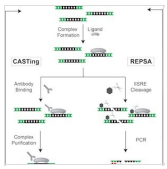

Jan 01, 2021

Expression and Purification of Thermostable Proteins Expressed in *E. coli*

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bq4jmyun

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ABSTRACT

Our laboratory studies putative transcription regulatory proteins from the extreme thermophile, *Thermus thermophilus* HB8. To do so, these proteins are often expressed in the mesothermic organism *Escherichia coli* and purified from whole-cell extracts. Here we describe our standard expression and purification scheme and its analysis using the thermostable protein His6-GFP-TEV. His6-GFP-TEV is usually used as an N-terminus tag for the bacterial expression of recombinant proteins. However, this surrogate provides a facile visual indicator of thermostable protein expression and purification and is especially amenable for laboratory instruction in a primarily undergraduate educational environment.

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GUIDELINES

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MATERIALS TEXT

Plasmid pET His6 GFP TEV LIC cloning vector (1GFP), Catalog #29663, Addgene

Recipes and instructions for bacterial culturing may be found at Addgene Protocols (https://www.addgene.org/protocols/). SOB medium recipe was from Cold Spring Harbor Protocols (http://cshprotocols.cshlp.org/content/2018/3/pdb.rec102723.full?rss=1).

Insights into protein expression using pET-based plasmids may be found in the Novagen PET System Manual TB055 (https://bit.ly/3pGgHAG).

Recipes and instructions for SDS-PAGE may be found in the Bio-Rad Bulletin_6040 (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf).

SAFETY WARNINGS

Protocol deemed safe for use in an undergraduate research environment.

ABSTRACT

Our laboratory studies putative transcription regulatory proteins from the extreme thermophile, *Thermus thermophilus* HB8. To do so, these proteins are often expressed in the mesothermic organism *Escherichia coli* and purified from whole-cell extracts. Here we describe our standard expression and purification scheme and its analysis using the thermostable protein His6-GFP-TEV. His6-GFP-TEV is usually used as an N-terminus tag for the bacterial expression of recombinant proteins. However, this surrogate provides a facile visual indicator of thermostable protein expression and purification and is especially amenable for laboratory instruction in a primarily undergraduate educational environment.

BEFORE STARTING

1. Plasmid pET His6 GFP TEV LIC cloning vector (1GFP) was a gift from Scott Gradia (Addgene plasmid # 29663). It is a pET-based vector that allows the expression of His6-GFP-TEV-tagged fusion proteins in suitable T7 RNA polymerase-containing *E. coli* strains (*e.g.*, BL21(DE3)). The His6-GFP-TEV tag provides the capability of fusion protein purification through immobilized metal affinity chromatography and enhanced protein expression and solubility through its appended optimized green fluorescent protein unit (see Pédelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and characterization of a superfolder green fluorescent protein. Nat Biotechnol. 2006 Jan;24(1):79-88. doi: 10.1038/nbt1172. Epub 2005 Dec 20. Erratum in: Nat Biotechnol. 2006 Sep;24(9):1170. PMID: 16369541). For our purposes, His6-GFP-TEV (NGFP) is stable to high-temperatures (70 °C), maintaining its solubility and fluorescent characteristics. Thus, we use this protein as a surrogate for other extreme thermophile proteins (*e.g.*, *T. thermophilus* HB8 transcriptional regulators) and as a training tool for undergraduate researchers.
2. *E. coli* BL21(DE3) was transformed with plasmid 1GFP following manufacturer's instructions (see doi: 10.17504/protocols.io.criv4d). Transformants were selected on LB agar plates containing 50 µg/mL kanamycin. Individual clones were sector streaked onto LB agar plates containing 50 µg/mL kanamycin and 1 mM IPTG and incubated overnight at room temperature to check for NGFP expression. Good expressors should be evident by their intense fluorescent green appearance. However, it is advisable to use clones from the uninduced plate for subsequent protein expression and purification, as recombinant protein overexpression can be toxic to bacteria.
3. Restreak best clones onto LB agar + 50 µg/mL kanamycin plates. Incubate overnight at 37 °C or longer at room temperature. Do not store at 4 °C.

Protein expression 8h

1 Inoculate 1 mL LB:50 µg/mL kanamycin culture in a 5 mL Falcon snap-cap tube with a ~1 cm swatch from a streak. 30m
Repeat for a second clone. Incubate tubes in a rack at 37 °C, 240 rpm, 30 min. Cultures will appear slightly cloudy when well dissociated.

