

Video Article

High-throughput Purification of Affinity-tagged Recombinant Proteins

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Abstract

X-ray crystallography is the method of choice for obtaining a detailed view of the structure of proteins. Such studies need to be complemented by further biochemical analyses to obtain detailed insights into structure/function relationships. Advances in oligonucleotide- and gene synthesis technology make large-scale mutagenesis strategies increasingly feasible, including the substitution of target residues by all 19 other amino acids. Gain- or loss-of-function phenotypes then allow systematic conclusions to be drawn, such as the contribution of particular residues to catalytic activity, protein stability and/or protein-protein interaction specificity.

In order to attribute the different phenotypes to the nature of the mutation - rather than to fluctuating experimental conditions - it is vital to purify and analyse the proteins in a controlled and reproducible manner. High-throughput strategies and the automation of manual protocols on robotic liquid-handling platforms have created opportunities to perform such complex molecular biological procedures with little human intervention and minimal error rates¹⁻⁵.

Here, we present a general method for the purification of His-tagged recombinant proteins in a high-throughput manner. In a recent study, we applied this method to a detailed structure-function investigation of TFIIB, a component of the basal transcription machinery. TFIIB is indispensable for promoter-directed transcription *in vitro* and is essential for the recruitment of RNA polymerase into a preinitiation complex⁶⁻⁸. TFIIB contains a flexible linker domain that penetrates the active site cleft of RNA polymerase⁹⁻¹¹. This linker domain confers two biochemically quantifiable activities on TFIIB, namely (i) the stimulation of the catalytic activity during the 'abortive' stage of transcript initiation, and (ii) an additional contribution to the specific recruitment of RNA polymerase into the preinitiation complex^{4,5,12}. We exploited the high-throughput purification method to generate single, double and triple substitution and deletions mutations within the TFIIB linker and to subsequently analyse them in functional assays for their stimulation effect on the catalytic activity of RNA polymerase⁴. Altogether, we generated, purified and analysed 381 mutants - a task which would have been time-consuming and laborious to perform manually. We produced and assayed the proteins in multiplicates which allowed us to appreciate any experimental variations and gave us a clear idea of the reproducibility of our results.

This method serves as a generic protocol for the purification of His-tagged proteins and has been successfully used to purify other recombinant proteins. It is currently optimised for the purification of 24 proteins but can be adapted to purify up to 96 proteins.

Video Link

The video component of this article can be found at <http://www.jove.com/video/4110/>

Protocol

PART A: High-throughput growth of bacterial cultures.

1. Grow Bacteria Overnight in 2 ml of Autoinduction Medium Using 24-well Plates

1. Sterilise the 24-well plates by microwaving.
2. Inoculate 1.5 ml of autoinduction medium (Overnight Express Medium) with freshly grown bacterial colonies or frozen glycerol stocks. We normally inoculate three wells per mutant with three individual cloned colonies. Reserve six wells for positive and negative controls. For the positive controls we grow up three wildtype clones and for the negative controls we grow up 2 clones which have been transformed with a non-expressing plasmid. Check for sufficient sterilization of the plate by leaving one well blank for the medium-only control.
3. Grow the cells for 18 hr at 37 °C and shaking at 250 rpm. We use lac-inducible BL21 (DE3) Rosetta 2 cells. Autoinduction medium contains a mixture of glucose and lactose. The bacteria initially feed on glucose and then begin to use lactose, which also induces the expression of the recombinant proteins.
4. Remove the lid and place the plate on the robotic platform.

PART B: Robotic purification of recombinant proteins.

