# His-tagged Protein Purification with Protease Inhibitor

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2:35 PM

**Sean Weise**

**Materials:**
1L flasks autoclaved (2 per protein/strain isolating from)
LB (at least 1 L)
IPTG (at least 4 ml) -20°C chest freezer
500 ml centrifuge bottles
Qiagen Lysis buffer (Qproteome cat# 37900) (at least 25 ml) fridge, -80°C
50 ml Falcon tubes (at least 5)
Ni-NTA agarose (Quiagen cat# 30210) (at least 2.5 ml) fridge
Wash buffer (at least 8 ml) deli case
Elution buffer (at least 6 ml) deli case
20 ml Bio-rad column with cap and ring stand to hold Ni-NTA agarose
Short microfuge tube holder to keep 2 ml tubes under column
Regeneration Solutions

**Labeled Tubes**
15 ml falcon tube – Pre induction E. coli (5 ml), **PRE**
15 ml falcon tube – Post induction E. coli (5 ml), **POST**
2 ml tube – Lysed cell supernatant (20 µl), **SUP**
2 ml tube – Lysed cell pellet (2 ml), **PELLET**
2 ml tube – Lysis buffer flow through (2 ml), **LWASH**
2 ml tube – Wash buffer flow through (2 ml), **WASH**
6, 2 ml tubes – Elution (≈ 1ml), **E1…..E6**

**Methods:**
**Growing *E. coli***

1. Prepare a 10 ml LB seed culture with your *E. coli* strain of interest allow to grow overnight at 30°C
2. In the morning (before 11:00am) prepare two 1 L flasks with 500 ml LB. Be sure to include appropriate antibiotic. Inoculate each flask with 5 ml of seed culture
3. Grow cultures until 5:00 pm at 37°C to an $OD_{600nm}$ of 0.8 – 1.0
4. Before inducing take 5 ml of culture and freeze so you have a pre induction protein sample
5. When culture reaches an OD of 0.6 – 1.0 induce with 1M IPTG (enough to bring flask concentration to 500 µM). **Turn off heat so that cultures are at room temperature**
6. Allow culture to grow until OD is between 3 – 6 (at least overnight at room temp)
7. You may need to adjust induction conditions, i.e. more or less IPTG and growing at lower temperature if you find your protein end up insoluble in the pellet. Be sure to carefully record your growing conditions and induction condition's including at what OD your cells were at when you harvest them. If things work you want to be able to repeat it!
8. Turn on centrifuge and cool it down to 4°C. Do this by setting temperature to 4°C and spinning the rotor with appropriate bottle holders at 100 rpm to mix air in centrifuge
9. Label empty 500 ml centrifuge bottles and weigh. You will need this to determine wet weight of *E. coli*
10. Pour flasks with culture into a 500 ml centrifuge bottles (you can combine samples if they are from the same strain with same protein etc.) Pour slowly and carefully here, this will fill up your bottles to the brim. If you do spill some culture clean up with 70% EtOH.

11. Centrifuge at 5,000 g for 10 minutes
12. Carefully pour off supernatant weigh bottle. This will tell you the wet weight of the *E. coli*
13. You can store in centrifuge bottle at -20°C or -80°C for a couple of days

**Lysing *E. coli***

1. If using Qiagen lysis buffer for the first time, dissolve the contents of a lysozyme vial in 600 µl Native Lysis Buffer, store – 80°C
2. Prepare the protease inhibitor lysis buffer according to the following formula
    a. 4 ml lysis buffer with protease inhibitor per gram wet weight
    b. 10 µl lysozyme per ml lysis buffer
    c. 1 µl Benzonase per ml lysis buffer
3. Thaw pellet in centrifuge bottle on ice for 15 min
4. Turn on centrifuge and cool it down to 4°C. Do this by setting temperature to 4°C and spinning the rotor with appropriate tube holders at 100 rpm to mix air in centrifuge
5. Suspend the cells in premixed protease inhibitor lysis buffer solution.
6. Transfer cells to a 50 ml falcon tube
7. Incubate on ice for 30 minutes to allow lysozyme to work
8. Sonicate on ice (slushy ice transfers heat away better) using a sonicator with a microtip (3 mm end for volumes < 10 ml, 5 mm end for 5 – 20 ml). Make sure sonicator tip end is in good shape and not pitted or broken, this can reduce the transferred power by 90%. If using advanced sonicator set total process time to 2:00 min, set pulse "on" time to 10 sec, set pulse "off" time to 15 sec, set initial output level to 4.0 (anywhere from 1.5 – 10 will work).
   If using a less advanced sonicator use the following procedure at a 50% duty cycle
   15 pulses output = 1
   Cool tip with ice water for 30 s
   15 pulses output = 1
   Cool tip with ice water for 30 s
   15 pulses output = 1
   Cool tip with ice water for 30 s
   15 pulses output = 1
   Cool tip with ice water for 30 s
   15 pulses output = 2
   Cool tip with ice water for 30 s
   15 pulses output = 2
9. Centrifuge the cells at 4,500 g for 30 min at 4°C. (Be careful not to set centrifuge for 30 hours)
10. Pipette off 100 µl of supernatant store at -80°C for SDS-PAGE Analysis later.
11. Pipette off remaining supernatant into a fresh 50 ml falcon tube
12. Re-suspend pellet in 10 ml native lysis buffer by up and down pipetting. Take 2 ml of this and save in -80°C. This can be used later in case a significant amount of your protein is in the insoluble fraction