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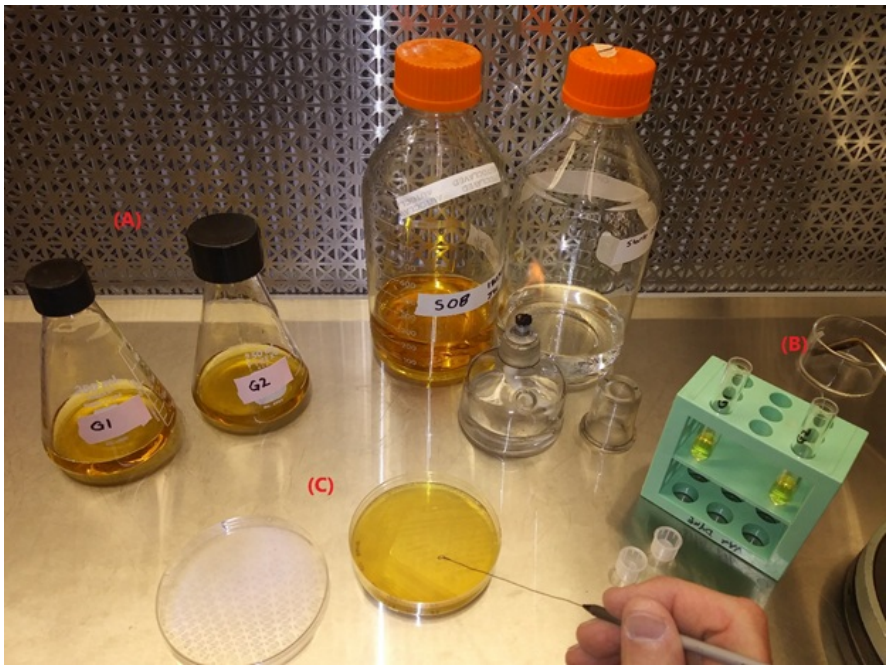

Figure 1. Workspace for bacterial culture preparation. (**A**) 250 mL flat-bottom flasks containing 50 mL SOB medium + 50 µg/mL kanamycin for mid-scale cultures. (**B**) 5 mL snap-cap Falcon tubes containing 1 mL LB medium + 50 µg/mL kanamycin for starter cultures. (**C**) Fresh LB agar + 50 µg/mL kanamycin plate with streaked BL21(DE3):1GFP clone that expresses NGFP highly.

2 Use each 1 mL culture to inoculate a 50 mL SOB:50 µg/mL kanamycin culture in a 250 mL flat-bottom flask. Culture should be relatively clear upon dilution. Incubate 37 °C, 220 rpm, for 2h. 2h

3 Cultures should become slightly cloudy. Remove 1 mL aliquot and measure absorbance at 600 nm (A_{600}). Values are typically 0.2-0.25 at this point. Continue incubation for an additional 30 min. 30m

4 Remove 1 mL aliquot and measure absorbance. A_{600} should be between 0.4-0.5. Add 500 µL 100 mM IPTG to culture and continue incubation at 37 °C, 220 rpm, for 4h. Process aliquot by transferring to 1.5 mL tube, microcentrifuging 1 min, decanting medium, resuspending with 20 µL 2x BEB (40 mM Tris-Cl [pH 7.5], 200 mM NaCl, 0.2 mM EDTA, 2 mM DTT, and 1 mM PMSF), and freezing pellet at –20 °C. The tube should be labeled with protein name, clone number, and time (*e.g.*, "NGFP G1 1330h). This is also referred to as the logarithmic growth ("log") sample. 4h

5 Cultures should have a slight yellow-green hue after 4h induction. Remove 1 mL aliquot and measure A_{600} (typically 1.8-1.9). Process aliquot as described in Step 4 above. This is also referred to as the IPTG-induced ("induced") sample. 10m

