

# Expression and purification of recombinant proteins

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## I. Description

This protocol describes how to isolate recombinant protein from an *E. coli* expression system. The protocol below is optimized for expression of yeast TBP mutant K97C. At the end of the expression and purification protocol, there is a list of possible factors to alter to optimize expression of a different recombinant protein. It is important to note that, for expression of K97C, it is highly common to have to try three or four expression attempts before one works successfully.

For your particular over-expression, you will be supplied with a set of optimal (or near optimal) expression conditions for your protein. Depending on the molecule you wish to isolate, you may use a different purification protocol (such as HPLC, affinity chromatography, etc.).

### A. Overexpression of K97C in BL21 (DE3) pARG electrocompetent cells

(p.31, notebook VI, ARK)

K97C is transformed into BL21(DE3) pARG electrocompetent cells. These cells contain a plasmid encoding the eukaryotic arginine tRNA, which is not present in prokaryotic cells. Plate 50  $\mu$ L and 500  $\mu$ L on LB agar containing 200  $\mu$ g/mL amp (to select for pK97C) & 50  $\mu$ g/mL kanamycin (to select for pARG) and incubate **12 hours** at 37°C. Directly proceed to the small growth – putting the colonies at RT or at 4°C slows the growth of the cells considerably, and I have found this affects the expression of K97C. Colonies should be visible. Inoculate a 60 mL growth in LB containing 200  $\mu$ g/mL amp and 50  $\mu$ g/mL kanamycin with one colony of K97C. Shake at 37°C until the small growth becomes slightly turbid (cloudy)- again, allowing this step to go overnight slows the growth of the cells and effects the expression of K97C.

(For a time schedule suggestion: plate cells at 4 or 5 pm, incubate 12 hours, start small growth at 4 or 5 am. You should be able to start the large growths in another 5-7 hours (around noon). The large growths can take 3-7 hours to reach the appropriate stage for induction of K97C expression, after which expression continues for 3 hours (yes, it's a long day).

1. Inoculate five 4 L flasks containing 1 L TB with:
  - 1 mL 100 mg/mL ampicillin
  - 1 mL 50 mg/mL kanamycin
  - 10 mL small growth

Shake at 37°C for 2 hours. Monitor growth by UV absorbance until  $OD_{600} = \sim 1.0$  (~4 or 5 hours on a good day). Collect 1 mL sample when  $OD_{600} = 1.0$  to set aside for phast gel.

2. Induce each flask with:
  - 500  $\mu$ L 1M IPTG (sterile filtered)

Shake at 37°C for 3 hours, collect a 1 mL sample after 3 hours to set aside for phast gel. Set flasks at 4 °C. Harvest cells by pelleting them in Sorvall centrifuge, GS-3 rotor, 8,000rpm, 10minutes, 4°C.

Phast gel sample preparation for 1 mL samples from growth: First pellet cells in a microcentrifuge and carefully decant the supernatant. Then resuspend pellet in 200  $\mu$ L 2xSDS buffer and heat shock the cells for 2-5 minutes at 95°C. Finally, run samples on Homo-20 or Homo-12 phast gel to verify that K97C was not expressed before addition of IPTG and was expressed after addition of IPTG. You may have to dilute the phast gel samples to be able to see bands clearly.

### **B. Harvesting protein from cells**

Done at 4 °C. After harvesting over-expressed protein, (p.32, notebook VI, ARK), resuspend cells in 1xRanish Buffer (65 mL total) on ice.

#### 1x Ranish Buffer

30mM Tris-HCl, pH 7.5 at 25°C

10% glycerol

50mM KCl

1mM EDTA

2mM DTT (Boehringer Mannheim), always added immediately before buffer is used!

In general, make a 2L stock of 5x Ranish, which is then diluted as needed throughout the purification process. Make sure to make 1x Ranish enough in advance of needing to use it that it can be sterile filtered, degassed and chilled to 4°C. Add DTT about 30 min before using the buffer.

Once the cells have been resuspended, lyse the cells by first freezing them to -70 °C on dry ice (can leave them at -70 °C until ready for next step), then thawing them on ice (takes about 2.5 hours). Sonicate the cell resuspension on Cycle 8, 80% duty, using the macrotip, and keeping the cells on ice. Sonicate for four 35 second bursts, with 2 minutes between each sonication. Collect a 100  $\mu$ L sample to set aside for phast gel.

Centrifuge to separate soluble and insoluble fractions by pelleting the insoluble fraction in Sorvall centrifuge, SA-600 rotor, 16,350 rpm, for one hour, at 4°C. Collect supernatant (yellow and translucent) and set aside a 100  $\mu$ L sample from supernatant for phast gel.

Run phast gel (Homo-12.5 or Homo-20 SDS PAGE) to check for expression of plasmid. To each collected sample, add 200  $\mu$ L 2xSDS buffer and denature at 95°C for 2 minutes.

## **II. Q and SP sepharose column purification (FPLC)**

The Q column is an anion exchange column that will bind negatively charged proteins. The SP column is a cation exchange column that will bind positively charge proteins and TBP.

Load soluble fraction on a Q column that is hooked in tandem to an SP sepharose FPLC column at 4°C, both equilibrated in 3 column volumes of 1x Ranish Buffer containing fresh DTT and hooked to a peristaltic pump at a rate of <2 ml/min. Run 1x Ranish buffer over the columns to remove excess protein until  $OD_{280} < 0.1$  (~300 mL).

