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Method Article

Optimization of production of recombinant gamma-tubulin in bacteria



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A B S T R A C T

Production of a protein of interest in bacteria and its purification from bacterial lysates are valuable tools for the purification of larger amounts of recombinant proteins. The low cost of culturing, and the rapid cell growth of bacteria make this host a good choice for protein production, but the folding and function of the purified protein might be altered due to the production of a eukaryotic protein in a prokaryotic host. Here, we provide a purification method for the purification of gamma (γ)-tubulin (TUBG) from soluble fractions of *Escherichia (E.) coli* lysates using affinity tags.

- This protocol describes a method that purifies soluble GST-TUBG1 from bacteria.
- Of the three tested induction conditions, the highest yield of recombinant GST-TUBG1 was obtained after the induction of *E. coli* with isopropyl-D-1-thiogalactopyranoside (IPTG) for 1 h at 37 °C followed by overnight incubation at room temperature.
- In comparison with other methodologies (Hoog et al., 2011), the technique described here retrieves larger amounts of recombinant TUBG1 from small-scale expression cultures.

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Resource availability:	NA

Protocol background

In mammalian cells, TUBG was first identified as an essential protein for microtubule nucleation [2,3]. Acentrosomal and centrosome-associated microtubules nucleate from the minus end on a complex formed of TUBG and various TUBG complex proteins, known as the TUBG ring complex [3]. TUBG is a ubiquitously expressed protein that forms a novel cytoskeleton/nucleoskeleton named the γ -tubulin meshwork [4–6]. The meshwork can interact with various proteins, such as E2F1 [1], lamin B1 [7], Rad51 [8], GCPs [3], PCNA [6], C53 [9], and α - and β -tubulin dimers [2,3], and influences various cellular processes [4,10,11]. The cellular folding of TUBG depends on chaperonin TCP1, which has hindered the purification of denatured protein in bacteria, and consequently, the use of recombinant TUBG in analysis in vitro [12]. As it is essential to understand the intrinsic features of this protein and to study the interaction between TUBG and other proteins in a cell-free system, the purpose of this study was to create a protocol for the purification of TUBG from soluble fractions of *E. coli* lysates.

Method details

Purification of GST- and His₆-tagged human TUBG1 from bacteria lysates

Step 1: Optimization of the culture conditions with small-scale expression cultures

Materials

- pGEX2T-*TUBG1* (Addgene Plasmid # 171967) [6]
- pET21-*TUBG1* (Addgene Plasmid # 101825) [13]
- *E. coli* DH5 α (gives high protein yield from pGEX2T-*TUBG1* plasmid; Thermo Fisher Scientific, cat. no. 18265017)
- *E. coli* BL21([DE3] optimized for protein expression from the T7 promoter included in a pET21 plasmid; Thermo Fisher Scientific, cat. no. C600003)
- Sterile Luria–Bertani liquid (LB) medium (Merck, cat. no. L2542)
- Sterile LB agar plates (Sigma-Aldrich, cat. no. L5667)
- Ampicillin (Thermo Fisher Scientific, cat. no. 11593027) dissolved in sterile, deionized distilled water
- Sterile 14 ml polypropylene round-bottom tubes (Fisher scientific; Falcon, cat. no. 10384641)
- Isopropyl- β -D- thiogalactoside (IPTG; Sigma-Aldrich, cat. no. I6758) dissolved in sterile, deionized distilled water
- Phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich, cat. no. 10837091001) dissolved according to the manufacturer's instructions
- Anti-TUBG (1:1000; Sigma-Aldrich, cat. no. T3320)
- Sterile phosphate-buffered saline (PBS; Sigma-Aldrich, cat. no. P4417)
- 1.5 ml microcentrifuge tubes (Sigma-Aldrich, cat. no. Z336777)
- Shaking incubator for culture growth (Merck, cat. no. CLS6791)
- Soniprep 150 Plus with exponential probe (240V; MSE, cat. no. MSS150.CX4.5)

- 5X loading buffer: 625 mM TRIS pH 6.5 (Sigma-Aldrich, cat. no. 10812846001), 10% (w/v) sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. GE17-1313-01), 25% (v/v) glycerol (Sigma-Aldrich, cat. no. G5516), 0.005% (w/v) bromophenol blue (Sigma-Aldrich, cat. no. GE17-1329-01), 250 mM dithiothreitol (Sigma-Aldrich, cat. no. D0632), and deionized distilled water

Note that the preceding list includes only the necessary bacteria strains and non-standard laboratory equipment.