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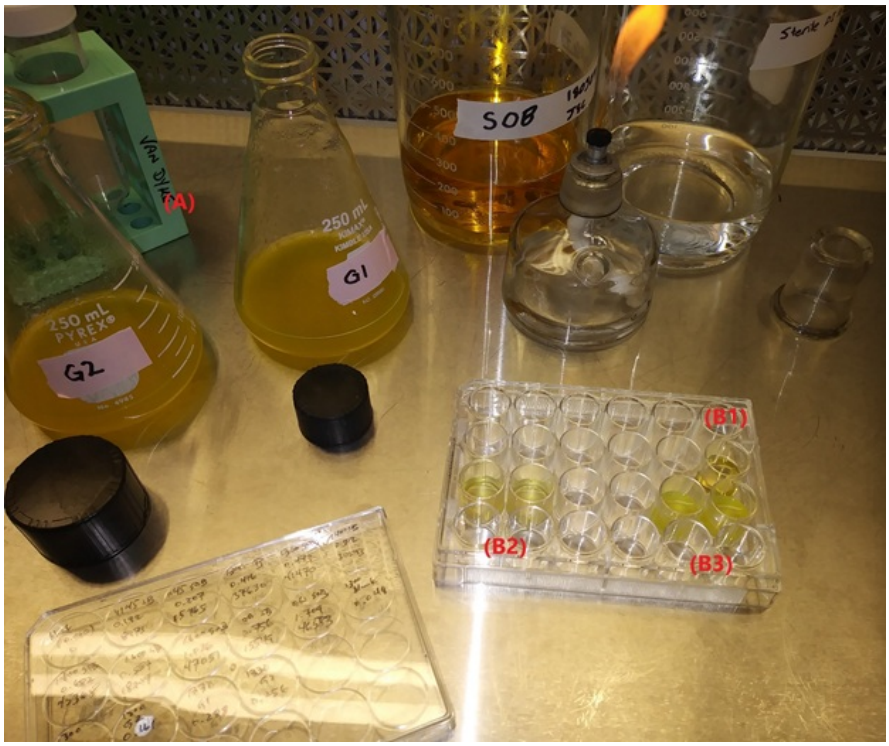

Figure 2. Assaying bacterial growth procession through A600 absorbance. (**A**) Mid-scale BL21(DE3):1GFP cultures 4h post-IPTG induction. (**B**) 24-well plate containing 1 mL bacterial culture aliquots: (**B1**) SOB blank, (**B2**) T +2.5h, logarithmic growth, (**B3**) T +7.0h, 4h post-IPTG induction.

6 Transfer remaining culture equally into two 50 mL conical screw-cap polypropylene centrifuge tubes. Centrifuge tubes at 4000 rpm for 10 min at 4 °C. Decant supernatant carefully, as pellets may be loose. Resuspend each pellet with 500 µL 2x BEB by mild vortexing. Pool and quick freeze (liquid N_2) before storage at –20 °C. 20m

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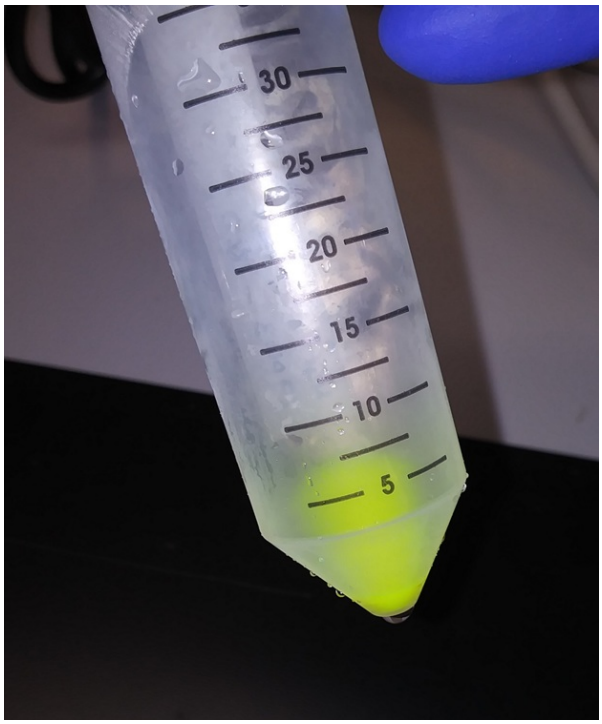

Figure 3. Bacterial pellet from 25 mL bacterial culture, 4h post-IPTG induction. Yellow-green fluorescence is from expressed NGFP protein.

