Expression and Purification of His-tagged Proteins expressed in *E. coli* (Price Lab)

Uses two liters of *E. coli* expressing the protein of interest.
Uses 4 ml Ni-NTA resin, (8 ml Ni-NTA slurry) for Ni-NTA Affinity Chromatography.
Finalizes the purification process with FPLC.

Transformation

In the mid to late afternoon, thaw *E. coli* DE3 competent cells.
Pipet 50 ul DE3 competent cells into 1.5 ml microcentrifuge tube.
Pipet 5 ng of selected DNA into the 50 ul DE3 competent cells, and gently mix with pipettor.
Incubate on ice 30 minutes. Heat shock at 42^{o}C for 30 seconds. Incubate on ice again two minutes.
Add 1 ml LB. Incubate at 37^{o}C, shaking at 250 rpm for one hour.
Spin at 13,000 rpm for 90 seconds in microfuge. Pour off supernatent. Add 100 ul room temperature LB, and resuspend pellet with pipettor.
LB/Ampicillin plates will have 1:1000 dilution of Ampicillin (stock: 100 mg/ml).
Plate 10 ul of the resuspended cells on a room temperature LB/Ampicillin plate.
Incubate plates overnight at 37^{o}C.

First thing in the morning, pick colonies and make 4 ml mini-cultures:
Add 20 ul Ampicillin (100 mg/ml) to 20 ml LB.
Pipet 4 ml of the LB/amp into each of four Falcon 14 ml round bottom tubes.
Transfer four colonies individually into each of the four tubes.
Incubate at 37^{o}C, shaking at 250 rpm for 8 – 12 hours.
Of the 4 cultures, select one that has highest density of *E. coli*.
Split the culture into two 2 ml microcentrifuge tubes, and spin at 13,000 rpm in a for 90 seconds.
Decant liquid, resuspend each pellet with 1 ml LB/amp, and spin again.
Thoroughly resuspend each pellet with 1 ml LB/amp.

Induction and Expression

Have two 2.8 L Fernbach flasks with 1 L LB each (autoclaved and cooled down). Add 1 ml of 100 mg/ml ampicillin per liter of LB.
Take an aliquot of the LB/amp and to use it to blank the spectrophotometer at 600 nm (A600).
Add 1 ml of the resuspended *E. coli* to each flask.
Cover top of flasks with aluminum foil. Incubate at 37^{o}C, shaking at 250 rpm.
Starting at about 1 hr. begin to read the OD A600 of the cultures versus the blank. When the OD reaches 0.5 – 0.6 (about 3.5 hr), turn the temperature down to 18^{o}C, and add 250 ul/L 0.4 M IPTG to each culture. Express overnight while shaking.

Lysis of cells

All the way through FPLC purification, work fast and keep the protein sample and all tools cold.
After expression, pellet the cells using a centrifuge comparable to a Beckman J-6B Centrifuge with a JS 4.2 swinging bucket rotor. Pour the cultures into 1 L containers that are made specifically for the rotor. Spin 15 minutes at 4,000 rpm, and discard the media.
Resuspend the pellets with ~12 ml/L **Lysis buffer** (PBS, 1% Triton X-100, 5 mM Imidizole pH 8.0, 0.1% PMSF saturated in Isopropanol added fresh).

Needed for sonication: Bucket of ice, three plastic beakers with ~500 ml capacity, one 30 ml dispo plastic beaker, chilled water, timer, and pipettor. Following this method of sonication will keep your protein lysate very cold, allowing thorough sonication, with minimal protein degradation due to heat.

Remember to tune the sonicator before use.

During the sonication, it is important to keep the sample as close to 4°C as possible.

Fill one plastic beaker with ice, and chilled water and submerge sonicator tip to keep it ice cold.
Insert the 30 ml beaker into a 500 ml beaker filled with packed ice water (it may be necessary to remove some of the ice water during sonication).
Pour the resuspended cell lysate into the chilled 30 ml beaker.
Raise sonicator tip out of ice water, and wipe dry with Kimwipe.
Carefully lower the sonicator into the 30 ml beaker until the sonicator tip lightly touches the bottom of the beaker, then raise the sonicator barely above the bottom of the beaker and fix sonicator in place.
Note: During sonication, don't allow the bottom of the tip to come into the top half of the lysate, as this can cause the lysate to turn to foam, effectively ending the sonication.
Sonicate 5 times for 15 seconds with 2 minutes between each burst. Cool the tip in ice water between bursts.
Save an aliquot of crude lysate for SDS-PAGE analysis.
Spin the sonicated lysate using a Beckman L7-55 Ultracentrifuge with a 55.2 TI rotor for 45 minutes @ 45,000 rpm at 4°C in two balanced #355618 Beckman tubes (Oakridge style).
During the 45 minute spin, prepare for Ni-NTA Affinity Chromatography.
Save an aliquot of spun lysate for SDS-PAGE analysis.
Add 5 M NaCl to make the lysate 500 mM NaCl (go to 1 M for RNA or DNA binding proteins).
Resuspend one pellet in 15 ml Lysis Buffer using a Dounce homogenizer. ***Save an aliquot***.