1. Transform the DH5 α and BL21 *E. coli* strains with the N-terminal-GST-tagged human *TUBG1* (pGEX2T-GST-*TUBG1*) and the C-terminal His₆-tagged human *TUBG1* gene (pET21-*TUBG1*-His₆), respectively, according to the manufacturer's instructions.
2. Plate the appropriate volume of the transformed bacteria on agar plates containing 100 μ g/ml ampicillin.
3. Incubate the bacteria at 37 °C until single colonies are visible. This can take longer than 16 h.
4. Pick a single colony, and inoculate 1 ml of LB-ampicillin (100 μ g/ml) with either DH5 α (DH5 α -GST-*TUBG1*) or BL21 (BL21-*TUBG1*-His₆) expressing recombinant *TUBG1* in a PRB tube. Shake the tube at 200 rpm overnight at 37 °C.
5. Dilute the overnight culture to 1:100 in 3 different tubes. Incubate the bacteria at 37 °C until the optical density measured at a wavelength of 600 nm (OD₆₀₀) of the sample reaches 0.6 to 0.8.
6. Induce protein expression by adding IPTG until the final concentrations for DH5 α -GST-*TUBG1* and to BL21-*TUBG1*-His₆ reach 0.2 mM and 1.0 mM, respectively. Incubate each tube following one of these conditions:
 - (a) Shake for 1 h at 37 °C;
 - (b) Shake overnight at room temperature; or
 - (c) Shake for 1 h at 37 °C followed by overnight incubation at room temperature.

Transfer the cultures into 1.5 ml microcentrifuge tubes, and pellet the bacteria by centrifugation (6000 \times g for 5 min at 4 °C).

7. Discard the supernatant, and drain the pellet. Then, place the tubes on ice.
8. Use one-twentieth of the original volume (50 μ l) of ice-cold PBS supplemented with 1 mM PMSF to resuspend the bacteria pellet.
9. Keep the bacteria lysates at -80 °C for at least 20 min. This step facilitates lysis of the bacteria but can be omitted.
10. Take the bacteria lysates out of the freezer, and thaw the tubes on ice.
11. Lyse the resuspended bacteria in ice-cold water using 3 sonication cycles of 20 s sonication followed by 30 s of cooling. The color of the bacteria suspension changes upon lysis.
12. Add the 5X loading buffer to obtain a final volume of 1X. Boil the sample at 95 °C for 20 min.
13. Analyze 20 μ l of each sample using Western blot [14].
14. Once the optimal culturing conditions are established, go back to Step 4.
15. Pick several colonies, and repeat Steps 5 to 13 only with the selected conditions.
16. Select the bacteria colony with the highest yield.
17. Prepare a glycerol stock by mixing 0.9 ml of an overnight culture of the selected colony with 0.1 ml of glycerol in a cryovial. Mix well before freezing. Keep the cryovial at -80 °C. This is the bacteria stock.

Note that the growth conditions (Fig. 1A) affect the expression levels of GST-*TUBG1* and *TUBG1*-His₆ differently in bacteria. The highest yield of GST-*TUBG1* was obtained in DH5 α -GST-*TUBG1* upon stimulation with 0.2 mM IPTG for 1 h at 37 °C followed by an overnight incubation at room temperature (Fig. 1A and B), whereas in BL21-*TUBG1*-His₆, the highest yield was achieved after stimulation with 1.0 mM IPTG for 1 h at 37 °C (Fig. 1A and C). Also, protein production may differ between different colonies within the same bacteria strain.

Step 2: Purification of human GST-*TUBG1*

The purification of *TUBG1*-His₆ is described elsewhere [13].

