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Recombinant protein expression and purification of codon-optimized Pfu-Sso7d V.1

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Protocol status: Working
This protocol was used to purify Pfu-Sso7d in triplicates. PCR tests demonstrated the enzymes were active as expected.

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Abstract

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 plasmid encoding the codon-optimized Pfu-Sso7d enzyme used here can be found on reclone.org

Materials

MATERIALS

- Sodium phosphate monobasic monohydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S9638
- PMSF **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P7626
- Sodium phosphate dibasic **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**7558-79-4
- Imidazole **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I5513
- NaCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**53014
- HiTrap Heparin HP affinity column **GE Healthcare Catalog #**17040701
- HisTrap FF Crude Column **GE Healthcare Catalog #**17528601
- Dextrose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D9434
- Nonidet P40 Substitute **Merck MilliporeSigma (Sigma-Aldrich) Catalog #** 74385
- EDTA **Merck MilliporeSigma (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 plate overnight at 37 °C 12h

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 [M] 0.05 mg/mL Kan. Grow overnight at 37 °C shaking at 250 rpm. 1d

DAY 3 – Protein Overexpression

1d

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

5 Upon reaching OD_{600} = 0.8, add [M] 0.5 millimolar (mM) IPTG and incubate overnight at 18 °C shaking at 160 rpm. 16h

DAY 4A – Protein Purification by IMAC

3h

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 [M] 0.5 millimolar (mM) PMSF and [M] 0.2 mg/mL lysozyme. 30m

7 Sonicate on ice for 00:04:00 using cycles of 00:00:01 ON and 00:00:04 OFF at 40% amplitude. 5m

8 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

9 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

You can regenerate your resin for another purification by washing with 10 c.v. buffer C (500 mM imidazole), 10 c.v. water, 10 c.v. stripping buffer (20 mM sodium phosphate buffer pH 8.0, 500 mM NaCl, 50 mM EDTA), 10 c.v. buffer A, 10 c.v. water, 0.5 c.v. 100 mM $NiSO_4$, 5 c.v. water and 5 c.v. 20% EtOH for storage.

10 To quickly pool the fractions containing the protein of interest, prepare an ELISA plate or 1.5 mL tubes with 40 µL of Bradford reagent and 160 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 1h

11 This method was preferred over protein dyalisis or Amicon protein concentration to avoid using large buffer volumes and proteins crashing out of the solution. 30m

Dilute the pooled fractions 3X in 50 mM Tris-HCl pH 8.0, such that the final concentration of NaCl is 100 mM. Then, load onto a **1 ml HiTrap Heparin column** previously equilibrated with 10 c.v. (here, 10 mL) **buffer HA.**
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

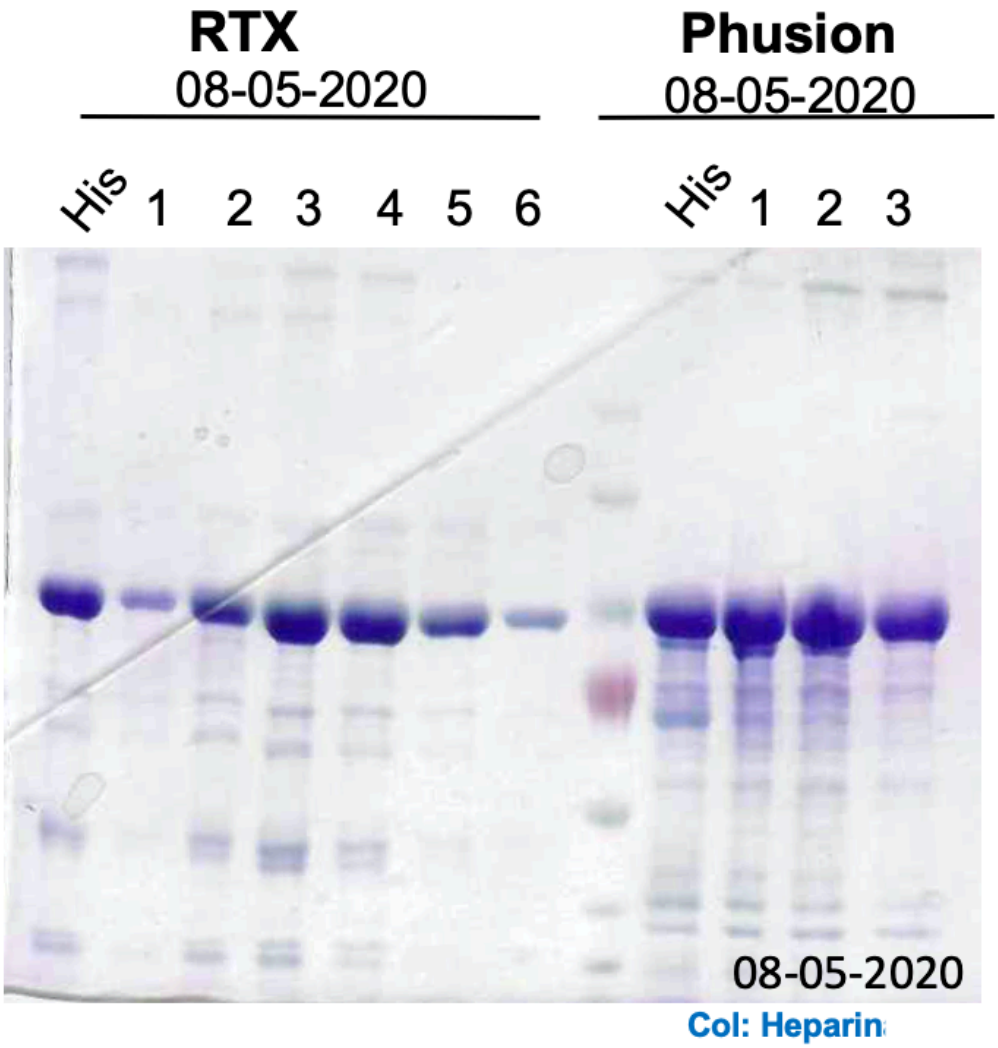
This linear gradient can be achieved by connecting two containers, one with 5 c.v. **buffer HA** and the other with 5 c.v. **buffer HB**, with a syphon or a tube, and withdrawing solution from the **buffer HA** container to the column using a cheap peristaltic pump or by gravity. (...Alternatively you could use a step gradient, but we have not tried it).

12 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. 10m

For storage, supplement your pooled fraction with [M] 0.2 % volume Nonidet P-40 and [M] 0.2 millimolar (mM) EDTA. Then, dilute the sample in an equivalent volume of 100% glycerol to achieve the final storage conditions: **25 mM Tris-HCl pH 8.0, ~250 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 50% glycerol.**

With this protocol, our usual final protein concentrations for storage are between [M] 0.2 mg/mL and [M] 0.6 mg/mL

Expected result



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).