# Purification of His Tagged Proteins

# from T7 Expression Vectors

## 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 expressed 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 16 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 50 ml pyrex beaker, chilled water, timer, and pipettor. Following this method of sonication will keep your protein lysate very cold, allowing thorough sonication, with minimal heat degradation.

Tune the sonicator.

Fill one plastic beaker with ice, and chilled water. Lower (flat) tip of sonicator into ice water 1".

Add ice to the second plastic beaker, and pack the ice down flat with the third plastic beaker. Pack the ice to a height in the beaker so that when you place the 50 ml pyrex beaker on top of the ice, the rim of the 50 ml beaker sits slightly higher than the rim of the plastic beaker.

Center the 50 ml beaker on the flat-packed ice, and surround it with ice.

Pour the resuspended cell lysate into the chilled 50 ml beaker.

Pour chilled water into the large plastic beaker to a height slightly lower than the top of the crude lysate.

Spin the 50 ml beaker containing your sample (not the large beaker) for two minutes, chilling it. During the sonication, it is important to keep the sample as close to 4C as possible.

Note: During the sonication process, it may be necessary to pipet water out of the large beaker as the ice melts, so that the 50 ml beaker does not tip over.

Raise sonicator tip out of ice water, and wipe dry with Kimwipe.

Carefully lower the sonicator into the 50 ml beaker until the sonicator tip lightly touches the bottom of the beaker; then raise the sonicator 1 cm above the bottom of the beaker and fix sonicator in place.

Note: During sonication, we don't want the tip come into the top half of the lysate, as this can cause the lysis to turn to foam, effectively ending the sonication. If more volume is needed in order to keep the sonicator tip out of the top half of the lysate, use a 30 ml pyrex beaker, or add more lysis buffer.

Sonicate for up to 45 seconds, carefully moving the large beaker in a horizontal circular motion (**never** up and down).

Place sonicator tip back into the ice water.

Spin the beaker with lysate with your finger for two minutes to chill.

Repeat the 45 second sonication and two minute spin/chill procedure a total of 4-5 times.

Save aliquot of crude lysate for SDS-PAGE analysis.

Spin the lysate using a Beckman L7-55 Ultracentrifuge with a 55.2 TI rotor for 45 minutes @ 45,000 rpm 4C in two balanced #355618 Beckman tubes.

During the 45 minute spin, prepare for Ni-NTA Affinity Chromatography.

The supernatent contains the protein.

Save aliquot of spun lysate for SDS-PAGE analysis.

Add 5 M NaCl to the Protein Lysate to make the lysate 500 mM NaCl.

Resuspend one pellet in 15 ml Lysis Buffer (PBS, 1% Triton X-100, 5 mM Imidizole pH 8.0, 0.1% PMSF saturated in Isopropanol added fresh).

Save aliquot of pellet for SDS-PAGE analysis.

## Ni-NTA Affinity Chromatography

Do the affinity chromatography in a 4C cold room. Add 8 ml QIAGEN Ni-NTA Agarose slurry to a 20 ml Biorad column. Allow flow through. You will be left with 4 ml resin in the bottom of the column.

Equilibrate column with 5 volumes (20 ml) **Equilibration Buffer**, which is **Lysis Buffer** that has 5 M NaCl added to make it 500 mM NaCl. Allow flow through.

Note: For proteins where RNA binding to the column is a problem, the salt content can be raised to as high as 1 M to lower the amount of RNA binding to the column. *It is important that the salt content of the* **Protein Lysate**, **Equilibration Buffer**, *and the* **High Salt Wash** *all be the same.*

Have 8 volumes (32 ml) **High Salt Wash** (10 mM Tris pH 7.8, 1% Triton X-100, 5 mM Imidizole, 500 mM NaCl, 0.1% PMSF saturated in Isopropanol) made fresh, and ready to use.

Have 5 volumes (20 ml) **Low Salt Wash** (10 mM Hepes, 1% Triton X-100, 70 mM KCl, 10 mM Imidizole, 0.1% PMSF saturated in Isopropanol) made fresh, and ready to use.

Have 15 ml **Elution Buffer** (10 mM Hepes, 1% Triton X-100, 70 mM KCl, 250 mM Imidizole, 0.1% PMSF saturated in Isopropanol) made fresh, and ready to use.