Materials

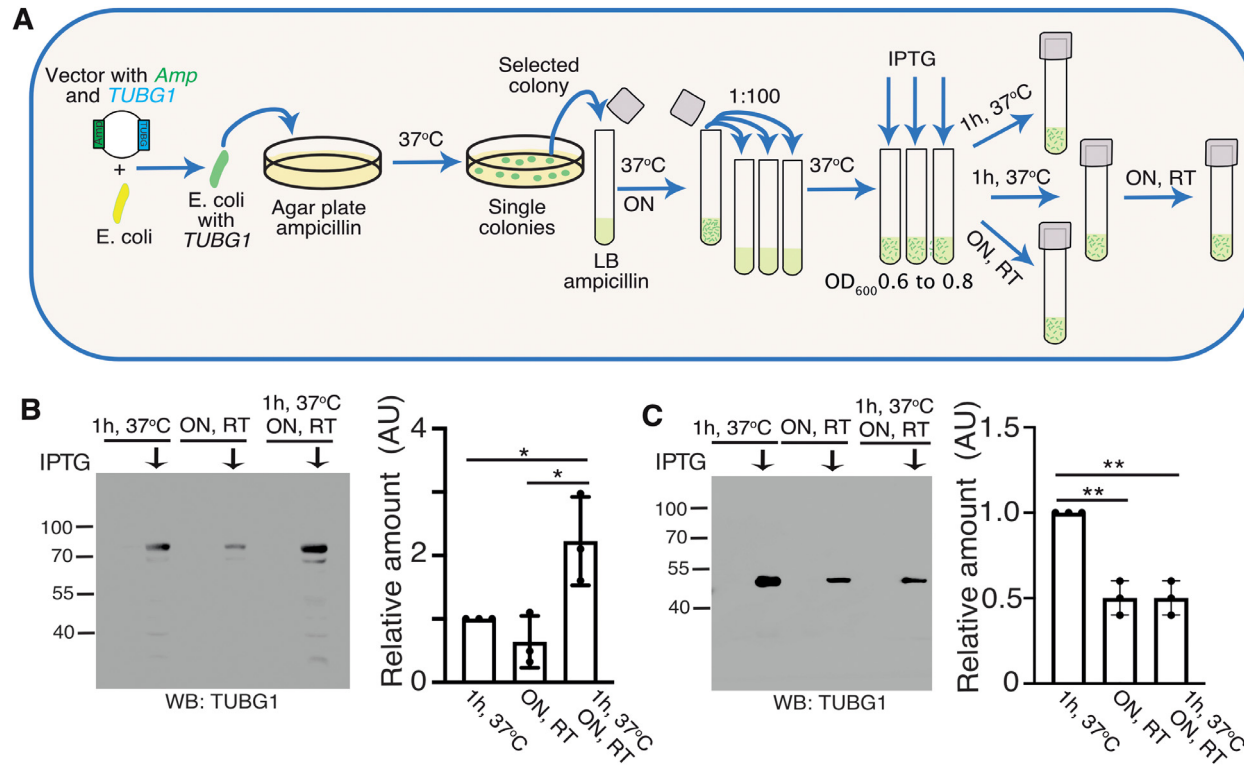


Fig. 1. Optimizing the conditions for expressing recombinant TUBG1: (A) a scheme for the optimization of culture conditions for the expression of TUBG1 using ampicillin (Amp) as a selection marker in transformed *E. coli*. In short, a selected *E. coli* colony carrying a vector containing the TUBG1 and Amp genes is cultured overnight (ON) in Luria-Bertani liquid supplemented with ampicillin. Then, the bacteria are diluted and cultured until the optical density measured at a wavelength of 600 nm (OD_{600}) reaches 0.6 to 0.8. The expression of recombinant TUBG1 is induced with isopropyl-1-thio- β -D-galactopyranoside (IPTG) during the indicated times and conditions. GST-TUBG1 (B) and TUBG1-His₆ (C) were induced in *E. coli* DH5 α containing the pGEX2T-TUBG1 vector with 0.2 mM IPTG (B) and in *E. coli* BL21 carrying the pET-TUBG1-His₆ with 1 mM IPTG (C), respectively. Thereafter, the bacteria were incubated under the following conditions: 1 h at 37 °C (1 h, 37 °C), overnight incubation at room temperature (ON, RT), and 1 h at 37 °C followed by overnight incubation at room temperature (1 h, 37 °C ON, RT). (B and C) The expression of recombinant TUBG1 was analyzed by Western blot (WB) using an anti-TUBG1 antibody. The highest yield of GST-TUBG1 was obtained upon stimulation with 0.2 mM IPTG for 1 h at 37 °C followed by overnight incubation at room temperature (B), whereas the highest yield of TUBG1-His₆ was reached after stimulation with 1.0 mM IPTG for 1 h at 37 °C (C). (B and C) The graphs illustrate densitometric analysis of the TUBG1 content in the Western blots presented (mean \pm SD; $N = 3$, ** $P < 0.01$, * $P < 0.05$).

- Frozen stock of the chosen DH5 α -GST-TUBG1 colony
- Sterile LB medium
- Ampicillin dissolved in sterile, deionized distilled water
- Sterile 14 ml PRB tubes
- Sterile Erlenmeyer flask
- Glutathione Sepharose 4B (GS beads; Amersham Pharmacia Biotech, cat. no. GE17075601)
- IPTG dissolved in sterile, deionized distilled water
- PMSF dissolved according to the manufacturer's instructions
- Anti-TUBG T3320
- Anti-GST (GE Healthcare, cat. no. 27-4577-50)
- Resuspension buffer (RB): PBS, 5 mM β -mercaptoethanol (β ME; Sigma-Aldrich, cat. no. M6250), 1 mM MgCl₂ (Sigma-Aldrich, cat. no. 208337), and 250 nM GTP (Sigma-Aldrich, cat. no. 10106399001)
- Wash buffer (WB): 50 mM TRIS pH 7.5, 5 mM β ME, 1 mM MgCl₂, 250 nM GTP, 300 mM NaCl (Sigma-Aldrich, cat. no. S9888), 1 mM PMSF, and 0.1% Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Sterile PBS
