Purification of His-tagged Proteins Under Denaturing Conditions Using PureCube His Affinity Agarose

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 200 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.

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. 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

- ☐ Ice bath
- ☐ Centrifuge for 50 mL tube (min 10,000 x g)
- ☐ 50 mL centrifuge tube
- ☐ Micropipettor
- ☐ Micropipetting tips
- ☐ Disposable gravity flow columns with capped bottom outlet, 2 ml, (e.g. Pierce / ThermoScientific #29920)
- ☐ Optional: 15 mL conical centrifuge tubes
- ☐ 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	300mM	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 pellet on ice.
2. Resuspend the cell pellet in 10 mL Denaturing Lysis Buffer.
3. Incubate at room temperature for 30 min on an end-over-end shaker.
4. Centrifuge the lysate for 30 min at room temperature and 10,000 x g. Collect the supernatant.
5. Resuspend the PureCube His Affinity Agarose by inverting the bottle until the suspension is homogeneous. Transfer 1 mL of the 50% suspension (corresponding to 0.5 mL bed volume) into a 15 mL conical centrifuge tube. Allow the resin to settle by gravity and remove the supernatant.
6. Add the cleared lysate to the resin and incubate the mixture for 1 h at room temperature on an end-over-end shaker.
7. Transfer the binding suspension to a disposable gravity flow column with a capped bottom outlet. Use Lysis Buffer to rinse the centrifuge tube and remove resin adhered to the wall.
8. Remove the bottom cap of the column and collect the flow-through.
9. Wash the column with 5 mL Denaturing Wash Buffer. Repeat the washing step at least 3 times.
10. Elute the His-tagged protein 5 times using 0.5 mL Denaturing Elution Buffer. Collect each eluate in a separate tube and determine the protein concentration of each fraction.
11. Analyze all fractions by SDS-PAGE.
12. Optional: Perform Western Blot experiment using PentaHis Antibody.

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.

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.

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

Tip: Alternatively, batch binding can be done directly in a gravity flow column with closed top and bottom outlet.

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

These are the **wash fractions.**

These are the **elution fractions.**

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

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

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.