Purification of His-tagged Proteins Under Denaturing Conditions Using PureCube His Affinity Agarose and PureCube 1-step batch Mini Columns

Overview

This protocol describes the generation of a cleared lysate from an *E. coli* cell pellet and the subsequent purification of His-tagged proteins under denaturing conditions using our PureCube His Affinity Agaroses, featuring NTA, IDA, or the novel EDTA-stable INDIGO ligands.

Reagent amounts given apply to 10 mL IPTG-induced bacterial culture of a well-expressed protein (approximately 10–50 mg/L). If other culture volumes are processed or protein expression is higher or lower, reagent volumes may need to be adjusted.

The protocol uses the revolutionary 1-step batch Mini Columns that feature the SelfSeal™ membrane technology, saving time and pipetting steps. They replace disposable gravity flow columns used in standard protocols. Volumes of up to 600 µL can be applied to a Mini Column. For larger scale experiments, 1-step batch Midi Plus Columns are available.

In this protocol cells are lysed with a high concentration of urea, which also aids to dissolve insoluble protein aggregates. The His-tagged protein is purified from the cleared lysate under denaturing conditions in a bind-wash-elute procedure. Binding occurs at slightly alkaline pH, while washing and elution are done with a stepwise pH decrease. Binding is performed in batch mode. This method is most efficient, especially when the target protein is present at low concentrations or the His-tag is not fully accessible. Batch binding can be done directly in the 1-step batch Mini column to simplify the procedure.

Please contact us if you have questions or need assistance optimizing a protocol for your application (contact@cube-biotech.com); other protocols can also be found at www.cube-biotech.com/protocols.

Equipment

- ☐ PureCube 1-step batch Mini Columns (Cube Biotech #63103)
- ☐ Ice bath
- ☐ Microcentrifuge with rotor suitable for 2 mL centrifuge tubes (min 10,000 xg)
- ☐ 2 mL centrifuge tubes
- ☐ Micropipettor
- ☐ Micropipetting tips
- ☐ pH meter
- ☐ End-over-end shaker
- ☐ SDS-PAGE equipment
- ☐ Optional: Western Blot equipment

Materials

- ☐ Cell pellet from a 200 mL culture (approx. 0.5 g)
- ☐ PureCube His Affinity Agarose, e.g.
- PureCube Ni-NTA Agarose (50 mL; #31105)
- PureCube Co-NTA Agarose (50 mL; #31405)
- PureCube 100 Ni-NTA Agarose (50 mL; #74105)
- PureCube 100 Co-NTA Agarose (50 mL; #74405)
- PureCube 100 INDIGO Ni-Agarose (50 mL; #75105)
- PureCube Ni-IDA Agarose (50 mL; #30105)
- ☐ Sodium phosphate monobasic (NaH_2PO_4)
- ☐ Sodium chloride (NaCl)
- ☐ Tris base
- ☐ Urea
- ☐ Hydrochloric acd (HCl)
- ☐ Sodium hydroxide (NaOH)
- ☐ Dithiothreitol (DTT)
- ☐ Glycerol
- ☐ Sodium dodecyl sulfate (SDS)
- ☐ Bromophenol blue
- ☐ Optional: Benzonase® nuclease (e.g. Merck Milipore, #707464)
- ☐ Optional: PentaHis Antibody (Cube Biotech #40040)

Solutions and buffers

Denaturing Lysis Buffer, pH 8.0, 50 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaH_2PO_4	100 mM	119.98	0.5 M	29.99 g/ 500 mL	10 mL
Tris base	10 mM	121.14	1 M	12.11 g/ 100 mL	0.5 mL
Urea	8 M	60.06	–	–	24 g
Instructions: Dissolve urea in 30 mL water and then add the remaining components. Adjust pH to 8.0 with HCl and add water to a total volume of 50 mL. Due to urea dissociation, adjust the pH immediately before use.					

Denaturing Wash Buffer, ph 6.3, 50 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaH_2PO_4	100 mM	119.98	0.5 M	29.99 g/ 500 mL	10 mL
Tris base	10 mM	121.14	1 M	12.11 g/ 100 mL	0.5 mL
Urea	8 M	60.06	–	–	24 g
Instructions: Dissolve urea in 30 mL water, then add remaining components. Adjust pH to 6.3 with HCl and add water to a total volume of 50 mL. Due to urea dissociation, adjust the pH immediately before use.					

Denaturing Elution Buffer, pH 4.5, 50 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaH_2PO_4	100 mM	119.98	0.5 M	29.99 g/ 500 mL	10 mL
Tris base	10 mM	121.14	1 M	12.11 g/ 100 mL	0.5 mL
Urea	8 M	60.06	–	–	24 g
Instructions: Dissolve urea in 30 mL water, then add remaining components. Adjust pH to 4.5 with HCl and add water to a total volume of 50 mL. Due to urea dissociation, adjust the pH immediately before use.					