- Freezing buffer (FB): 50 mM TRIS pH 7.5, 150 mM NaCl, 50% glycerol, 1 mM DTT, and 1 mM PMSF
- Thrombin (1 unit/ μ l in PBS; GE Healthcare, cat. no. 27-0846-01)
- Gelcode Blue Stain Reagent (Thermo Scientific, cat. no. 24590)

Note that the preceding list includes only necessary bacteria strains and non-standard laboratory equipment.

1. Take a sterilized pipette tip, scratch bacteria from the frozen (-80 °C) bacteria stock, and inoculate 1 ml of LB-ampicillin (100 μ g/ml) in a PRB tube. Shake the tube at 37 °C overnight.
2. Dilute the overnight culture to 1:100 (into fresh, pre-warmed LB medium supplemented with 100 μ g/ml ampicillin in an Erlenmeyer flask). Shake at 37°C until the OD₆₀₀ reaches 0.6–0.8.
3. To induce the production of GST-TUBG1, add IPTG for a final concentration of 0.2 mM and continue shaking for 1 h at 37 °C.
4. Then, continue shaking the bacteria at room temperature overnight.
5. After incubation, transfer the culture to centrifuge tubes and pellet the bacteria by centrifugation (5000 \times g for 15 min at 4 °C).
6. Discard the supernatant, drain the pellet by inverting the tubes, and allow it to dry on absorbent paper.
7. Place the tube on ice.
8. Resuspend the pellet from 50 ml culture in 1 ml of cold RB by pipetting up and down until no lumps of bacteria are present in the suspension.
9. Keep the bacteria at -80 °C for at least 20 min or overnight.
10. Take up the resuspended bacteria, and thaw it on ice.
11. Lyse the resuspended bacteria (Lysate 1) in ice-cold water using 6 sonication cycles of 20 s sonication followed by 30 s of cooling. The color of the bacteria suspension changes upon lysis of the bacteria. Repeat this step with the remaining tubes
12. Clarify the lysate from the bacterial debris by centrifugation (12,000 \times g for 15 min at 4 °C). Transfer the supernatant to a new tube, and discard the pellet.
13. Resuspend the GS beads, and take 50 μ l of the slurry (25 μ l bed volume) into 2 tubes (Tubes 1 and 2) containing 1 ml of PBS each to wash away the ethanol.
14. Centrifuge Tube 1 (500 \times g for 5 min at 4 °C), and remove the supernatant.
15. Add Lysate 1 from Step 12 to the washed GS beads into Tube 1.
16. Incubate (Lysate 1 in Tube 1) under rotation for 2 h at 4 °C.
17. Centrifuge Tube 2 (500 \times g for 5 min at 4 °C), and remove the supernatant.
18. Spin down the bacteria lysate/GS bead slurry in Tube 1, and transfer the supernatant (Lysate 1) to the washed GS beads in Tube 2.
19. Incubate Lysate 1 in Tube 2 under rotation 2 h at 4 °C