    If you know you will need to stop and cannot run your sample on Ni-NTA column then divide your sample in two and freeze half by freezing quickly in liquid nitrogen and storing at -80°C. Do not add any glycerol to the sample, the high protein concentration in the sample should act as a cryoprotectant. With the other half carry it through to step 5 below and allow to incubate with the Ni-NTA agarose overnight at 4°C. The next day thaw your frozen sample and carry it through to step 5. Keep these sample independent though the rest of the protocol.

**Purification of His-tagged proteins under native conditions**

1. Determine the amount of Ni-NTA agarose: volume of lysate ÷ 4 = volume of Ni-NTA agarose to use

2. The Ni-NTA agarose comes in 30% ethanol. Remove the ethanol by adding the amount of Ni-NTA needed directly from bottle to a 50 ml falcon tube and centrifuging at 3,000 g for 5 minutes
3. Remove the supernatant and add double the volume protease inhibitor lysis buffer to the slurry. i.e. if you started with 3 ml of Ni-NTA slurry add 6 ml of protease inhibitor lysis buffer. Mix by gently inverting
4. Centrifuge again at 3,000 g for 5 minutes and remove supernatant
5. Add lysate to the tube and incubate on a rocker at 4°C for an hour. If you have to stop this is a place you can stop if you have to and allow your sample to incubate overnight. If you can it is better to go on with the protocol.
6. Label 8 two ml microfuge tubes that you will collect your protein in. 1 will be for collecting flow through from the lysis buffer, 1 will be for collecting flow through from the wash buffer, and 6 will be for collecting your actual eluate from the elution buffer
7. Load the lysate-Ni-NTA mixture into a 20 ml column with the bottom outlet capped. A 1 ml pipette with the tip cut off works well.
8. Remove cap from bottom on column and collect 1 – 2 ml of flow through. This can be useful for SDS-PAGE analysis later
9. Wash the column twice with 5 times the bed volume protease inhibitor wash buffer. Ex. If you used 3 ml of the Ni-NTA slurry the bed volume is half this (1.5 ml) so you would wash twice with 7.5 ml wash buffer. Collect 1 – 2 ml of the first wash. This can be useful for SDS-PAGE analysis later
10. Elute your protein by adding 1 ml of protease inhibitor elution buffer 6 times and collect eluate. The protein usually comes off in the third or fourth eluate fraction.
11. To each 1 ml fraction add 150 µl glycerol, to each 2 ml fraction remove about 300 µl and add 300 µl glycerol (15%) freeze all samples in $LN_2$ if possible and store at -80°C
12. To store column wash column with 5 volumes of the elution buffer, 5 volumes $dH_2O$, and 2 volumes 20% EtOH, store column in 20% EtOH
13. **DO NOT** reuse column for a different protein unless you regenerate it, unless we are very short of money regeneration is a waste of time and potentially hazardous, $NiSO_4$ is a carcinogen and can be absorbed through the skin, only use in a fume hood!
14. Discard used Ni-NTA as hazardous waste

**His-tagged Protein Purification from *E. coli* Reagents**

**LB Liquid, 1 L**
In 1 L $dH_2O$
+ 25 g LB Broth (Difco, Luria-Bertani)

In 1.75 L $dH_2O$
+ 43.75 g LB Broth

Autoclave for 25 min

| Antibiotic | Stock (mg/ml) | Working (µg/ml) | µl of stock in 5 ml | µl of stock in 20 ml | µl of stock in 40 ml | ml of stock in 200 ml | ml of stock in 500 ml | ml of stock in 1 L |
|---|---|---|---|---|---|---|---|---|
| **Ampicillin** | 10 | 50 | 25 | 100 | 200 | 1 | 2.5 | 5 |
| **Chloramphenicol** (methanol) | 10 | 20 | 10 | 40 | 80 | 0.4 | 1 | 2 |
| **Kanamycin** | 10 | 25 | 12.5 | 50 | 100 | 0.5 | 1.25 | 2.5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Rifampicin (methanol)** | 30 | 150 | 25 | 100 | 200 | 1 | 2.5 | 5 |
| **Spectinomycin** | 10 | 100 | 50 | 200 | 400 | 2 | 5 | 10 |
| **Streptomycin** | 10 | 30 | 15 | 60 | 120 | 0.6 | 1.5 | 3 |
| | | | | | | | | |