Protein purification 2h 5m

7 Thaw frozen pellets on ice. Measure volume (in this example, 1250 µL) and transfer to a 2 mL microcentrifuge tube. Save 100 µL aliquot of freeze/thawed sample ("F/T"). To the remainder, add 0.02x volume (23 µL) 10 µg/µL freshly prepared lysozyme and mix by inversion. Incubate 10 min on ice, mixing every 5 min. 20m

8 Using a microprobe, sonicate at minimum power (2.5) for 10 sec., then chill on ice for 50 sec. Repeat a total of five times. Note that clarification of the suspension is apparent after the third burst. If sudsing occurs, microfuge briefly (4 sec) to reduce before sonicating again. 10m

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Figure 4. Disruption of lysozyme-treated IPTG-induced BL21(DE3):1GFP cells.

9 Microcentrifuge samples for 10 min at 4 °C. Transfer supernatant to a 1.5 mL microcentrifuge tube. Measure volume 15m
(1100 µL). The supernatant should be clear and intensely yellow-green; pellet, relatively small (20 µL) and slightly green, indicating efficient bacterial lysis and release of soluble NGFP. Save 100 µL aliquot of lysis/soluble sample ("L/S").

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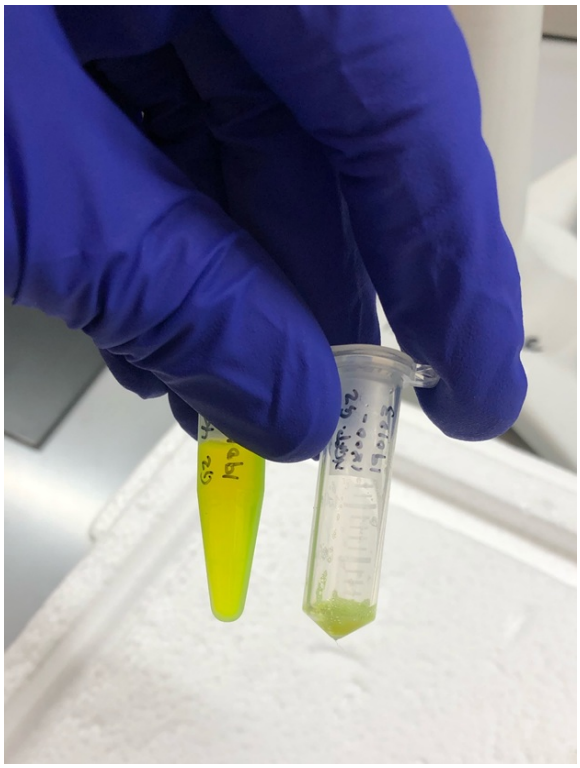

Figure 5. NGFP purification, post-lysis and microcentrifugation. (Left) Supernatant; (right) pellet.

10 Place samples into a 70 °C hot block. Remember to add a small amount of water to each well to facilitate good heat transfer. Incubate 15 min with samples mixed by inversion after 8 min. Samples will become cloudy due to the denaturation of *E. coli* proteins. An intense yellow-green color should be retained. 20m

11 Microcentrifuge samples for 15 min at 4 °C. Transfer supernatant to a 1.5 mL microcentrifuge tube. Measure volume (900 µL). The supernatant should be intensely yellow-green; pellet, larger (100 µL) and beige in color, indicating efficient *E. coli* protein denaturation and soluble NGFP protein recovery. Save 100 µL aliquot of the heat-treated sample ("Δ"). 20m

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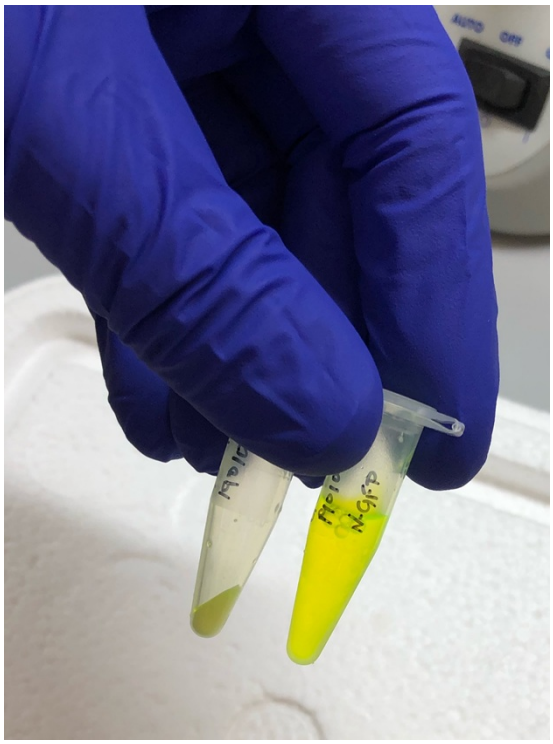

Figure 6. NGFP purification, post-heat-treatment and microcentrifugation. (Left) pellet; (right) supernatant.