2. Prepare the Robotic Platform

1. Make up the wash buffer consisting of 20 mM imidazole, 0.1% Triton X-100, 0.5 M NaCl, 20 mM Tris-acetate, pH 7.9, 10 mM MgOAc₂, 0.7 mM ZnOAc₂, 10% glycerol, and the elution buffer consisting of 0.5 M imidazole, 0.1 % Triton X-100, 0.5 M NaCl, 20 mM Tris-acetate, pH 7.9, 10 mM MgOAc₂, 0.7 mM ZnOAc₂, 10% glycerol. These buffers will be used to wash the beads after the tagged proteins have been bound to them, or to elute the proteins from the beads, respectively.
2. Make up the bacterial diluent (100 ml distilled water with 15 µl antifoam reagent). This solution will be used to make dilutions of the bacterial overnight cultures for optical density (A600) measurements. The presence of antifoam in the diluent prevents the formation of air bubbles that would interfere with the plate reader measurements.
3. Make up the lysis solution consisting of 10x FastBreak reagent, 2 µl lysonase per sample and 15 mM MgOAc₂. FastBreak contains a mix of detergents and salts that break the bacterial cell walls and facilitate the release of intracellular proteins. Lysonase is a proprietary mix of lysozyme and a nuclease. Lysozyme assists in disrupting the cell wall and the nuclease digests the released bacterial nucleic acids.
4. Optional: Make up a 6 M guanidine hydrochloride solution. Some recombinant proteins tend to stick to the Teflon-coated pipetting needles. The guanidine hydrochloride solution denatures proteins and washes them off more effectively than water that is routinely used to rinse the washable robotic pipetting tips after each step.
5. Prepare the BCA (bicinchoninic acid) protein quantitation reagent by mixing bicinchoninic acid solution (reagent A) and the copper sulphate solution (reagent B): Peptide bonds reduce Cu²⁺ from the CuSO₄ component present in the BCA reagent to Cu¹⁺ whereby the amount of Cu¹⁺ is proportional to the number of peptide bonds present in the solution. In a second step, two molecules of bicinchoninic acid chelate Cu¹⁺ resulting in an absorbance shift to 562 nm, resulting in a purple colour. The colour reaction is time-dependent and usually needs several hours to be definitive. After that the colour is stable for several hours.
6. Fill troughs or plates of the right size and place them in their pre-allocated positions on the robotic platform.
7. Place one 24-well plate for the guanidine hydrochloride waste, two clear 96-well plates for OD₆₀₀ and absorbance measurements and one blue 96-well plate for the purified proteins on their positions on the platform. Place one 96-deepwell plate on the magnetic stand.
8. Dilute MagneHis Ni-particles which bind proteins by forming chelates with their His-tags 5-fold in distilled water and fill them into the bead-stirring unit. Switch the stirrer on to keep the beads in suspension.
9. Switch on the robotic platform and flush the pipetting needles for several minutes to remove air bubbles which would otherwise interfere with the pipetting accuracy. The re-usable pipetting needles are flushed in between individual pipetting steps. Alternatively, disposable tips could be used. All subsequent steps are carried out robotically. The robotic protocol is available on request.

3. Cell Growth is Checked by Measuring the OD600

1. 10 µl of overnight culture is diluted in 90 µl of diluent solution.
2. Measure the OD₆₀₀ to ensure that the bacteria have grown to similar densities (Figure 1).

4. The Cells are Broken up to Release the Proteins and to Allow Bead-binding

1. 100 µl of magnetic Ni-bead suspension is distributed into each well of a second 24-well plate.
2. 900 µl of each small-scale bacterial culture is transferred into the 24-well plate containing the beads. Add 100 µl of the 10x FastBreak/lysonase mix.
3. The 24-well plate is transferred to the shaking platform for rapid shaking (800 rpm) for 30 min at room temperature. Bacterial cell walls are disrupted by a combination of mechanical forces and chemical action and the recombinantly produced proteins are released into solution. With their affinity tags they immediately bind the paramagnetic chelating nickel beads.