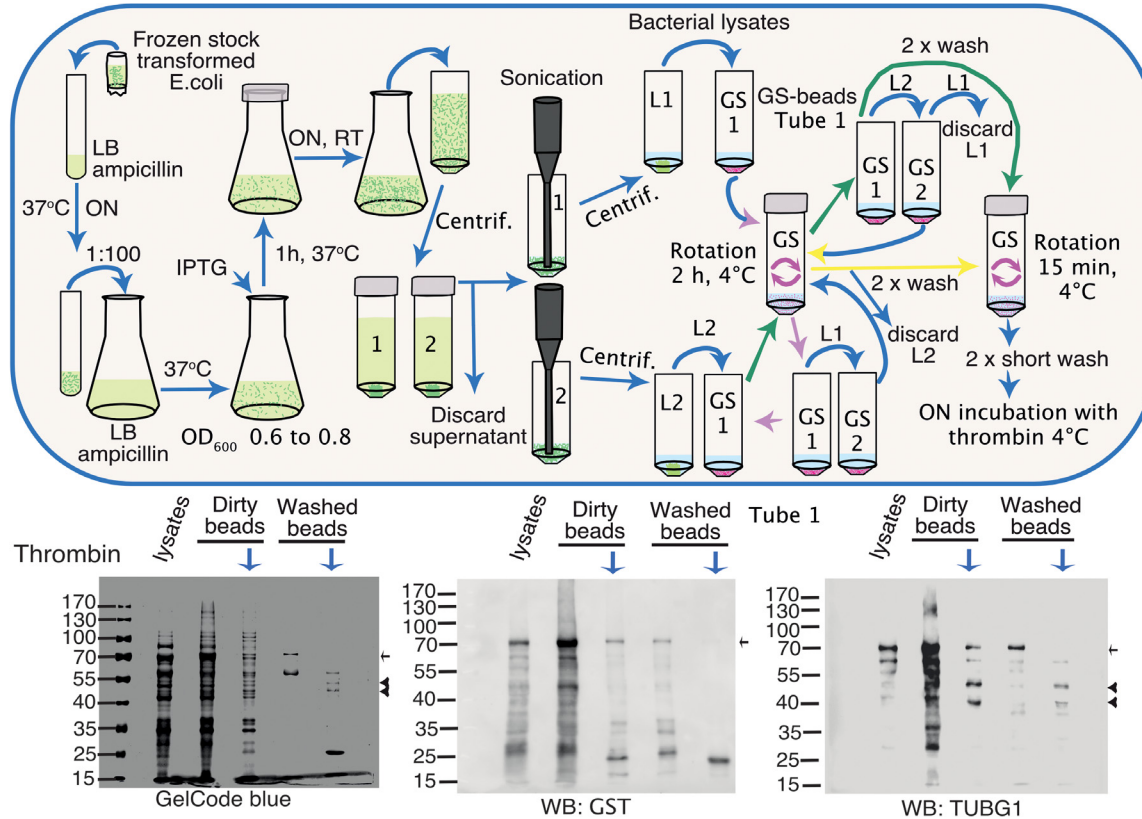


Fig. 2. Purification of GST-TUBG1 and removal of the GST tag: a scheme of the production and affinity purification of GST-TUBG from *E. coli* DH5 α carrying the pGEX2T-TUBG1 vector and using ampicillin (Amp) as a selection marker. In short, the *E. coli* colony from a pre-selected colony is cultured overnight (ON) in Luria-Bertani liquid supplemented with ampicillin. Then, the bacteria are diluted and cultured until the optical density measured at a wavelength of 600 nm (OD_{600}) reaches 0.6 to 0.8. The expression of recombinant TUBG1 is induced with 0.2 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 1 h at 37°C followed by overnight incubation at room temperature. The bacteria are harvested and lysed by sonication. After preclearing the lysates by centrifugation (centrif.), the recombinant GST-TUBG1 in the resulting bacterial lysates (lysates) is affinity purified with Glutathione Sepharose 4B beads. The GST tag is thereafter cleaved with thrombin after removal of PMSF (Dirty beads) or after extensive washing (Washed beads). The expression of recombinant TUBG1 and the efficient removal of GST were analyzed by SDS-page stained with Gelcode Blue and by Western blotting (WB) using an anti-TUBG1 antibody and anti-GST antibodies. The color of the arrows shows the sequence of events in the scheme (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

20. Thaw a new tube containing the resuspended bacteria on ice. Repeat Steps 11 and 12. Add the resulting bacteria lysate (Lysate 2) to the GS beads in Tube 1 from Step 18.
21. Incubate Lysate 2 in Tube 1 under rotation for 2 h at 4 °C.
22. Thereafter, spin down Tube 1 and Tube 2. Discard Lysate 1 from Tube 2, and transfer Lysate 2 to Tube 2.
23. Incubate Lysate 2 in Tube 2 under rotation for 2 h at 4 °C.
24. Spin down Tube 2, and discard Lysate 2. Take 5 μ l (bed volume) of the GS beads from Tube 2, and label them as dirty beads.
25. Wash the GS beads in Tubes 1 and 2 twice with 1 ml of WB under rotation for 15 min at 4 °C each time. If you want to analyze the yield of recombinant protein in the beads, go to Step 27.
