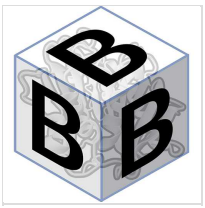

2

Nov 05, 2021

Recombinant protein expression and purification of codon-optimized Pfu-Sso7d V.2

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dx.doi.org/10.17504/protocols.io.bzusp6we

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This protocol has been optimized for the recombinant expression of a codon-optizimed Pfu-Sso7d DNA polymerase. This is a fusion protein composed of the Pfu enzyme from *Pyrococcus furiosus* for DNA amplification by PCR fused to a small 7 kDa protein from *Sulfobulus solfataricus* that binds to double-stranded DNA without any preference for specific sequences, thus enhancing polymerization processivity without affecting the catalytic activity or thermal stability of the enzyme.

The goal of this protocol was to eliminate the use of large volumes for dyalisis and potential issues with the protein crashing out of the solution due to the use of concentrators for buffer exchange of this enzyme into storage conditions. We also eliminated the use of DTT, which is often found in other similar protocols.

The sequence plasmid encoding the codon-optimized Pfu-Sso7d enzyme used here can be found at https://benchling.com/s/seq-2TcUPjO2uMbDG5ufTQN4

DOI

dx.doi.org/10.17504/protocols.io.bzusp6we

Maira Rivera, Javiera Reyes, Paula Blazquez-Sanchez, Cesar A Ramirez-Sarmiento 2021. Recombinant protein expression and purification of codon-optimized Pfu-Sso7d . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bzusp6we Cesar A Ramirez-Sarmiento

ANID Millennium Science Initiative Program
Grant ID: ICN17_022

ANID CONCYTEC
Grant ID: covbio0012

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Nov 05, 2021

Nov 05, 2021

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MATERIALS

Sodium phosphate monobasic monohydrate **Sigma Aldrich Catalog #S9638**

PMSF **Sigma Aldrich Catalog #P7626**

Sodium phosphate dibasic **Sigma Aldrich Catalog #7558-79-4**

Imidazole **Sigma Catalog #I5513**

NaCl **Sigma Aldrich Catalog #53014**

HiTrap Heparin HP affinity column **Ge Life Sciences Catalog #17040701**

HisTrap FF Crude Column **Ge Healthcare Catalog #17528601**

Dextrose **Sigma – Aldrich Catalog #D9434**

Nonidet P40 Substitute **Sigma Aldrich Catalog # 74385**

EDTA **Sigma Aldrich Catalog #ED2SS**

Buffer A, pH 8.0

[M] **50 millimolar (mM)** NaPO4, pH 8.0

[M] **50 millimolar (mM)** dextrose

[M]**300 millimolar (mM)** NaCl

[M]**1 millimolar (mM)** EDTA

[M]**0.1 % volume** Nonidet P-40

[M]**40 millimolar (mM)** Imidazole, pH 8.0

Buffer B, pH 8.0

[M]**50 millimolar (mM)** NaPO4, pH 8.0

[M]**50 millimolar (mM)** dextrose

[M]**300 millimolar (mM)** NaCl

[M]**1 millimolar (mM)** EDTA

[M]**0.1 % volume** Nonidet P-40

[M]**150 millimolar (mM)** Imidazole, pH 8.0

Buffer C , pH 8.0

[M]**50 millimolar (mM)** NaPO4, pH 8.0

[M]**50 millimolar (mM)** dextrose

[M]**300 millimolar (mM)** NaCl

[M]**1 millimolar (mM)** EDTA

[M]**0.1 % volume** Nonidet P-40

[M]**500 millimolar (mM)** Imidazole, pH 8.0

Buffer HA, pH 8.0

[M]**50 millimolar (mM)** Tris-HCl, pH 8.0

[M]**100 millimolar (mM)** NaCl

[M]**0.1 % volume** Nonidet P-40

Buffer HB, pH 8.0

[M]**50 millimolar (mM)** Tris-HCl, pH 8.0

[M]**2000 millimolar (mM)** NaCl

[M]**0.1 % volume** Nonidet P-40

DAY 1 – Plasmid transformation 1d

1 Transform **100 ng** of plasmid containing codon-optimized into *E. coli* C41 competent cells using either heat shock or electroporation. 2h

2 Spread transformed cells in LB Agar plates supplemented with [M]**0.05 mg/mL** Kan. Grow 12h

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plate overnight at **37 °C**

