Protein Production and Purification Partnership in Europe

Contents

Procedure

Expressed cell stock before protocol development

For protocol development prepare big stock of induce cells. Keep aliquot cell pellet at -80ºC

The idea is to aliquot and keep pellet cells at -80ºC after induction. Conditions are first optimized at small scale before scale-up.

1) Grow 1L culture
2) Induction (IPTG, salt induction, etc)
3) Spin cell culture 10min 8000rpm 4ºC, discharge supernatant
4) Resuspend cell pellet at 4ºC very gently with 100ml cold PBS buffer. Aliquot as following:

a) 10 tubes (1.5ml plastic tubes) with 1ml suspension (it means 10ml original culture per tube);

b) 4 tubes (15ml plastic tubes) with 10ml suspension (it means 100ml original culture per tube)

c) 1 tube (50ml plastic tube) with 50ml suspension (it means 500ml original culture).

5) Spin 10min 8000rpm 4ºC, discharge supernatant
6) Keep cell pellet at -80ºC

Buffer for Protein Extraction

Buffer selection is very important for many proteins. Salt concentration, pH, detergents and additives must be consider for prone to aggregate protein (see Protocol: "A screening methodology for purifying proteins with aggregation problems") while type and quantity of reducing agents depends of the presence or absence of free Cysteines or disulfide bridges

Suggested buffer: 25mM TrisHCl/$NaPO_4$ pH 8.0 + 0.3M NaCl + "optional" additives
Alternative buffers: 20-50mM TrisHCl, MOPS, HEPES or Phosphate buffers from pH 7.0 to 8.0 and NaCl or KCl from 25mM to 1M

Optional additives to the lysis buffer

a) ßME up to 15mM for only for proteins with free cysteines residues, in order to maintain reduced cysteines and avoid the formation of nonspecific disulfide bridges that can cause aggregation (not recommended if you want to maintain disulfide bridges). In case of the presence of both, disulfide bridges and free cysteines, a compromise use of 2mM ßME can be consider
b) 0.1-2% Triton X-100, NP40, or any other detergent that do not affect the biological activity of your protein. Add just if necessary
c) 0.02% NaN_3 (azide) to avoid bacterial contamination in the buffer
d) Up to 5-40mM Imidazole to reduce non-specific binding to the column. Initial Imidazole concentration can be check previously at low scale: see alternative protocol
e) Glycerol 10% to stabilize protein and avoid aggregation in some cases

Reagents compatible with the purification

Each IMAC supplier could have different reagents compatibility, mainly at the level of reducing agents and chelants as EDTA. Most of the different IMAC suppliers are compatible with:

6M Guanidine HCl	*2% Tween 20*	*50% Glycerol*	*4M $MgCl_2$*
8M Urea	*1% CHAPS*	*20% Ethanol*	*5mM $CaCl_2$*
2% Triton X-100	*20mM ßME*	*2M NaCl*	*Up to 20mM Imidazole*

Recommended additives to the lysis buffer:

f) Dnase 100U/ml or 25-50ug/ml (SIGMA DN-25). Incubate 10min 4°C in the presence of 10mMMgCl₂
g) Lysozime 0.2mg/ml. Incubate 10min 4°C
h) 1mM PMSF and/or protease inhibitor cocktail 1:200 (cocktail for bacterial cells #P-8849 from Sigma or any other commercial cocktail without EDTA)

Limitations

Some IMAC matrices lost the charge metal in the presence of reducing agents as DTT or DTE (you can use up to ßME 20mM) and chelating agents such as EDTA and EGTA. Avoid NH_4^+ buffers and amino acids as Arg, Glu, Gly or His.

Equilibration IMAC resin low scale

Place 50ul beads (100ul suspension) of IMAC beads in 1.5ml plastic tube.
Wash with 1 x 1.5ml H_2O and 1 x 1.5ml lysis buffer (washing: mix, spin 3min 3500rpm, discharge supernatant).

Protein Extraction low scale

1) Resuspend pellet of 10ml cell culture in 1ml lysis buffer (or 100ml bacterial culture for very low expression level).