Ni-NTA Affinity Chromatography

Do the affinity chromatography in a 4°C cold room. Add 4 ml QIAGEN Ni-NTA Agarose slurry to a 20 ml plastic Biorad column. Allow unbound material to flow through.
Wash the resin with 5 volumes (10 ml) **Equilibration Buffer**, which is **Lysis Buffer** with the extra NaCl.
Prepare 10 column volumes of:
High Salt Wash (10 mM Tris pH 7.8, 1% Triton X-100, 5 mM Imidizole, 500 mM NaCl, 0.1% PMSF)
Low Salt Wash (10 mM Hepes, 1% Triton X-100, 70 mM KCl, 35 mM Imidizole, 0.1% PMSF)
Elution Buffer (10 mM Hepes, 1% Triton X-100, 70 mM KCl, 300 mM Imidizole, 0.1% PMSF)
After equilibrating the column, cap the bottom of the column.
Carefully resuspend Ni-NTA resin with 4 ml of the lysate using a 10 ml pipet. Pipet the resuspended protein lysate/resin mixture into a 50 ml conical tube. Using up to 10 ml of the protein lysate at a time, rinse and collect all Ni-NTA resin from the column and pipet it into the 50 ml conical. Cap the top of the column, and save at 4°C. Incubate the protein lysate/resin mixture at 4°C gently rocking for one hour.
Set up column on a ringstand with a 50 ml conical below to collect the flow through. Uncap column and add incubated protein lysate/resin to column as quickly as allowed.
Save an aliquot of flow through and store the rest at -80°C.
Wash the resin with 10 volumes of **High Salt Wash**. Using a pipettor, carefully add the **High Salt Wash** to the column, using the pipettor and liquid as a tool to actually *wash* the sides of the column. Avoid creating bubbles – if they occur, remove with a pipet. ***Save an aliquot.***
Wash the resin with 10 volumes of **Low Salt Wash**. Apply in the same manner as the High Salt Wash.
Elute the protein with 5 volumes of **Elution Buffer.** ***Save an aliquot.***
Spin the eluted material in the J2-21 @ 12,000 rpm for 15 minutes. ***Save an aliquot.***

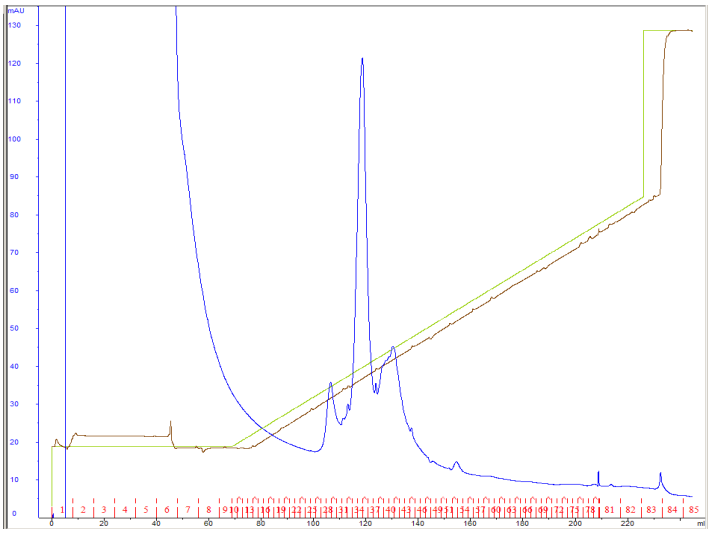
FPLC Column Chromatography

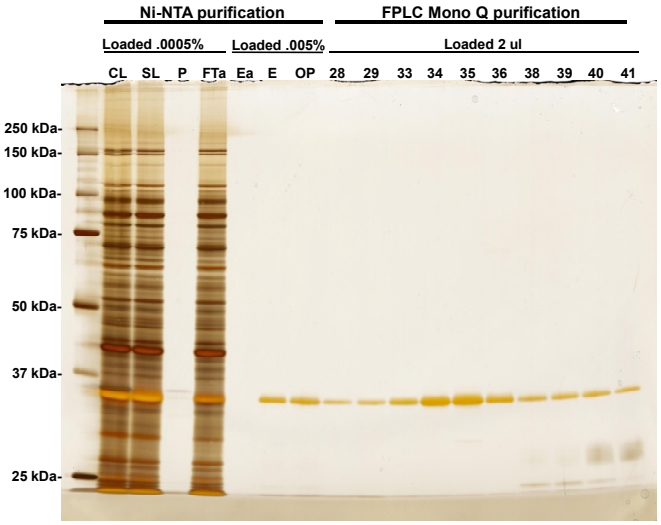
The FPLC buffers (HGE and HGKE) can be made in advance, several liters at a time; however, DTT, and PMSF must be added right before use. For many uses the glycerol and EDTA can be omitted