DAY 2 – Preinoculum 1d

3 Select a single colony from the LB agar plate to prepare a preinoculum in **10 mL** LB media supplemented with **0.05 mg/mL** Kan. Grow overnight at **250 rpm, 37°C** 1d

DAY 3 – Protein Overexpression 1d

4 Use the full volume of the preinoculum to inoculate **1 L** of LB (or TB) media supplemented with **0.05 mg/mL** Kan (1% inoculation). Grow at **160 rpm, 37°C** until reaching an optical density at 600 nm (OD_{600}) = 0.8 4h

5 Upon reaching OD_{600} = 0.8, add IPTG to a final concentration of **0.5 millimolar (mM)** and incubate overnight at **160 rpm, 18°C** 16h

DAY 4A – Protein Purification by IMAC 4h

6 Centrifuge the cell culture **4000 x g, 4°C, 00:20:00** .Then, resuspend the cell pellet in **50 mL** of **Buffer A** freshly supplemented with **0.5 millimolar (mM)** PMSF and **0.2 mg/mL** lysozyme. 30m

7 Incubate the resuspended cells at **80 rpm, Room temperature , 00:20:00** 30m

8 Sonicate on ice for **00:04:00** using cycles of **00:00:01** ON and **00:00:04** OFF at 40% amplitude (Qsonica Q125, 125W). 10m

9 On an ultracentrifugation tube, Incubate the unclarified lysate at **70 °C** for **00:30:00** to precipitate most of *E. coli* proteins, and then place on ice for **00:05:00** . Centrifuge **20000 x g, 4°C, 00:20:00** and collect the supernatant. You might want to collect a small sample for SDS-PAGE afterwards. 1h

10 On a **1 mL HisTrap column** preequilibrated with 10 column volumes (c.v.) (here, 10 mL) of **Buffer A**, load the supernant.
Wash with 10-20 c.v. of **Buffer A.**
Then, elute with 5 c.v. of **Buffer B**, collecting the eluted fractions every **0.5 mL** in 1.5 ml tubes. 1h

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11 To quickly pool the fractions containing the protein of interest, prepare a 96-well plate or 1.5 mL tubes with **40 µL** of Bradford reagent and **150 µL** of distilled water. Then, add **10 µL** of each protein fraction and compare against a blank reference sample corresponding to **10 µL** of **Buffer B**. You can determine your protein-containing fractions either by absorbance at 595 nm on a plate reader or visually by comparing the blue coloration of each fraction against the blank reference. Pool your fractions and collect a **10 µL** sample for SDS-PAGE 5m

DAY 4B – Second purification and buffer exchange by Heparin 2h

12 This method was preferred over protein dyalisis or Amicon protein concentration to avoid using large buffer volumes and proteins crashing out of the solution. 5m

Dilute the pooled fractions 3X in 50 mM Tris-HCl pH 8.0, such that the final concentration of NaCl is 100 mM.

13 Next, load the diluted sample onto a **1 ml HiTrap Heparin column** previously equilibrated with 10 c.v. (here, 10 mL) of **Buffer HA.** 30m
Then, elute the protein using a 10 c.v. linear gradient against **Buffer HB**, collecting the eluted fractions every **1 mL** in 1.5 mL tubes. The protein will elute at high concentrations between 300 and 600 mM NaCl

This linear gradient can be achieved by connecting two containers, one with 5 c.v. **Buffer HA** and the other with 5 c.v. **Buffer HB**, with a syphon or a tube, and withdrawing solution from **Buffer HA** container to the column using a cheap peristaltic pump or by gravity.

14 Again, determine your protein-containing fractions using the Bradford assay. Pool your fractions and determine its protein concentration using the same method and collect a **10 µL** sample for SDS-PAGE. 5m

15 For storage, supplement your pooled fraction with **0.2 % volume** Nonidet P-40 and **0.2 millimolar (mM)** EDTA. Then, dilute the sample by adding glycerol up to **50 % volume** to reach final storage conditions: **25 mM Tris-HCl pH 8.0, ~250 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 50% glycerol.** 5m

16 Generate **200 µL** aliquots of the enzyme and store it at **-20 °C** until required. Usual final protein concentrations for storage are between **0.2 mg/mL** and **0.6 mg/mL** 30m

SDS-PAGE Result

17

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The unlabelled lane corresponds to the PageRuler protein ladder. His = Pooled fraction from HisTrap IMAC column (20 µl), 1-2-3 correspond to eluted fractions from Heparin column (5 µl).

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