26. At this point, you can resuspend the beads in FB and store the beads at -80 °C long-term.
27. To release TUBG1 from GST, take 5 μ l (bed volume) of the GS beads from one of the tubes and label them as washed beads. Take the washed and dirty beads, and wash them twice with 300 μ l PBS to remove residual PMSF from the beads by inverting the tubes up and down for 10 s each time. PMSF inhibits the proteinase activity of thrombin.
28. To remove the GST tag, add 10 μ l of thrombin (1 unit/ μ l in PBS) to 5 μ l of the washed and dirty beads (bed volume).
29. Incubate for least 16 h at 4 °C.
30. Stop the remaining proteinase activity by adding PMSF for a final concentration of 1 mM.
31. Analyze the resulting cleavage products by loading 5 μ l of the slurry into two different SDS-PAGE gels. Stain one of the gels with Gelcode Blue Stain according to the manufacturer's instructions. Transfer the second gel onto a nitrocellulose membrane, and develop the membrane using Western blot with anti-TUBG1 and anti-GST antibodies [14].
32. Once the yield is known, take the necessary amounts of frozen beads and repeat Steps 27 to 30.
33. Spin down the beads/thrombin slurry, and transfer the supernatant containing the non-tagged TUBG1 into a new tube. TUBG1 is now ready for further in vitro analysis [7].

Note that the purest fraction of recombinant TUBG1 is obtained from the washed beads (Fig. 2). For a large-scale culture, scale up the above protocol.

Additional information

Different studies describe that the correct folding of tubulin in mammalian cells depends on Tcp1 [15,16], which is an obstacle for the refolding of non-native recombinant proteins after purification from bacteria. In almost all bacteria and archaea, the protein FtsZ is the homolog of tubulins and provides the basis for correct protein folding in bacteria. With the above methodology, recombinant soluble non-tagged TUBG1 is purified from small-scale expression bacteria cultures.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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