Amicon® Pro Affinity Concentration Kit - Ni-NTA

Purification of His-tagged recombinant proteins by metal chelation chromatography.

Catalog Nos. ACR5000NT, ACK5003NT, ACK5010NT, ACK5030NT, ACK5050NT, ACK5100NT

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Introduction

The expression and purification of recombinant proteins is central to the study of protein regulation, structure and function. Purification has been greatly facilitated by incorporation of small affinity tags into the protein sequence. The Amicon® Pro Affinity Concentration Ni-NTA Kit is optimized for rapid centrifugal-based purification of polyhistidine-tagged proteins from cellular extracts. With Ni-NTA technology, purification is based on the affinity between residues of the His-Tag sequence and Ni^{2+} cation-coordinated reactive groups on the agarose resin. Ni-NTA resins use covalently attached nitrilotriacetic acid (NTA) as the chelating agent; each NTA group has four metal ion coordination sites. NTA chemistry minimizes metal ion leaching thereby reducing precipitation while increasing overall purity. Following resin capture of the target protein, unbound lysate components are removed by spin-based clearing and washing steps. The target protein is recovered by elution in the presence of imidazole.

By condensing the entire purification workflow into one device, the Amicon® Pro device eliminates the need for multiple sample transfers thereby minimizing protein loss. The large exchange device reservoir (up to 9ml) accommodates a range of sample capacities as well as reduces the need for multiple spin steps during the wash and elution phases. Direct coupling to an Amicon® Ultra-0.5 device further provides simultaneous concentration during the elution phase. Lastly, the Amicon® Pro device offers highly efficient diafiltration in a single 15 minute spin. The kit contains sufficient Ni-NTA resin, optimized buffers, and Amicon® Pro devices for 12 standard reactions.

Sample Preparation Guidelines

- Optimizing lysis parameters is critical for protein extraction and downstream purification. The Amicon® Pro Ni-NTA kits are compatible with a range of buffer conditions including BugBuster™ Protein Extraction Reagent (Cat. No. 70584), a proprietary mixture of non-ionic detergents offering a rapid cost-effective alternative to mechanical cell lysis methods such as sonication.
- Irrespective of extraction method, we recommend inclusion of rLysozyme™ Solution (Cat. No. 71110) and Benzonase® Nuclease (Cat. No. 70746) during protein extraction for increased *E. coli* cell lysis (overall yield) and reduction in sample viscosity, respectively.
- EDTA-free Protease Inhibitor Cocktails (Cat. Nos. 539134, 539137) can also be added to minimize protein degradation.
- High concentrations of reducing agents (DTT or DTE) will disrupt the binding of Nickel-based resins. Ni-NTA resin is compatible with up to 20 mM β-mercaptoethanol for purification under reducing conditions.
- Cells should be lysed without chelating agents (EDTA, EGTA) or ionic detergents such as SDS. Buffers can be supplemented with non-ionic detergents (0.1% Tween® 20 or Triton® X-100) or high salt concentration (2 M NaCl) to reduce non-specific binding to the Ni-NTA matrix.
- The Amicon® Pro Ni-NTA reagents are optimized for purification under native conditions. In certain systems, high protein expression can lead to aggregation in the form of inclusion bodies. Strong denaturants such as 6 M guanidine or 8 M urea can be used to solubilize aggregates greatly enhancing yield. It may be necessary to perform all purification steps under denaturing conditions to prevent protein precipitation.
- For more information, consult the Ni-NTA His•Bind® Resin User Guide available at http://www.emdmillipore.com/chemicals. Search using the Cat. No. 70666.

Kit Components

- CS211420-Ni-NTA Resin (3 mL) – Nickel-charged NTA immobilized onto crosslinked agarose is supplied as 50% slurry. The binding capacity is typically 5-10 mg/mL settled resin.
- Reaction Buffers:
  - CS211413-4X Ni-NTA Bind Buffer (25 mL) – 200 mM NaH_2PO_4 pH 8, 1.2 M NaCl, 40 mM Imidazole
  - CS211414-4X Ni-NTA Wash Buffer (25 mL) – 200 mM NaH_2PO_4 pH 8, 1.2 M NaCl, 80 mM Imidazole
  - CS211431-4X Ni-NTA Elution Buffer (5 mL) – 200 mM NaH_2PO_4 pH 8, 1.2 M NaCl, 1 M Imidazole
- Amicon® Pro Devices – The kit includes 12 complete assemblies. Each device consists of an exchange device, holder tube, 50 mL collection tube, and Amicon® Ultra-0.5 centrifugal filter device (AU-0.5). A 2 mL collection tube is included for sample recovery from the AU-0.5 device by reverse spin. The kit is available in five formats based on the nominal molecular weight limit (NMWL) of the AU-0.5 device: 3 (ACK5003NT), 10 (ACK5010NT), 30 (ACK5030NT), 50 (ACK5050NT), and 100 kDa (ACK5100NT). Consult the Amicon® Pro (http://www.emdmillipore.com/psp, search keywords "Amicon® Pro") and Amicon® Ultra-0.5 mL Centrifugal Device User Guides (http://www.millipore.com/catalogue/module/c82301) for proper assembly/disassembly and additional product information.