12 NGFP can be stored frozen at –20 °C. To avoid repeated freeze/thaw cycles, NGFP can be diluted with an equal volume of glycerol and mixed by inversion, then gentle rocking for 30 min at 4 °C before storage at –20 °C. A 1x BEB:50% glycerol solution will not freeze under these conditions. 40m

Protein analysis: Fluorescence

13 NGFP is a highly fluorescent protein. Thus, fluorescence can be used to measure recovery at each step during purification.

14 Dilute 1 µL each sample with 200 µL 2x BEB in a 0.45 mL Corning Axygen super-clear microfuge tube. The control contains only 2x BEB. Remember to label only the tube caps.

15 Fluorescence assays using an Invitrogen Qubit 3 fluorometer:

1. Turn on the Qubit 3 fluorometer.
2. From Home Screen, choose “Fluorometer” mode.
3. Next, choose Blue [470 nm] excitation.
4. Insert sample and close lid.
5. Press “Read tube.”
6. Record green emission [510 – 580 nm] value shown.
7. Remove the tube and insert a new sample. Repeat steps 15.4 – 15.7.

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A	B
Sample	Qubit Fluorescence
2xBEB (blank)	29.32
1330h (log)	64.03
1800h (induced)	109729.28
F/T	131246.60
L/S	147702.10
Δ	143622.60

Table 1. NGFP Expression and Purification: Fluorescence.

Results: Induction was substantial. Fluorescence values increased slightly with purification. Fluorescence recovery was near quantitative through purification.

Protein analysis: SDS-PAGE

17 Our laboratory routinely performs SDS-PAGE analyses using homemade 12% Mini-Protean SDS-PAGE gels and associated reagents. Recipes and instructions may be found in the Bio-Rad Bulletin_6040 (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf).

18 Dilute 2 µL each sample with 20 µL 1x Laemmli sample buffer + BME in a 0.45 mL microfuge tube. Heat sample at 95 °C for 10 min (unless otherwise noted), minispin briefly to consolidate sample, then keep at room temperature until needed.

19 SDS-PAGE analysis using a Bio-Rad Mini-Protean gel system:

1. Assemble gel in electrophoresis cell.
2. Fill inner and outer chambers with 1x Tris-glycine/SDS running buffer.
3. Slowly, load 20 µL of the sample into gel well. Samples containing whole bacteria (*e.g.*, “log” and “induced”) may exhibit properties (viscosity, stringiness) that make them more difficult. Repeated pipetting before loading may be beneficial.
4. Electrophorese at 50 V for 20 min, then 200 V until bromophenol blue indicator dye band is near the bottom of the gel (60 min total).
5. Disassemble electrophoresis cell, then gel.
6. To image native NGFP directly, we use a GelDoc EZ Imaging System, Blue tray, and the fluorescein option. Details may be found at https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10019683.pdf.
7. Afterward, the gel is stained with Coomassie Brilliant Blue R250 and destained until the background is clear. Imaging is performed with a GelDoc EZ Imaging System, White light tray, and the Coomassie option.