2) Sonicate in ice bucket 3 x 10sec or more if the cells are not completely disrupted (Lysis is complete when the cloudy cell suspension becomes translucent. Avoid protein denaturation by frothing, and extensive sonication).

3) Spin 10min 13000rpm 4°C. Separate soluble proteins (supernatant) from insoluble or inclusion bodies proteins (pellet). Use supernatant for next step. Keep a 40ul sample of supernatant for PAGE-SDS: **soluble proteins**

4) Resuspend pellet in another 1ml buffer and keep sample of 40ul for PAGE SDS: **insoluble proteins, or unlysed cells**.

Protein Purification low Scale

5) Mix the supernatant of last step gently with the equilibrated resin for 60-90 min at 4°C.

6) Spin 3min 3500rpm 4°C. Discharge supernatant and *keep sample* of 40µl for PAGE-SDS: **unbound proteins**

7) Wash beads with 1ml buffer several times (washing: mix, spin 3min 3500rpm, keep supernatant aside, be careful not to take the resin) up to OD280nm <0.05. *Keep sample* of 40µl for PAGE-SDS of each **washing**.

8) Elute recombinant protein with 3x100ul buffer + 350mM Imidazol (incubate 3 to 5min each time before spinning 3min, 3500rpm at 4°C). *Keep sample* of 40ul for PAGE-SDS of each **elution**.

9) Resuspend beads in 50ul H_2O + 20ul 5x sample buffer. Mix and spin. *Keep sample* of 40ul for PAGE-SDS: **protein not eluted (or SDS extracted beads)**.

10) Run on PAGE-SDS: crude supernatant; resuspended pellet; unbound, washings, elutions, and SDS extracted beads.

11) Optimize purification according to results before large scale purification (see Troubleshootings and Alternative protocol)

Alternative protocol for low scale purification if target protein is not pure enough

1) Perform parallel purification procedures where you include 10-20-30-40 or 50mM Imidazole in the lysis,binding and washing buffer.

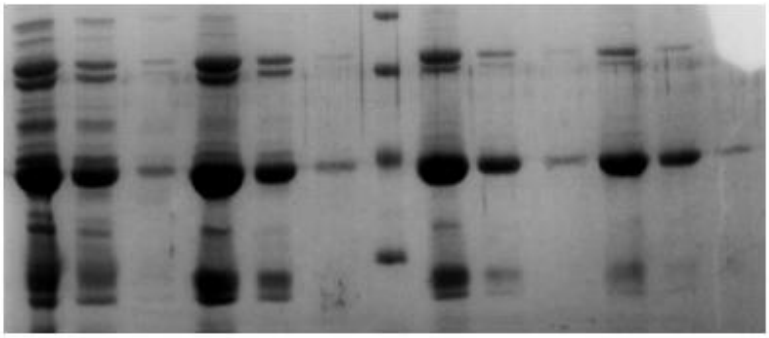
2) Elute directly with 3x100ul elution buffer + 300mM Imidazole.

3) Check eluted proteins on PAGE-SDS. Expect lower yields but higher purification by increasing the Imidazole concentration.

4) Results of this protocol can be use to choose the Imidazole concentration to be use during binding and step washings in large scale purification using IMAC columns

Ni-NTA equilibration (mM Imidazol)	10	10	10	20	20	20		30	30	30	40	40	40
[Imidazol] for binding (mM Imidazol)	10	10	10	20	20	20		30	30	30	40	40	40
[Imidazol] for washing (mM Imidazol)	10	10	10	20	20	20		30	30	30	40	40	40
[Imidazol] for elution (mM Imidazol)	250	250	250	250	250	250		250	250	250	250	250	250
Elution number	1	2	3	1	2	3	MW	1	2	3	1	2	3

- *Cells from 250ml culture. Sonication 3x20sec in 7.5ml Lysis buffer + Dnase + Lysozyme and Protease inhibitors.*
- *Spin 30min 20000g 4°C.*
- *Divide supernatant in 4 fractions, add to each one different [Imidazol].*



