚体分离，可以先使用BufferD 然后再使用 Buffer E.

Table 1. Compatibility of reagents with Ni-NTA

Reagent	Effect	Comments
Buffer reagents		
Tris, HEPES, MOPS	Buffers with secondary or tertiary amines may reduce nickel ions	Up to 100 mM can be used, however sodium phosphate or phosphate-citrate buffer is recommended
Chelating reagents		
EDTA, EGTA	Strip nickel ions from resin	Up to 1 mM has been used successfully in some cases, but care must be taken
Sulfhydril reagents		
β-mercaptoethanol	Prevents disulfide cross-linkages. Can reduce nickel ions at higher concentration	Up to 20 mM can be used. Do not store resin under reducing conditions
DTT, DTE	At high concentrations (>1 mM) resin may turn reversibly brown due to nickel reduction. Up to 10 mM has been tested and shown not to compromise purification or increase nickel leaching.	Up to 10 mM DTT has been used successfully. Do not store resin under reducing conditions.
Detergents		
Nonionic detergents (Triton®, Tween NP-40, etc.)	Removes background proteins and nucleic acids	Up to 2% can be used
Cationic detergents		Up to 1% can be used
Nonionic detergents (®-OG, DM, DDM, Cymal 6, Apo12 9, NG and others)	Resolubilization and purification of membrane proteins	Up to 2% can be used
Zwitterionic detergents (LDAO, CHAPS, CHAPSO)	Removal of background proteins and nucleic acids; purification of membrane proteins	Up to 1% can be used
Anionic detergents (SDS, sarkosyl)		Not recommended, 0.3% has been in some cases

Triton X-114	Removes endotoxins	Up to 2% can be used
Denaturants		
GuHCl Urea	Solubilize proteins	Up to 6 M Up to 8 M
Amino acids		
Glycine		Not recommended
Glutamine		Not recommended
Glutamic acid		Up to 100 mM
Arginine		Not recommended
Histidine	Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	Can be used at low concentrations (20 mM) to inhibit nonspecific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Other additives		
NaCl	Prevents ionic interactions	Up to 2 M can be used, at least 300 mM should be used
$MgCl_2$		Up to 4 M
$LiCl_2$		Up to 50 mM
$CaCl_2$		Up to 5 mM
$MgSO_4$		Up to 1 M
Glycerol	Prevents hydrophobic interaction between proteins	Up to 50%
Ethanol	Prevents hydrophobic interactions between proteins	Up to 20%
Triethanolamine	Prevents hydrophobic interactions	Up to 100 mM
Sorbitol, betaine, ectoine	Prevents hydrophobic interactions	Up to 500 mM
Dextran sulfate	Prevents hydrophobic interactions	Up to 0.05% (w/v)
Imidazole	Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	Can be used at low concentrations (20 mM) to inhibit non specific binding and, at higher concentrations (>100

		mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Sodium bicarbonate		Not recommended
Hemoglobin		Not recommended
Ammonium		Not recommended
Citrate		Up to 60 mM has been used successfully

Troubleshooting Guide

	Comments and suggestions
Protein does not bind to the Ni-NTA Spin column	
a) 6xHis tag is not present	Check expression construct. Sequence ligation junctions to ensure that the reading frame is correct. Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal tag).
b) 6xHis tag is inaccessible	Purify protein under denaturing conditions. Move tag to the opposite end of the protein.
c) 6xHis tag has been degraded	Check that the 6xHis tag is not associated with a portion of the protein that is processed.
d) Binding conditions incorrect	Check pH of all buffers. Dissociation of urea often causes a shift in pH. The pH values should be checked immediately prior to use Ensure that there are no chelating or reducing agents present and that the concentration of imidazole is not too high
Protein elutes in the wash buffer	
a) Wash stringency is too high	Lower the concentration of imidazole or increase the pH slightly.
b) 6xHis tag is partially hidden	Purify under denaturing conditions.
c) Buffer conditions incorrect	Check pH of denaturing wash buffer.
Protein precipitates during purification	
a) Temperature is too low	Perform purification at room temperature.
b) Protein forms aggregates	Try adding solubilization reagents such as 0.1% Triton® X-100 or Tween®-20, up to 20 mM ®-ME, up to 2 M NaCl, or stabilizing cofactors such as Mg^{2+}. These may be necessary in all buffers to maintain protein solubility.
Protein does not elute	
Elution conditions are too mild(protein may be in an aggregate or multimer form)	Elute with decreased pH or increased imidazole concentration.
Protein elutes with contaminants	
a) Binding and wash conditions	Include 10–20 mM imidazole in the binding not stringent enough and wash buffers
b) Contaminants are associated with tagged protein	Add-mercaptoethanol to a maximum of 20 mM to reduce disulfide bonds.

	Increase salt and/or detergent concentrations, or add ethanol/glycerol to wash buffer to disrupt nonspecificinteractions (see Table 1, page 11).
c) Contaminants are truncated	Check for possible internal translation starts forms of the tagged protein (C-terminal tag) or premature termination sites (N-terminal tag). Prevent protein degradation during purification by working at 4°C or by including protease inhibitors

Appendix A: Preparation of Guanidine-Containing Samples for SDS-PAGE

Since the fractions that contain GuHCl will form a precipitate when treated with SDS, they must either be diluted with water (1:6), dialyzed before analysis, or separated from the guanidine hydrochloride by trichloroacetic acid (TCA) precipitation. TCA-precipitation: Bring the volume of the samples up to 100 µl with water, add an equal volume of 10% TCA, leave on ice 20 min, spin 15 min at 15,000 x g in a microcentrifuge, wash pellet with 100 µl of ice-cold ethanol, dry, and resuspend in 1x SDS-PAGE sample buffer (5x SDS-PAGE sample buffer is 0.225 M Tris·Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT). In case there is still any GuHCl present, samples should be loaded immediately after boiling for 7 min at 95°C.

Appendix B: Buffer Compositions

Bacterial media and solutions

LB medium	10 g/liter tryptone; 5 g/liter yeast extract 10 g/liter NaCl
LB agar	LB medium containing 15 g/liter agar
Psi broth	LB medium, 4 mM $MgSO_4$; 10 mM KCl
Kanamycin stock solution	25 mg/ml in H_2O, sterile filter, store in aliquots at –20°C
Ampicillin stock solution	100 mg/ml in H_2O, sterile filter, store in aliquots at –20°C
IPTG (1 M)	238 mg/ml in H_2O, sterile filter, store in aliquots at –20°C