5. The Beads are Washed

1. The cell-lysates containing the resuspended magnetic beads are transferred to a 96-deepwell plate which is positioned on a stand with magnetic rods that slide between the wells. The 96-well plates have got a square cone-shaped bottom which allows easier removal of the supernatant. The wells can hold volumes of up to 2.1 ml but are filled with much smaller volumes. This enables us to perform vigorous shaking steps without sample cross-contamination by splashing.
2. The pipette tips are washed between individual pipetting steps with 6 M guanidine-HCl. This step is crucial for the purification of TFIIB and other "sticky" proteins to avoid cross-contamination. With other proteins it may be sufficient to rinse the needles extensively with water between the pipetting steps.
3. The magnetic rods of the magnetic stand attract the paramagnetic beads, pulling them away from the centres of the wells and allow the pipetting needles free access to the supernatant. The supernatant is discarded.
4. 500 µl of wash buffer is first added to the 24-well plate and subsequently transferred to the 96-well plate to ensure the complete transfer of the beads. The 24-well plate is removed from the shaker.
5. Another 500 µl of wash buffer is added directly to the 96-well plate, the plate is transferred to the shaker and vigorously shaken for 1 min. The plate is moved back to the magnetic stand and the wash buffer discarded.
6. This step is repeated twice and the wash procedure is finished by removing any buffer remnants from the plate.

6. Elute the Proteins in Elution Buffer

1. 100 µl of elution buffer is added to the beads, the plate is moved to the shaker and vigorously shaken for 30 min at room temperature.
2. The plate is moved back to the shaker and the eluate, containing the purified recombinant protein, is transferred to a new plate.

7. Measure the Concentrations of the Purified Proteins

1. 190 μ l of BCA reagent mix is transferred to a clear 96-well plate and 10 μ l of the protein solution added.
2. After several hours of incubation (typically 5-6 or overnight), measure the absorbance and compare it to BSA standards to determine the concentrations of each of the purified protein preparations (**Figure 2**).
3. The purified proteins can now be used for downstream applications at the appropriate concentrations (**Figure 3**).

8. Representative Results

The purification protocol offers two quality control stages, examples of which are shown. We are able to identify and document potential problems at the bacterial growth stage (**Figure 1**) and later on upon assessing the yields of the purified proteins (**Figure 2**). We typically purify proteins and test them in triplicate. This, in combination with the two quality control steps, gives us confidence that any variation we observed in our functional assays are due to the mutant phenotype (**Figure 3**) and not caused by experimental variations or failed purifications. The yields obtained typically range from 50-200 μ g and are more than sufficient for various functional assays.

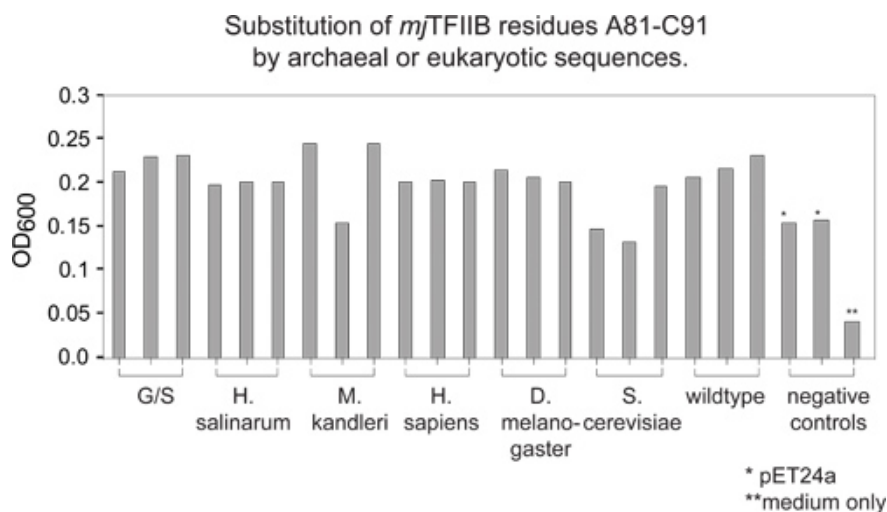


Figure 1. Histogram of the OD measurements of a 24 well plate with overnight cultures. Three clones of six TFIIIB mutant variants as well as of the wildtype TFIIIB have been grown. Two clones carrying a non-expressing plasmid and a well with medium only serve as negative controls. The OD measurements show that there are small variations in the growth rates between individual cultures.