- *Incubate each fraction (batch binding) 60min 4°C with 150µl Ni-NTA (equilibrated with different [Imidazol]).*
- *Spin 3min 3000rpm 4°C. Discharge unbound material.*
- *Wash resin 6x1ml washing buffer + different [Imidazol].*
- *Elution 3x300µl elution buffer (250mM Imidazol). Run 12%PAGE-SDS of each elution.*

Large Scale Extraction and Purification

Scale-up using open columns or FPLC equipment with resins that can be used at high pressure: like Ni-NTA superflow resin from QIAGEN , BD Talon™ Metal Affinity

from CLONTECH, Chelating Sepharose Fast Flow or Ni Sepharose High Performance from GE-Healthcare , Ni-NTA Hi-Bind or Metal Chelate Resins from NOVAGEN-MERCK or others. The use of FPLC equipment will allow greater operational flexibility and simple optimization:
A) gradient or step gradients elutions
B) optimization of flow rate, column dimension, washing conditions, etc.
C) rapid and convenient comparison of protein purification by the use of columns charged with other metal ions with different strength of binding, e.g. Zn^{2+}, Co^{2+}, Fe^{2+} and Cu^{2+}
You can consider to use IMAC as a first capture step, or as an intermediate step after a capture step with ion or hydrophobic exchange chromatography: very important to eliminate interferents to IMAC binding in crude materials.

VERY IMPORTANT

A. MANTAIN PROTEIN AT 4ºC DURING ALL THE PROCEDURE
B. AVOID PROTEIN DENATURATION BY FROATHING (FOAM)
C. WORK AS QUICKLY AS YOU CAN TO AVOID PROTEOLYTIC DIGESTIONS
D. Keep sample for PAGE-SDS from each step

1. Equilibrate IMAC column with buffer. Protein capacity, flow-rate and pressure limit depends on the supplier resin. Equilibration is confirmed by measuring pH and conductivity. Flow-rate and pressure limit: depends of the commercial resin

2. Resuspend cell culture pellet with suggested lysis buffer, (lysis in 1/10 or less, of original culture medium). Initial Imidazole concentration during binding and washing steps, can be check previously at low scale: see alternative protocol. After resuspension incubate 10min 4°C in the presence of 10mM$MgCl_2$. Gross filter to eliminate not resuspended particles, **and inmediately**:

3. Cell disruption: Microfluidizer or French Press lysis at 21000psi 4°C . Sonication in ice bucket 3 x 10sec or more, is a less recommendable procedure since cells are not completely disrupted, is less reproducible and slightly denaturative for proteins. Lysis is complete when the cloudy cell suspension becomes translucent. Avoid protein denaturation by frothing.

4. Spin 20min 10,000rpm 4°C. Separate soluble proteins (supernatant) from insoluble or inclusion bodies proteins (pellet). Filter supernatant with GF/D (Whatman) and 0.45 mm filter (Whatman). Keep sample of 40µl of supernatant for PAGE-SDS: **soluble**

proteins. Use resuspended pellet to keep sample **from insoluble proteins** (and some un-disrupted cells)

5. Load supernatant on equilibrate IMAC column at low flow rate (Suggestion: loading time ~1.5hr); wash with buffer at higher flow rate up to low 280nm optical density. Keep sample of 40µl of supernatant for PAGE-SDS: **unbound and wash fractions**. Imidazole concentration during washing steps, can be check previously at low scale: see alternative protocol

