Expression and purification of recombinant Bsu polymerase V.1

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We use this protocol and it's working

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ABSTRACT

The DNA polymerase I or *Bsu* is an enzyme from the Gram (+) bacteria *Bacillus subtilis.*
The *Bsu* is used as part of an isothermal DNA amplification based on the recombination process, the RPA reaction.
RPA uses 4 enzymes: UvsX, UvsY, Bsu and Gp32. It's an isothermal amplification technique that can run at 37°C. In this protocol we are producing a recombinant *Bsu* that has a 6xHIS-tag using a *E. coli* expression system.
The protocols for the production of the other proteins are also available in protocols.io.

MATERIALS

Binding buffer, pH 7.9

[M] 50 millimolar (mM) Tris-HCl, pH 7.9
[M] 20 millimolar (mM) Imidazole, pH 7.9
[M] 1 Molarity (M) KCl
[M] 5 % (v/v) Glycerol
[M] 0.025 millimolar (mM) PMSF
[M] 0.01 % (v/v) 2-Mercaptoethanol (BME)

Buffer with lower [KCl], pH 7.9

[M] 50 millimolar (mM) Tris-HCl, pH 7.9
[M] 20 millimolar (mM) Imidazole, pH 7.9
[M] 100 millimolar (mM) KCl
[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) BME

Elution buffer (for resin purification), pH 7.9

[M] 50 millimolar (mM) Tris-HCl, pH 7.9

[M] 150 millimolar (mM) Imidazole, pH 7.9
[M] 100 millimolar (mM) KCl
[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) BME

Elution buffer (for FPLC purification), pH 7.9
[M] 50 millimolar (mM) Tris-HCl, pH 7.9
[M] 500 millimolar (mM) Imidazole, pH 7.9
[M] 100 millimolar (mM) KCl
[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) BME

Storage buffer pH 7.4
[M] 25 millimolar (mM) Tris-HCl, pH 7.4
[M] 50 millimolar (mM) NaCl
[M] 1 millimolar (mM) DTT
[M] 0.1 millimolar (mM) EDTA

Ladder:
Pageruler Prestained Protein Ladder **Thermo Fisher Scientific Catalog #26616**

Equipment:
Sonicator OMNI Sonic Ruptor 400
Protein purification system FPLC AKTA START

DAY1: Transformation of competent cells

1d

1 Quantify the plasmid containing the Bsu DNA polymerase gene and determine the volume that contains [100 ng] of the plasmid.

2 Defrost the aliquot of BL21(DE3) chemically competent cells [On ice]. Softly pipette [100 ng] of the plasmid in the aliquot and let the tube rest [On ice] for [00:30:00]. 30m

3 Incubate the tube at 42 °C for 00:00:30. 30s

4 Quickly return the tube On ice and incubate for 00:05:00. 5m

5 Add the mixture to a microcentrifuge tube with 800 µL SOC medium or LB broth and incubate at 37 °C for 00:45:00 45m

6 Centrifuge the tube 4500 rpm, Room temperature, 00:08:00. 8m

7 Discard 800 µL of the supernatant and gently resuspend the pellet with the remaining supernatant.

8 Add the resuspension to an LB agar plate previously supplemented with [M] 0.05 mg/mL Kanamycin and spread the recently transformed cells. Incubate plate Overnight at 37 °C.

DAY2: Preparation of pre-inoculum 1d

9 For verification that the colonies in the plate contain the desired plasmid with the protein sequence, perform a PCR colonyusing universal T7 primers and the PCR protocol for Phusion DNA Polymerase

Phusion High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0530L**

. Use the following thermocycling procedures for the *Bsu* polymerase plasmid:

Step	Temperature (C°)	Time	Cycles
Initial denaturation	98	3 min	1
Denaturation	98	30 sec	25
Annealing	60	30 sec	
Extension	72	58 sec	
Final Extension	72	5 min	1
Hold	4	∞	

Run the PCR product in a 1% agarose gel and verify if there is a band in the desired weight (Bsu insert = 1949 bp).

10 Select an isolated bacterial colony from the plate and inoculate a test tube with 10 mL LB medium and [M] 0.05 mg/mL Kanamycin. Incubate the tube Overnight at 220 rpm, 37°C.

DAY 3-A: Protein expression in small scale

2d

11 Inoculate 50 µL from the pre-inoculum to an Erlenmeyer flasks with 50 mL LB medium (ratio 100:1) and [M] 0.05 mg/mL Kanamycin. Incubate at 220 rpm, 37°C until OD_{600} = 0.5 - 0.6 (3-4 hours).

12 Add IPTG to a final concentration of [M] 0.5 millimolar (mM) and incubate 20:00:00 at 220 rpm, 18°C. 20h

13 Centrifuge the cell culture 8000 rpm, 4°C, 00:05:00. Discard the supernatant. At this point, you may store the cells pellet at -20°C until you are ready to run the purification. 5m

DAY 4-A:Protein purification in resin

14 Resuspend the cell pellet in 5 mL Binding buffer. Then add lysozyme to a final concentration of [M] 0.1 µg/µL.

15 Incubate the cells at 220 rpm, Room temperature , 00:20:00 and add 10% SDS to a final concentration of 0.02%.

16 Add ~ 100 µL of glass beads and shake vigorously in a vortex for 00:20:00 at room temperature. You can do this by fixing a 15 mL tube to the vortex rubber platform with tape. 20m

17 Centrifugate at 13500 rpm, 4°C, 00:07:00. Collect the supernatant and label it as a Soluble fraction. The pellet is the Insoluble fraction. Collect small fractions of each one to run an acrylamide gel afterwards.