After equilibrating the column, cap the bottom of the column.

Carefully resuspend Ni-NTA resin with 4 ml of the **Protein Lysate** using a 10 ml pipet. Pipet the resuspended protein lysate/resin mixture into a 50 ml conical. Using up to 10 ml of the protein lysate at a time, wash 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 4C. Incubate the protein lysate/resin mixture at 4C gently rocking for one hour.

Set up column on a ringstand with a 50 ml conical below to collect the flow through. Uncap column, add incubated protein lysate/resin to column as quickly as allowed.

Save aliquot of flow through for SDS-PAGE analysis.

Save 50 ml conical of flow through at -80C.

**Note:** Regarding salt content and Conductivity relating to the Ni-NTA **Low Salt Wash**, **Elution Buffer**, and the FPLC buffers **HGEDP** and **HGKEDP**.

The **Low Salt Wash** washes the **High Salt Wash** from the Ni-NTA column, and equilibrates the salt content to match the salt of the **Elution Buffer.**

The salt content of the **Elution Buffer** (measured as conductivity) needs to match that of the equilibrated FPLC column – in this case, the FPLC column is equilibrated to 100 mM KCl using FPLC buffers **HGEDP** and **HKGEDP**.

We have found in our lab that the Ni-NTA **Low Salt Wash**, and the **Elution Buffer** need 70 mM KCl to match the Conductivity of the 100 mM KCl equilibrated FPLC column.

This is because ingredients other than KCl in the Ni-NTA column buffers increase the overall conductivity, and it is necessary to lower the KCl in the **Low Salt Wash**, and the **Elution Buffer** to bring down the Conductivity to the level of the equilibrated FPLC column.

If the salt level is too high in the **Elution Buffer**, it can lead to unsuccessful binding of the protein to the FPLC column.

It would be advisable to check the conductivity of these buffers that you make.

Directly after flow through, it's time for the 5 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 transfer pipet.

Immediately following the **High Salt Wash** will be the 5 volumes of **Low Salt Wash**. Apply in the same manner as the High Salt Wash.

Next, we elute. Pipet 1 ml **Elution Buffer** into the column, and collect it in a 1.5 ml microcentrifuge tube.

There will almost certainly be no protein in the 1 ml elution, but save it anyway, along with an aliquot for SDS-PAGE analysis.

Collect a 10.3 ml elution into a centrifuge tube that will work in a Beckman J2-21 Centrifuge with a JS-13.1 swinging bucket rotor. This 10.3 ml elution fraction will be the onput to the FPLC.

Save aliquot of onput for SDS-PAGE analysis.

Spin the onput in the J2-21 @ 12,000 rpm for 15 minutes to pellet any impurities we don't want in the FPLC.

During the Ni-NTA washes, and the 15 minute spin, you should be preparing the FPLC, so that it's ready to go when the onput is done spinning, and loaded into the Superloop.

## FPLC Column Chromatography

The FPLC buffers can be made in advance, several liters at a time; however, two ingredients need to be added fresh: 100 ul 1 M DTT, and 100 ul 0.1% PMSF. Add both of these ingredients fresh to 100 ml each of FPLC Buffers A and B .

FPLC Buffer A: **HGE** (25 mM HEPES, 15% Glycerol, 0.1 mM EDTA, adjust pH to 7.6, and filter through 0.45 µm filter)

FPLC Buffer B: **HKGE** (25 mM HEPES, 15% Glycerol, 0.1 M EDTA, 1 M KCl, adjust pH to 7.6, filter through 0.45 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: I load the 10 ml Superloop in a way that works for me. I feel there is less waste, and is a quicker way to load – end result being better protein yield.

I take the 10.3 ml elution fraction, of which my tests have shown contain no protein in the last 2-3 ml. I assemble the Superloop manually, wetting the moving parts with **HGE**; and pour the 10.3 ml elution fraction into the glass chamber. Since the capacity of that chamber is 10 ml, when I push the last inner end piece in towards the eluent, I push harder just before it seats. This creates a suction that pulls out any bubbles, and fills the short tube with eluent.

Install Superloop to the FPLC, and run.

When the run is finished, save aliquots of the fractions of interest from the Fraction Collector. Select suitable fractions based upon a quick study of the Cromatogram. 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.