6. Protein is eluted by step-gradient, using buffer + 300mM Imidazole as elution buffer:

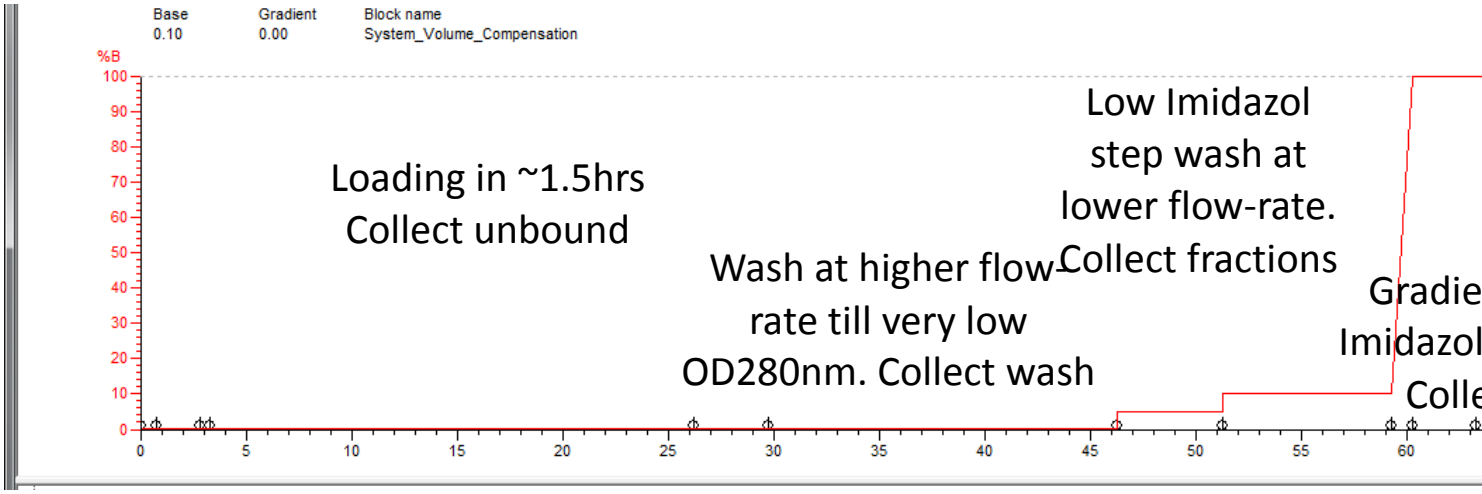
a) Low concentration Imidazole steps at lower flow-rate and start collecting fractions (the goal is to eliminate low binding contaminant proteins in these steps).

Suggested steps: 10, 20 and 30mM Imidazole in buffer.

b) Short gradient up to 300mM Imidazole at very low flow-rate in order to obtain sharp peak, up to low 280nm optical density. Collect smaller fractions.

c) Samples from each step and each fraction are analyzed for protein content by SDS–PAGE. Protein-containing fractions are then pooled according to the profile obtained.

d) Optimize next purification and scale-up, according to results (optimization by adding, changing or eliminating steps)



Further Purification

7. Following this single-capture step, protein is 70–90% pure. In order to achieve a higher degree of purification, which is often required for downstream applications such as structural studies, one should add additional purification steps. Ion exchange (IEX), hydrophobic exchange (HIC), mixed mode (MMC) and size exclusion chromatography (SEC). IEX is essential as an intermediate step for separating target proteins from protein contaminants such as chaperons and other host cell proteins. It also allows separation of the target protein from heterogeneously folded forms that are a consequence of the expression and purification conditions used and from heterogeneity in posttranslational modifications. Sometimes purification techniques that separate proteins according to their charge are deficient or inadequate, so other approaches based on different principles could be consider: HIC, MMC or hydroxyapatite.

8. As a final polishing step, it is often recommended to use SEC, not only to eliminate protein contaminants and low molecular weight molecules but also to obtain a homogeneous oligomeric form. An added value of SEC step is that the protein will elute in the final desired buffer.

9. When His-Tag fusion proteins are purified by affinity chromatography without further purification columns, dialysis after affinity purification is enough to eliminate Imidazole from the protein solution.

10. Detergent exchange: bound the fusion protein to IEX, HIC, MMC or hydroxyapatite. Wash extensively with the replaced detergent before protein elution.

Regeneration of IMAC resin

According to QIAGEN the resin may be reused many times when regenerated promptly after use.

Short wash after each run

1) Highly suggested: NaOH 0.5M 5cv (column volumes) and buffer up to neutral pH
2) Water 5cv
2) If storage for more than 1-2 days: 20% Ethanol wash with 3cv and keep at 4°C.
   If storage for less than 1-2 days: Wash or lysis buffer + 0.02%NaAzide, wash with 3cv and keep at 4°C.