HGE (25 mM HEPES, 15% Glycerol, 0.1 mM EDTA, adjust pH to 7.6, and filter through 0.22 µm filter) **HGKE** (25 mM HEPES, 15% Glycerol, 0.1 mM EDTA, 1 M KCl, adjust pH to 7.6, filter through 0.22 µm filter).

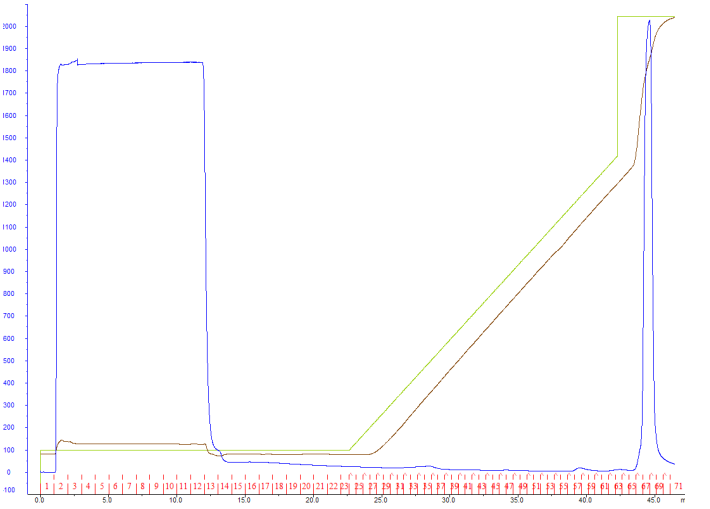
Wash the FPLC column, and equilibrate to 100 mM using 10% Buffer B. Prepare the fraction collector, turn on the UV lamp, and whatever other preparatory things your FPLC needs.

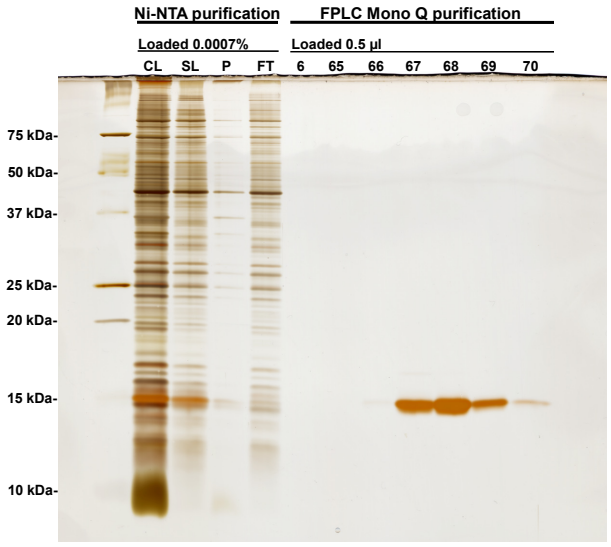
Load the Loop: Assemble the Superloop manually, wetting the moving parts with **HGE**; and pour the elution fraction into the glass chamber.
Install Superloop to the FPLC, and run.
When the run is finished, save aliquots of the fractions of interest. Select suitable fractions based upon a quick study of the chromatogram. Saving aliquots now will allow SDS-PAGE analysis, and determination of absorbance by spectrophotometry, without having to freeze/thaw the major portion of your protein.





Analysis of a truncated HEXIM1 protein. The Mono Q chromatogram shows protein eluting over several peaks, which are analyzed by SDS-PAGE. The gel includes the Crude Lysate (CL), Spun Lysate (SL), Pellet (P), Ni-NTA column Flow Through (FTa), A second Elution from pouring the Spun Lysate over a second Ni-NTA column again (Ea), Elution from the NI-NTA column (E), Onput to the Mono Q column (OP), and Mono Q fractions 28-41.





Analysis of another truncated HEXIM1 protein. The Mono Q chromatogram shows protein eluting at very high salt. The gel includes the Crude Lysate (CL), Spun Lysate (SL), Pellet (P), Ni-NTA Flow Through (FT), and Mono Q fractions 6 and 65-70.