Buffers should be stored at room temperature; Ni-NTA resin should be stored at 2° to 8°C (do not freeze). All 4X buffers should be diluted to 1X with sterile deionized H2O shortly before use. 1X solutions may be stored at 2° to 8°C for up to 1 week. The Amicon® Pro devices can be stored separately at room temperature.

Procedures for using the Amicon® Pro Affinity Concentration Kit - Ni-NTA

The protocol is based on purification of His-tagged protein from 0.5 mL of *E. coli* lysate using 200 µL of Ni-NTA resin slurry (100 µL packed resin). The protocol is linearly scalable for 50-1000 µL of resin slurry. Due to large variability among sample preps, parameters which may require optimization include bead input, binding time, wash, and elution parameters. This protocol includes steps for simultaneous concentration during the elution step as well as buffer exchange using the Amicon® Ultra-0.5 centrifugal filter device.

Note: Given the collection tube's capacity, it is not necessary to remove filtrate between the various centrifugation steps. However, if process samples need be retained for analytical purposes, the collection tube should be cleared.

Bead Preparation

1. To ensure uniform suspension, vortex the Ni-NTA resin thoroughly before adding it to the device.
2. Remove the collection tube cap and open the exchange device cap.
3. Add 200 µL of resin slurry to the base of the exchange device. Close the exchange cap.
   - Up to 500 µl packed resin (1000 µl slurry volume) may be added per device. We recommend using wide-bore tips (Cat. No. 02-707-134, Fisher Scientific) for resin transfer.
4. To remove storage buffer, centrifuge in a swinging bucket rotor at 1000 g X 1 min.
5. Add 500 µL of 1X Bind Buffer. Centrifuge at 1000 g X 1 min.

Protein Binding

1. Add 500 µL of sample to the exchange device.
   - Up to 9 mL of sample can be added. The volume loaded is determined by the target protein's expression level and resin's binding capacity.
2. Incubate for 60 min at room temp with gentle agitation.
   - We recommend upright agitation on a plate shaker at low setting.
   - End-over-end mixing, particularly with small volumes or for extended time, may result in substantial bead loss to the sides of the feeder tube.
   - The duration of binding time may vary with application.
3. Centrifuge the device at 1000 g X 1 min in a swinging bucket rotor. Recover the sample flow-through from the 50 mL collection tube (optional).
   - To ensure maximal protein capture, collect all resin into solution prior to centrifugation.
4. Add 1.5 mL of Wash Buffer. Centrifuge at 1000 g X 1 min. Recover the wash fraction from the 50 mL collection tube (optional).
   - Due to the large capacity of the exchange device, the volume of the wash can be increased for greater sample purity. There is no need for multiple wash steps.

Sample Elution

Samples can be eluted without concentration by adding elution buffer and centrifuging (1000g X 2 minute) directly into a clean 50 ml collection tube. Given the limited volume processing capacity of the AU-0.5 device, we recommend this protocol if elution volumes > 1.5 ml are required.

For simultaneous elution with concentration, attach the Amicon® Ultra-0.5 device and follow the steps outlined below.

1. Remove the exchange device and insert it into the AU0.5 device.
2. Place the exchange device/AU-0.5 assembly back in the holder and return the device to the collection tube.
3. Add up to 1.5 mL of Elution Buffer, gently resuspend the resin, and incubate for 5 min.
   - Under standard conditions, one elution is sufficient for recovery of 90-95% of captured protein.
   -
4. Close the exchange device cap and screw on the collection tube cap to ensure a proper seal.
5. Centrifuge at 4000 g X 15 min in a swinging bucket rotor. Concentrated samples can be buffer exchanged or recovered from the AU-0.5 device by reverse spin (see below).
   - Depending on the starting elution volume, NMWL of AU0.5 device employed, and the degree of concentration desired, the length of the spin time can range for 10-30 minutes. Please consult the **Performance Characteristics** section in the Amicon® Pro Affinity Concentration System User Guide (http://ww.emdmillipore.com/psp, and search keywords "Amicon Pro") for recommended guidelines.
   - , consult.
6. Recover the concentrated fraction by reverse spin or proceed to Buffer Exchange (see below).

Buffer Exchange (Optional if samples have been collected in the Amicon® Ultra-0.5 device)

1. After sample concentration, add 1.5ml desired buffer to the exchange device/AU-0.5 assembly.
2. Centrifuge device at 4000g X 15 minutes in a swinging bucket rotor. Concentrated samples can be recovered from the AU-0.5 device by reverse spin (see below).

Collect sample from the AU0.5 device by Reverse Spin (following Concentration or Buffer Exchange)

1. Disassemble the exchange device/AU-0.5 assembly from the holder tube.
2. Using a gentle twisting motion, detach the AU-0.5 from the exchange device.
3. If there is residual sample in the exchange device tip, depress the exchange device cap to expel the remaining sample volume into the AU-0.5.
4. Hold AU-0.5 upright and slide the 2 ml collection tube on top of it.
5. Invert the assembly and centrifuge (in a microcentrifuge) with a fixed angle rotor 1000g X 2min.