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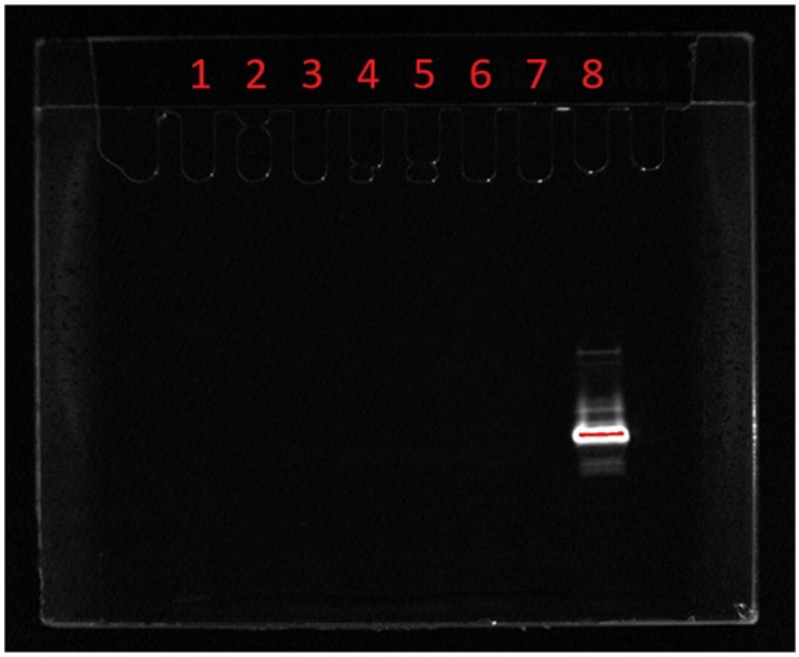

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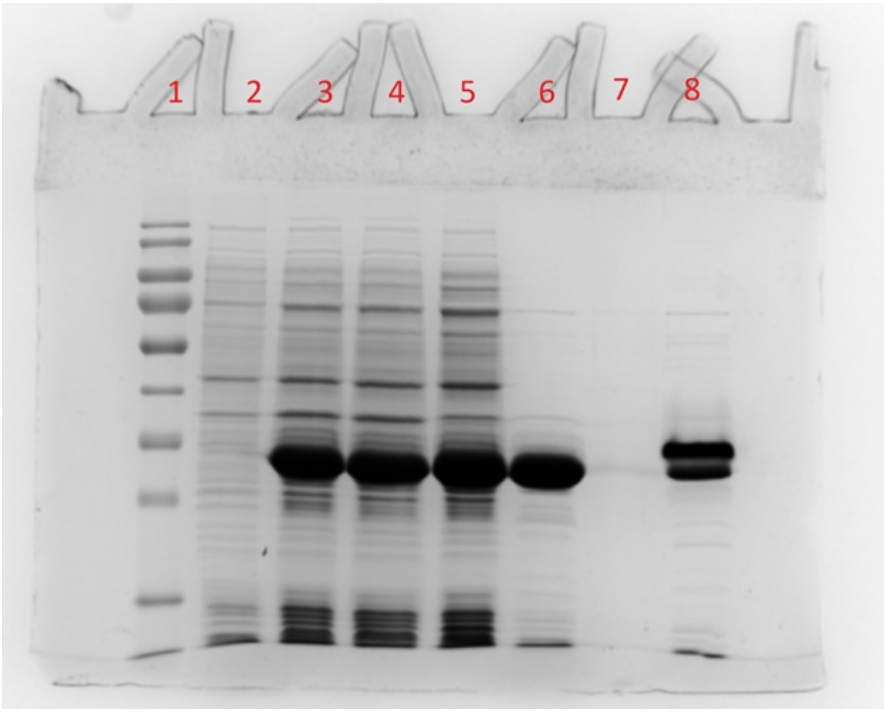

Figure 7. SDS-PAGE analysis of NGFP purification. (**Top**) Blue light plate, fluorescence; (**bottom**) white light plate, Coomassie-stained proteins. Lanes: (**1**) ThermoFisher PageRuler Prestained Protein Ladder (2.5 µL). Marker protein molecular masses: 10, 15, 25, 35, 40, 55, 70, 100, 130, and 180-kDa. (**2**) T +3.0h BL21(DE3):1GFP culture, logarithmic growth. (**3**) T +7.0h culture, 4h post-IPTG induction. (**4**) Freeze-thawed BL21(DE3):1GFP bacterial extract, post-induction, "F/T." (**5**) Supernatant, post-lysis and microcentrifugation, "L/S". (**6**) Supernatant, post-heat-treatment and microcentrifugation, "Δ." (**7**) Blank. (**8**) As in (5), except that the sample was not heat-treated at 95 °C for 10 min.

Results: NGFP is a 266 amino acid protein with a molecular mass of 29.8 kDa. NGFP induction was substantial, constituting the majority of bacterial protein. NGFP recovery was near quantitative through purification. Final purity was estimated at > 95 % with an expected yield of > 12 mg NGFP protein/50 mL bacterial culture. Native NGFP exhibited a slightly reduced mobility in SDS-PAGE and could be directly visualized through its fluorescence.

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