Note: If using multiple antibiotics you can cut the concentration of each by half

**10 mg/ml Antibiotic, 10 ml**
In 10 ml of sterile $dH_2O$
+ 0.1g Antibiotic
Filter sterilize using 0.2 - 0.45 mm syringe filter
Aliquot into sterile 1.5 ml microfuge tubes and store at -20°C

**1 M IPTG, 15 ml**
In 15 ml of sterile $dH_2O$
+ 3.5 g IPTG
Filter sterilize using 0.2 - 0.45 mm syringe filter
Aliquot into sterile 1.5 ml microfuge tubes and store at -20°C

**Lysis Buffer, 250 ml**
Normally we use the lysis buffer from the Qiagen QProteome kit but if the kit is missing the following lysis buffer should work equally well
In 250 ml $dH_2O$
+ 1.73 g $NaH_2PO_4 \bullet H_2O$, 50 mM (FW 137.99 g/mol)
+ 4.39 g NaCl, 300 mM (FW 58.44 g/mol)
+ 0.34 g Imidazole, 10 mM (FW 68.08 g/mol)
pH to 8.0 using NaOH store at 4°C

**Lysis Buffer with Protease Inhibitor, 100 mL**
+100 mL Qiagen QProteome kit Lysis Buffer, or Lysis buffer made above
+10 cOmplete Mini EDTA-free Roche Protease Inhibitor Tablets
* The solution is stable 1-2 weeks stored at +2-8°C, or 12 weeks at -15-25°C

**2X SDS-PAGE sample buffer, 150 ml**
In 105 ml of $dH_2O$
+ 1.6 g Tris, 90 mM (FW 121.14 g/mol)
pH to 6.8 using HCl
+ 30 ml glycerol, 20%
+ 15 ml 20% SDS, 2%
+ 0.03 g bromphenol blue, 0.02%

**1 M DTT:**
In 10 ml $dH_2O$
+ 1.54 g DTT (fw 154.2; Sigma D-5545)
Divide into 1.5 ml microfuge tubes and store in -80° C

**1.09 µg/µl, 2.73 U/µl DNase1**
Normally we use the Benzonase (DNase and RNase) from the Qiagen Qproteome kit but if we are out you can use DNase1

Using Qiagen DNase Set (cat# 79254)

1. Prepare DNase I stock solution by injecting 550 µl RNase free water into vial sing an RNase-free needle and syringe

**** Do not vortex DNase I, DNase I is sensitive to physical denaturation. Mix by gently inverting the tube ****

2. Label tubes and aliquot DNaseI solution into 3, 150 µl aliquots and 1, 100 µl aliquot, label tubes and store at -20 or -80°C for up to 9 months, Thawed aliquots can be stored at 4°C for 6 weeks, **Do not refreeze**

**Wash Buffer, 250 ml**

In 250 ml $dH_2O$
+ 1.73 g $NaH_2PO_4 \cdot H_2O$, 50 mM (FW 137.99 g/mol)
+ 4.39 g NaCl, 300 mM (FW 58.44 g/mol)
+ 0.34 g Imidazole, 10 mM (FW 68.08 g/mol) (Use 50 mM for IDI, Amy recommends 20 mM for other enzymes)
pH to 8.0 using NaOH store at 4°C

**Wash Buffer with Protease Inhibitor, 40 mL**

+ 40 mL Wash Buffer
+4 cOmplete Mini EDTA-free Roche Protease Inhibitor Tablets
* The solution is stable 1-2 weeks stored at +2-8°C, or 12 weeks at -15-25°C

**Elution Buffer, 250 ml**

In 250 ml $dH_2O$
+ 1.73 g $NaH_2PO_4 \cdot H_2O$, 50 mM (FW 137.99 g/mol)
+ 4.39 g NaCl, 300 mM (FW 58.44 g/mol)
+ 4.25 g Imidazole, 250 mM (FW 68.08 g/mol)
pH to 8.0 using NaOH store at 4°C

**Elution Buffer with Protease Inhibitor, 30 mL**

+ 30 mL Elution Buffer
+3 cOmplete Mini EDTA-free Roche Protease Inhibitor Tablets
* The solution is stable 1-2 weeks stored at +2-8°C, or 12 weeks at -15-25°C

**20% Ethanol, 250 ml**

In 200 ml $dH_2O$
+ 50 ml 100% EtOH (molecular biology grade)
Store on bench