Short regeneration after several runs

1) Water 5cv (column volumes)
2) EDTA 100mM pH8.0 5cv
3) Water 5cv
4) $NiSO_4$ 100mM 2cv (Recharging)
5) Water 5cv
6) If storage for more than 1-2 days: 20% Ethanol wash with 3cv and keep at 4°C.
   If storage for less than 1-2 days: Wash or lysis buffer + 0.02%NaAzide, wash with 3cv and keep at 4°C.

More stringent regeneration for a highly contaminated column (According to QIAGEN)

1) Regeneration buffer (6M GuHCl or 0.2M Acetic acid) 2cv (column volumes)
2) Water 5cv
3) 2%SDS 3cv
4) 25% Ethanol 1cv
5) 50% Ethanol 1cv
6) 75% Ethanol 1cv
7) 100% Ethanol 5cv
8) 75% Ethanol 1cv
9) 50% Ethanol 1cv
10) 25% Ethanol 1cv
11) Water 5cv (column volumes)
12) EDTA 100mM pH8.0 5cv
13) Water 5cv
14) NiSO4 100mM 2cv (Recharging)
15) Water 5cv
16) If storage for more than 1-2 days: 20% Ethanol wash with 3cv and keep at 4°C. If storage for less than 1-2 days: Wash or lysis buffer + 0.02%NaAzide, wash with 3cv and keep at 4°C.

Quality Control

See Protocol: "Guidelines for Protein Production for Biophysical and Biochemical Studies"

Analysis of results - Troubleshooting

Expect over-expressed protein to be found only in the crude supernatant and in the elution of the IMAC resin.

For prone to aggregate proteins see Protocol: "A screening methodology for purifying proteins with aggregation problems"

If most of the protein remains insoluble after extraction, try

a) To change lysis buffer by adding additives as ßME, glycerol, detergents or more NaCl.
b) Re-extract pellet with more buffer,
c) Use more lysis buffer during extraction,
d) Perform a more intensive sonication,
e) Incubate with lysozyme before sonication.
f) Try the denaturating protocol

If protein does not bind to the Ni-NTA resin, there are several options to choose:

a) Check the IMAC resin: binding of a cell sonicate containing a control protein
b) If only partially bound, use more resin, or bind for longer time (The longer the duration of purification, the greater the risk of protein degradation).
c) Try additives as glycerol, different detergents up to 2% or increase ionic strength up to 1.5M NaCl or KCl in the lysis buffer. Try low concentration of denaturants like Urea up to 1.5M or Guanidine HCl up to 1M (*see Protocol: "A screening methodology for purifying proteins with aggregation problems"*)
d) His-tag is inaccessible, purify protein under denaturating protocol
e) Check by western-blot if the His-tag has been degraded; if this is the case, try to work all the time at 4°C and use more protease inhibitors during lysis
f) Construct a new vector with the tag in the opposite end of the protein.
g) According to (A.Magnusdottir et al.) a serious drawback of IMAC is the often-experienced failure to purify low-abundance His-tagged proteins from E. coli lysates; increasing the culture size and thereby increasing the amount of available His-tagged protein does not result in increased yield. They examined this issue and propose that it is tightly linked to metal-ion leakage from the columns induced by periplasmic material from the E.coli lysate, and this periplasmic fraction can be removed by osmotic shock (A.Magnusdottir et al.B) . Another possibility is to perform a purification step before the IMAC column through IEX or HIC.