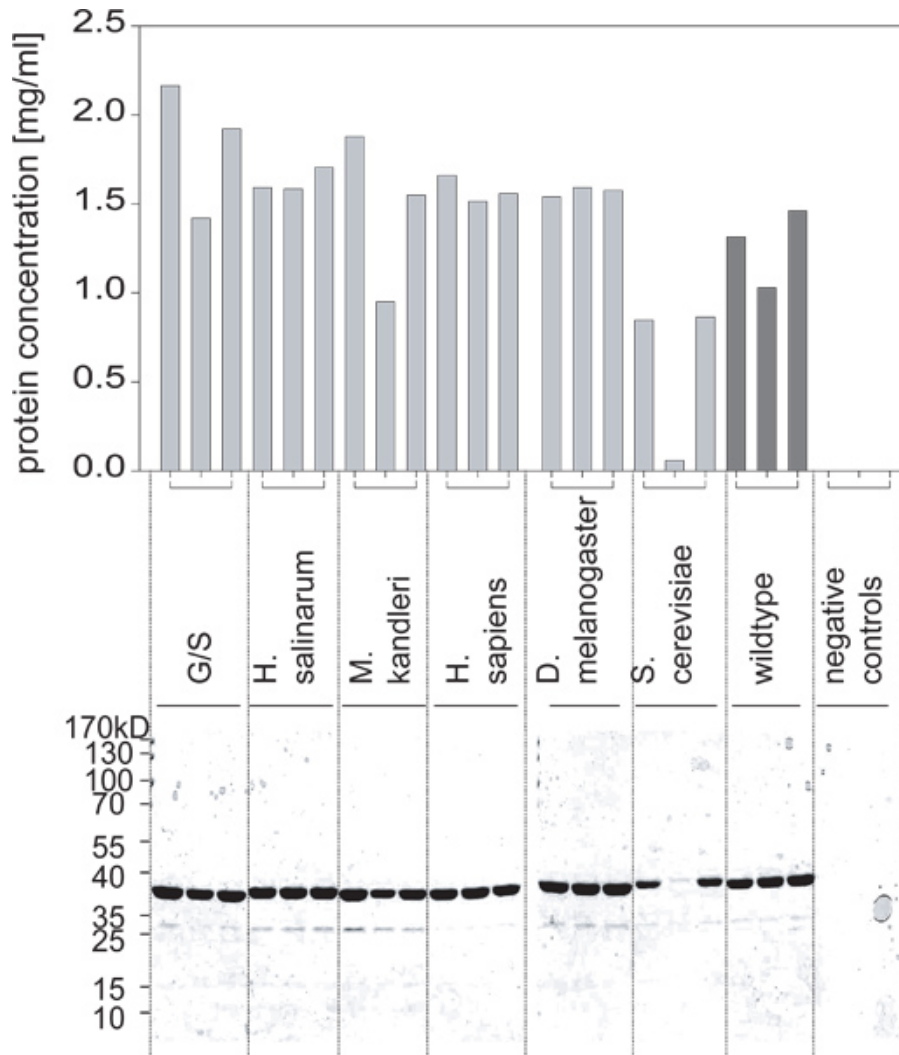


Figure 2. Protein yields obtained from these cultures as determined by a BCA assay and confirmed by SDS PAGE. One of the variants is not expressed at high levels. In combination with **Figure 1**, we can conclude that this was not due to differential cell growth but due to protein expression not being induced properly.