Disconnect the SP column from the Q column and connect the SP column to the FPLC at 4°C. Separate fractions using a salt gradient from 50mM to 600mM KCl over 120 mL. Collect 4 mL fractions, run column at 2 mL/min, with backpressure on FPLC set to 2. yTBP elutes around 360 mM KCl (around fractions 17-20). Monitor fractions for protein concentrations with the UV monitor on the FPLC. Check fractions showing protein concentration on phast gel (or check all fractions on a Coomassie-stained filter paper "dot

blot"). For the phast gel, mix 3  $\mu$ L of fraction with an equal amount of 2x SDS buffer, run a HOMO-20 phast gel. Select TBP-containing fractions.

Concentrate/buffer exchange fractions in Centriprep-10 concentrators at 4°C. Reduce KCl concentration to < 100mM by buffer exchanging with 1x Ranish. Reduce total volume to less than 5 mL.

#### **A. Heparin Hi-Trap column purification**

Load concentrated TBP-containing fractions on a Heparin Hi-Trap column (1 mL) that has been equilibrated in 1x Ranish (DTT freshly added). Load protein using a 5 mL syringe that is connected to the column with a luerlock/screw piece. Collect flow-through and load again, to ensure that everything is on column. Connect Heparin-HiTrap column to FLPC.

Purify TBP using a salt gradient from 50 mM to 600 mM over 80 mL. Collect 2 mL fractions, run column at 1 mL/min, backpressure on FPLC set at 2. yTBP elutes around 375 mM KCl (around fractions 24-26). Monitor fractions for protein concentrations with UV monitor on FPLC. Check fractions showing protein concentration on phast gel by mixing 3  $\mu$ L of fraction with equal amount of 2x SDS buffer and running a HOMO-20 phast gel. Select TBP-containing fractions

Concentrate/ Buffer exchange fractions in Centriprep-10 concentrators at 4°C. Reduce KCl concentration to < 100 mM as above. Reduce total volume to ~3 mL. Estimate final concentration using by checking absorbance at 280 nm and using the following equation:

$$[\text{conc}]^a = \frac{A_{280} - 0.0051}{0.0177}$$

Store at -70°C until ready to use.

#### **B. Expression optimization**

The following is a list of different factors in recombinant protein expression that can be altered to achieve higher expression of the protein.

##### *1. Transformation*

Different cell lines can be used for protein expression. The general cell line used in our lab is BL21 (DE3) cells, which can be purchased from Novagen in a competent form. We also currently have in the lab BL21 (DE3) pARG cells which contain a plasmid encoding the eukaryotic arginine tRNA that is not naturally present in E. coli cells. There are many other variations on expression systems as well.

##### *2. Small Growth*

A small growth (5-10 mL per 1 L culture) is typically started 24 hours or more after the transformation of the plasmid into the cells. (Often a transformation is done one afternoon, and plates are incubated overnight, then the small growth is started the next afternoon and incubated overnight.) I have found greater success in starting the small growth 12 hours after the transformed cells have been plated. The small growth often takes 3-5 hours to become cloudy when the colony used to start it is fresher, and the expression of the recombinant protein is more robust as a result of fresher cells.

##### *3. Large Culture*

Try inoculating the large growth (usually 1 L growths in 4 L flasks) when the small growth first becomes cloudy. As I stated above, keeping the cells multiplying at a healthy rate seems to result in the best expression.

#### 4. *Temperature of Growth*

Some proteins may be less stable and therefore will need to be expressed at a lower temperature. Try expression at 30 °C rather than 37 °C. This will greatly increase the time required for growth, but it may be a more stable environment for the recombinant eukaryotic protein in a prokaryotic environment.

#### 5. *Media for growth*

Sometimes minimal media encourages cell growth, and sometimes a rich media does better. Maniatis lists several different broths that may be used for growing cultures.

#### 6. *IPTG concentration*

With IPTG, sometimes less is more and other times, brute force (lots of IPTG) is necessary to get acceptable expression of recombinant proteins. Usually, 0.4 to 1 mM final concentration of IPTG in your large growth is a good range to test.

#### 7. *OD<sub>600</sub>*

If aggregation is a problem (i.e., you get expression but see little or nothing when you purify your protein), perhaps inducing the expression of your protein earlier may help reduce the aggregation. If you see no expression at all, perhaps your cells need to grow longer before recombinant protein expression can be induced. A good range to test is  $OD_{600} = 0.6 - 1.0$  (log phase growth).

#### 8. *Duration of protein expression*

If a protein is fragile or has problems folding, it may degrade during a long expression period. After induction of the recombinant protein, expression can be as short as 0.5 hours and last up to 3 hours.

#### 9. *Troubleshooting:*

- ⌚ Do you need to use a protease inhibitor?
- ⌚ Are you truly meticulous about keeping cell cultures / cell pellets / protein solutions on ice when they are not growing? You should be!!!
- ⌚ Do you need to increase the volume of the growth to increase expression (500mL – 5L)?
- ⌚ Are you achieving complete cell lysis?
- ⌚ Perhaps a protein cannot survive a PEI or ammonium sulfate precipitation – perhaps it needs to stay in solution?
- ⌚ Do you see overexpression in pre-induction? In post-induction?
- ⌚ Do you see protein in significant concentration in any discarded fractions during the purification process?