If multiple proteins bands are seen in the elution try:

a) If multiple protein bands are present after elution, then protein degradation is to be suspected. Western blot analysis can be performed to verify if proteolysis is occurring. Conducting all purification steps at 4°C, reducing the overall time taken to carry out the procedure, and using protease inhibitors during the cell disruption process, can all help to reduce proteolysis.
b) If the additional bands visible on SDS–PAGE are not the result of target protein degradation, there are two main reasons that usually explain the presence of cellular protein contaminants:
(i) contaminating proteins are binding nonspecifically to the resin,
(ii) contaminants are sticking to the target protein.
If contaminants are bound nonspecifically to the resin, consider decreasing the resin volume to increase competition, or increasing the ionic strength of the buffers (up to 1 M NaCl or KCl), to reduce hydrophobic interactions with the resin; or use more stringent conditions during binding and washing by increasing Imidazole concentrations: see alternative protocol
c) If contaminants stick to the target protein, increasing the washing step is the first option that should be considered. If this does not work, consider increasing the ionic strength of the buffers (up to 1 M NaCl or KCl), adding additives such as glycerol, adding up to15mM *ß*-ME as reducing agents in order to disrupt nonspecific intermolecular disulfide bonds, or adding detergents as Triton X-100, NP40 or Tween 20 up to 2% that might reduce hydrophobic interactions.
d) If taking these options does not reduce the presence of contaminants, additional purification steps should be performed before or after affinity purification. In some cases, columns charged with other metal ions instead of Ni^{2+} (Zn^{2+}, Co^{2+}, Fe^{2+} and Cu^{2+}) can give a different purification pattern, or IMAC resins of different suppliers.
e) For large scale production, the use of FPLC equipment with the proper resins will allow simple optimization and rapid and convenient comparison of protein purification conditions.
f) Robichon C. et al (APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2011, p. 4634–4646 Vol. 77, No. 13) engineer Escherichia coli BL21(DE3) derivative strains to minimize E. coli protein contamination after purification by Immobilized Metal Affinity Chromatograph [two new strains, NiCo21(DE3) and NiCo22(DE3)], which express the endogenous Ni contaminants proteins fused at their C terminus to a chitin binding domain (CBD), so then can be eliminated by chitin resins; and replace with alanines six surface histidines of another contaminant protein. They claim a significant reduction of Ni impurities using these strains (pdf)

Protein degradation:

a) Discern if degradation happen or start during expression, or take place only during purification, or both of them.
b) Start during expression: can be seen by western-blot analysis of the protein integrity before purification (add sample buffer directly on pellet cells). In this case, the options are: to change expression conditions, or to change bacteria strains or to modify amino acid sequence in the degradation site
c) Degradation start during purification: work quickly, 4C all the time, try to load on affinity column as soon as possible after lysis, since most of the proteases are in the crude lysate. If necessary, add more protease inhibitors (PI), or check which one avoid degradation, and strength only it (how to do it?: incubate in parallel a few hours crude lysate with or without different PI, and see which one is the better to avoid degradation. Once you know which PI, you can strength your cocktail with this specific PI .Information about PI:
(http://wolfson.huji.ac.il/purification/extraction_and_clarification.html#Protease_Inhibitors)
d) If you have a mix of problems and nothing helps, change construct for MBP, SUMO, etc

How to eliminate yellow color, endotoxins, and nucleic acids:

a) Yellow color in concentrated proteins can be eliminated with 100mM n-Octyl B-D-glucopyranoside (OGP): incubate overnight and run a Gel Filtration column. It can be used to remove yellow pigments and endotoxins (LPS) too.
b) Endotoxins clearence: wash Ni column with 50 column volumes of buffer containing 0.1% (v/v) of TritonX-114 (Sigma-Aldrich) followed by 20 column volumes of buffer without detergent at 4 °C *{Timo Zimmerman et. al. Journal of Immunological Methods 314 (2006) 67–73}*
c) Endotoxins can be removed with anion exchange columns, gel filtration, immobilized polymixin B, and other methods (see Protocol: Endotoxin Removal)
d) Nucleic acids are eliminated by enzymes as DNase, RNase or Benzonase. Or by chemicals as Polyethyleneimine (PEI) precipitation, Streptomycin sulfate precipitation or Polyethyleneimine (PEI) precipitation (see Protocol: Nucleic Acid Removal)

If the protein does not elute from the column

a) Use higher Imidazole concentrations (up to 1M), or additives
b) Reduce elution flow-rate
c) Elute under denaturating condition