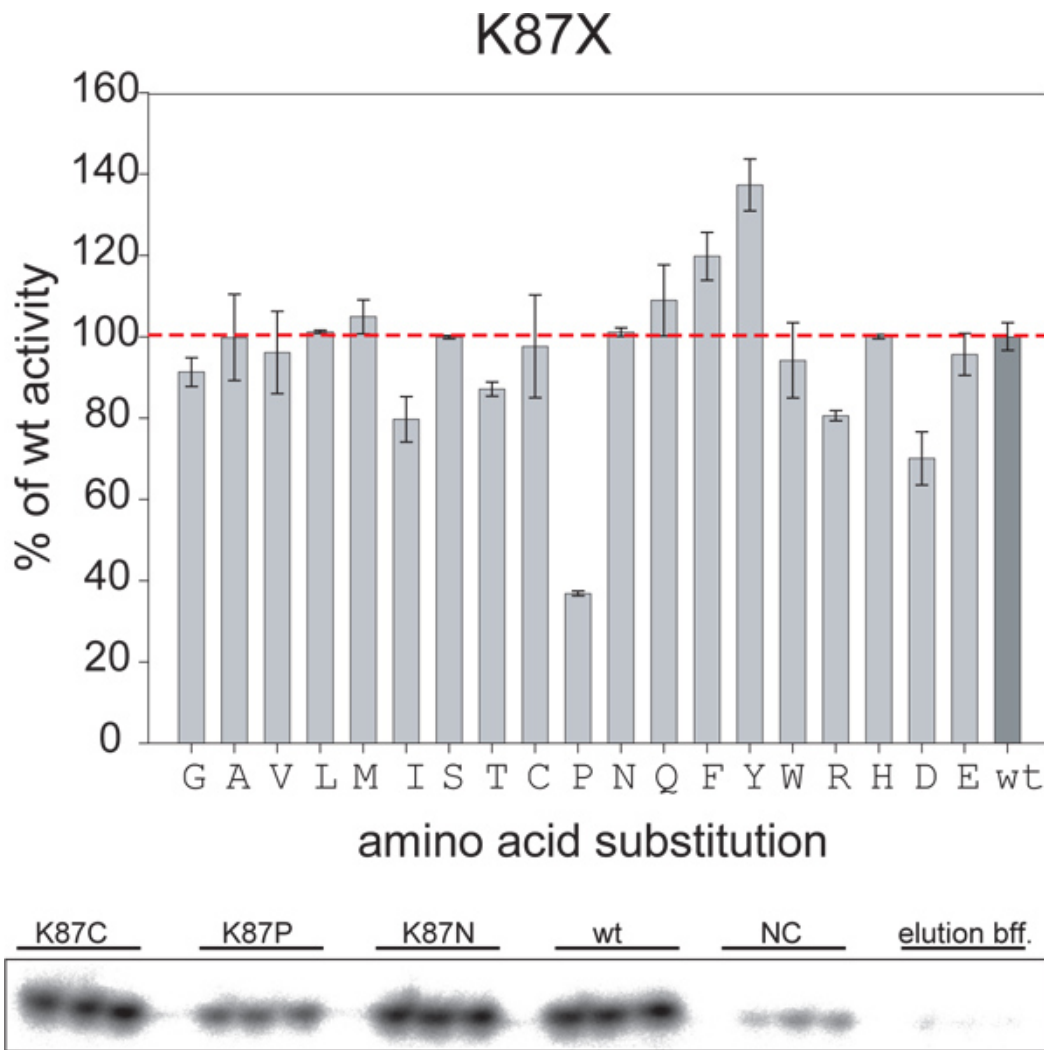


Figure 3. Representative result of a transcription assay. We measured the stimulation activity of TFIIIB variants on the production of small abortive transcripts by RNAP. Here, the stimulation effects of a full library of single amino acid substitutions of TFIIIB residue K87 are shown. The high degree of reproducibility is confirmed by small error rates. A sample gel showing the performance of three mutants as compared to wildtype (wt), negative controls (NC) and elution buffer only controls is depicted underneath.

Discussion

The automated recombinant protein purification method described here allows the production and purification of a large number of mutant proteins in a small-scale format under highly reproducible conditions with minimal human intervention. **Figures 1 and 2** show results of systematic quality-controls and examples of the purified proteins. **Figure 3** shows that the purified transcription factors used in this example perform in a highly reproducible manner in functional assays.

Even though the procedure was developed for the purification of archaeal TFIIIB, it is widely applicable for the purification of affinity-tagged proteins. The use of such automated purification protocols will thus significantly facilitate the biochemical analysis of recombinant proteins and will thus further our understanding of protein-protein interactions on a scale that is difficult to achieve manually.

Disclosures

No conflicts of interest declared.

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