5X SDS-PAGE Buffer, 10 mL

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Tris-HCl, pH 6.8–7.0	300 mM	121.14	1 M	121.14 g/ 1 L	3 mL
Glycerol	50% (v/v)	–	100% (v/v)	–	5 mL
SDS	5% (w/v)	–	–	–	0.5 g
Bromophenol blue	0.05% (w/v)	–	4%	–	125 µL
DTT	250 mM	154.25	1 M	1.54 g/ 10 mL	125 µL/aliquot
Instructions: Make sure to prepare a 1 M Tris-HCl stock by dissolving Tris base in 500 mL deionized water, adding HCl to a pH of 6.8–7.0, and adding water to a final volume of 1 L. For the SDS-PAGE Buffer, mix all components listed **except DTT** and add water to a total of 10 mL. Freeze 20 aliquots (375 µL each) at –20°C. Before use, add DTT to the needed single aliquots.					

Procedure

1. Thaw the *E. coli* cell pellets corresponding to 10 mL bacterial culture on ice for 15 min.
2. Resuspend the cell pellet in 1 mL Denaturing Lysis Buffer and pour it into a 2 mL microcentrifuge tube.

Optional: Benzonase® can be added to the lysate to reduce viscosity caused by nucleic acids (3 U/mL bacterial culture). **Read: "About Denaturation"**. Nucleic acids can also be sheared by passing the lysate 10 times through a fine-gauge needle.

3. Incubate on an end-over-end shaker at room temperature for 30 min, or at 4°C for 1 h, depending on the temperature stability of the protein.

Optional: Up to 20 mM EDTA and 20 mM DTT can be used with INDIGO Ni-Agarose; NTA and IDA agaroses tolerate up to 1 mM EDTA and 10 mM DTT.

4. Centrifuge the lysate at 10.000 x g for 30 min and carefully collect the supernatant without touching the pellet.

Note: The supernatant contains the **cleared lysate fraction. We recommend to take aliquots of all fractions for SDS-PAGE analysis.**

5. Resuspend the PureCube His Affinity Agarose by inverting the bottle until the suspension is homogeneous. Transfer 200 µL of the 50% suspension (corresponding to 100 µL bed volume) into the batch incubation chamber of the spin column barrel. Close the chamber and spin the resin at 10.000-14.000 x g for 20 sec.
6. Add 600 µL of Denaturing Lysis Buffer and centrifuge again at 10.000-14.000 x g for 20 sec.
7. Repeat the step to completely remove any residual ethanol that might interfere with protein binding to the affinity resin.
8. Immediately before loading, filter the cleared lysate prepared in step 4 through a 0.2 µm filter (e.g. syringe filter) to remove any solid material that might clog the column.

Note: It is critical to perform this filter step immediately before loading the column.

9. Empty the 2 mL centrifuge tube and place the spin column barrel containing the equilibrated purification resin back into it.
10. Load the lysate filtered in step 8. The maximum loading volume is 600 µL. Invert 2-3 times to mix sample and resin. Incubate at 4°C for 1 h on an end-over-end shaker.
11. Centrifuge at at 10.000-14.000 x g for 20 sec, or until the lysate has completely passed through, and collect the flow-through.

This is the **flow-through fraction**.

12. Wash twice with 600 µL each of Denaturing Wash Buffer.

These are the **wash fractions.**

13. Replace the 2 mL microcentrifuge tube with a fresh one. Elute the His-tagged protein by adding 50-600 µL Denaturing Elution Buffer and centrifuging for 20 sec at 10.000 - 14.000xg.

Tip: If the target protein is acid-labile, elution can be performed with 250-500 mM imidazole.

14. Repeat step 13 four times, for a total of five elutions. Collect each elution fraction separately.

These are the **elution fractions.**

15. Determine the protein concentration of the elution fractions with Bradford assay, using BSA as protein standard.

Note: Do not boil membrane proteins. Instead, incubate samples at 46°C for 30 min in preparation for SDS-PAGE analysis.

16. Analyze all fractions by SDS-PAGE.
17. Optional: Perform Western Blot experiment using PentaHis Antibody.

About denaturation:

In some cases 8 M urea is not sufficient to completely solubilize inclusion bodies. In these cases the urea in the Denaturing Lysis Buffer can be replaced with 6 M guanidine hydrochloride (Gu-HCl). **Important:** Samples containing Gu-HCl cannot be directly applied to SDS-PAGE. Dilute the sample or subject it to a precipitation step (e.g., using trichloracetate (TCA) or similar) to remove the denaturant.
If using Benzonase to remove nucleic acids, the concentration of urea in the Denaturing Lysis Buffer must be decreased. Benzonase is active only at urea concentrations ≤7 M. In contrast, Gu-HCl inactivates Benzonase even at low concentrations.