18 **Prepare the resin**. Homogenize resin with its storage buffer by shaking the bottle and transfer it to a new tube. You will use 330 µL of resin for each 1 mL of soluble fraction. Let the slurry sediment or spin it down. Remove the storage buffer and wash the resin in **Binding buffer.** Wash the resin with the same volume as the obtained soluble fraction. Repeat this wash step 3 times.

19 Add the soluble fraction to the resin. Homogenize the mixture gently in an orbital shaker for 20 min (~60 RPM) at room temperature.

20 Let the resin sediment for 10 minutes. Collect a small fraction of the supernatant to run an acrylamide gel afterwards, and discard the remainder. Resuspend resin with 1 mL of **Binding buffer.** Homogenize the tube gently with finger taps. Don't flip the tube (1st washing step).

21 Spin down for a few seconds and discard supernatant. Resuspend resin with 1 mL of **Binding buffer.** Homogenize the tube gently with finger taps. Don't flip the tube (2nd washing step).

22 Spin down for a few seconds and discard supernatant. Resuspend resin with 1 mL of **Elution buffer (150mM Imidazole).** Homogenize the tube gently with finger taps. Don't flip the tube. Incubate for 00:10:00 . 10m

23 Spin down for a few seconds and collect the supernatant. Resuspend resin with 1 mL of **Elution buffer (500mM Imidazole).** Homogenize the tube gently with finger taps. Incubate for 00:10:00. Collect small fractions of elutions to run an acrylamide gel afterwards. 10m

Run a 12% acrylamide gel at 200 V to evaluate all the samples you just generated:Lysis sample, Soluble fraction, Insoluble fraction, Flowthrough, 1st washing step, 2nd washing step and Eluted fraction.

DAY 3-B: Protein expression in medium scale 2d

24 Inoculate 2.5 mL from the pre-inoculum to an Erlenmeyer flasks with 250 mL LB medium and [M] 0.05 mg/mL Kanamycin, use 4 flasks to obtain 1L of cell culture. Incubate at 220 rpm, 37°C until OD_{600} = 0.5 - 0.6 (3-4 hours).

25 Add IPTG to a final concentration of [M] 0.5 millimolar (mM) to each flask and incubate at 220 rpm, 18°C, 16:00:00.

26 Centrifuge the cell culture 4000 rpm, 4°C, 00:20:00. Discard the supernatant. At this point, you may store the 1-2 grams of cell pellet at -20°C until you are ready to run the purification. 20m

DAY 4-B: Cells Lysis 1d

27 Resuspend all the cell pellets (from a total of 1 L of culture) in 100 mL Binding buffer. Add PMSF to a final concentration of [M] 0.1 millimolar (mM). Add lysozyme to a final concentration of [M] 0.1 µg/µL.

28 Incubate the cells on an orbital shaker at 220 rpm, Room temperature , 00:20:00.

29 Sonicate on ice until the lysate turns translucid. Use 5 cycles of 00:15:00 power ON, pulse 10. Then 00:15:00 power OFF, with the tube on ice. 30m

30 Centrifuge 6000 rpm, 4°C, 00:20:00 to separate the insoluble fraction (pellet) from the soluble fraction. Transfer the soluble fraction to a new and clean tube on ice. Collect small fractions of each one to run an acrylamide gel afterwards. 20m

DAY 4-B: Protein Purification with FPLC

31 Prepare the 5 mL HisTrap column in the FPLC system. Wash the tubes, pumps system and the column with 7 column volumes (c.v.) of distilled and filtrated water. Then equilibrate the column with 7 c.v. of **Binding buffer**.

32 Load the soluble fraction to the FPLC system at a flow of 1 mL/min. Collect a small fraction of each step and signal change to run an acrylamide gel afterwards. Wash the column with 5 c.v. of **Binding buffer**, until the UV and conductivity signal stabilizes. Then load the **Buffer 100mM KCl** at 2 mL/min to reduce the salt concentration until the UV and conductivity signal stabilizes (5-7 c.v).

33 **Elution:** Load the column with 27% of pump B (**Elution Buffer**), which is equivalent to ~150 mM Imidazole, until the signal stabilizes.

Start collecting the elution in 8 mL tube fractions immediately after the UV signal increases. After approximately 40 mL, the UV signal will stabilize at a low value. Then load the column with 3 c.v. of 100% of pump B (**Elution Buffer**), which is equivalent to 500 mM Imidazole, until the signal stabilizes again.

34 **Wash the column for storage.** Load the column with **Buffer 100mM KCl** at 2 mL/min 5 c.v. Wash the FPLC system with distilled and filtrated water. Load the column with 7 c.v of distilled and filtrated water. To storage the column, load it with 5 c.v. of **ethanol 20%** and storage it at 4°C. Finally, remove the rest of the water from the system with **ethanol 20%** and keep the system with it until next use.

35 Determine the fractions with the Bsu polymserase by running a SDS-PAGE in a 8% acrylamide gel. The Bsu polymerase weights ~66.9 kDa.

36 Concentrate the eluted fractions with the protein with an Amicon Ultra-15 Centrifugal Filter Unit **Emd Millipore Catalog #UFC910024** **10kDa**. Reconstitute the concentrate so it is stored with the components detailed in **Storage Buffer** and decrease the Imidazol to 20 mM or less. Add glycerol to a 20%, homogenize, make aliquots of

400 µL of the protein and storage them at -80°C.