Sample protein yield can be determined by Mid IR-based spectrometry using the DirectDetect™ biomolecular quantitation system and DirectDetect™ Assay Free Sample Cards.

Troubleshooting

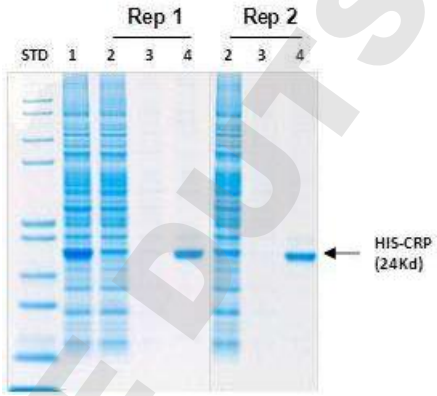
Issue: Recombinant Protein is present in low amount in eluate	
Possible Cause	**Solution**
Protein is insoluble, formed inclusion bodies.	After lysate clearance, check both the supernatant and pellet for protein. Perform lysis and binding procedures under denaturing conditions.
The His-tag is not exposed for binding to the affinity resin.	The protein may require denaturing conditions for binding.
The His-tag is not present.	Sequence the 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).
Recombinant protein is degraded during cell lysis.	Add protease inhibitors to the cell lysate.
Protein does not elute from the Ni-NTA Resin.	After binding, check the filtrate for depletion of the target protein. Increase the concentration of imidazole in the Elution Buffer.
Protein forms aggregates.	Add solubilizing agents such as detergents (0.1% Triton® X-100, TWEEN®-20) or increase salt concentration.
pH of the Lysis or Binding Buffers is incorrect.	Check the buffer pH; the acceptable range is 7-8. Acidic buffers will prevent binding.
Protein expression is insufficient.	Optimize the growth/induction conditions.
Cell Lysis is incomplete.	Optimize the Cell Lysis Protocol.
Cell Lysate is too viscous.	If possible, dilute the lysate in Bind Buffer. Alternatively, include Benzonase® Nuclease during lysis to remove free RNA/DNA.
Protein is lost during sample concentration while using the AU0.5 device.	Check the protein's expected size and MWCO of AU0.5 device used. AU0.5 is offered in 5 different MWCO formats - 3, 10, 30, 50, and 100 kDa.
Protein precipitates during sample concentration while using the AU0.5 device due to over-concentration.	Reduce the duration of the centrifugation time during the elution/concentration step.
Protein elutes in the Wash Buffer.	Ensure that there are no chelating or reducing agents present in the Lysis, Bind, or Wash Buffers. The protein may bind weakly to Ni-NTA and thus requires less (or no) imidazole in the Lysis/Wash Buffers.

Issue: High Non-specific binding	
Possible Cause	**Solution**

Ionic strength of the Lysis, Bind, and Wash Buffers is insufficient.	Salt concentrations up to 2M of NaCl are tolerable.
Binding and Wash conditions are not stringent enough.	Increase the imidazole concentration. In certain cases, 50 mM of imidazole may be required.
Insufficient washing.	Increase the volume of Wash Buffer used or the number of wash steps.
Contaminants interact directly with the His-tagged protein.	Add β-mercaptoethanol to 20 mM to reduce disulfide bonds. Add detergents to disrupt non-specific interactions.
His-tagged protein is degraded.	Degraded/truncated forms of the recombinant protein will still bind the Ni-NTA resin and appear as contaminating bands in SDS-PAGE. Perform a lysis procedure on ice and include protease inhibitors.
Cell lysate is too concentrated.	Dilute the lysate in Bind Buffer before purification.

Performance

Purification with Amicon® Pro-Affinity Concentration Kit – Ni-NTA



Replicate purifications of a 24 kDa His-tagged protein from *E. coli* lysate using the Amicon® Pro Affinity Concentration Ni-NTA kit. The numbered lanes are:
1 – lysate
2 – flowthrough fraction
3 – wash fraction
4 – eluted fraction

Product Ordering Information

	No Devices	Amicon® Pro + AU 0.5 mL with MWCO:				
		3k	10k	30k	50k	100k
Amicon® Pro Affinity Concentration Kit - Ni-NTA	ACR5000NT	ACK5003NT	ACK5010NT	ACK5030NT	ACK5050NT	ACK5100NT
Amicon® Pro Affinity Concentration System 12PK		ACS500312	ACS501012	ACS503012	ACS505012	ACS510012
Amicon® Pro Affinity Concentration System 24PK		ACS500324	ACS501024	ACS503024	ACS505024	ACS510024

The Amicon® Pro Affinity Concentration Kit contains reagents and devices sufficient for 12 standard reactions. Amicon® Pro devices are also sold separately in 12 and 24 packs.

Additional Reagents	Catalogue Number	Qty
Ni-NTA His•Bind® Resin	70666-3/4/5	10/25/100 mL
Ni-NTA Buffer Kit	70899	1 Kit

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