Jun 10, 2020

Recombinant expression and purification of codon-optimized M-MLV and Mashup

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2 *Works for me* This protocol is published without a DOI.

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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

PROTOCOL CITATION

Maira Rivera, Javiera Reyes, Paula Blazquez-Sanchez, Cesar A Ramirez-Sarmiento 2020. Recombinant expression and purification of codon-optimized M-MLV and Mashup. **protocols.io** https://protocols.io/view/recombinant-expression-and-purification-of-codon-o-bhcvj2w6

CREATED

Jun 09, 2020

LAST MODIFIED

Jun 10, 2020

PROTOCOL INTEGER ID

38005

MATERIALS

NAME	CATALOG #	VENDOR
Sodium phosphate monobasic monohydrate	S9638	Sigma Aldrich
PMSF	P7626	Sigma Aldrich
Sodium phosphate dibasic	7558-79-4	Sigma Aldrich
DTT	D0632	Sigma Aldrich
Imidazole	I5513	Sigma
NaCl	53014	Sigma Aldrich
HiTrap Heparin HP affinity column	17040701	Ge Life Sciences
HisTrap FF Crude Column	17528601	Ge Healthcare

NAME	CATALOG #	VENDOR
Glycerol	G5516	Sigma Aldrich
Dextrose	D9434	Sigma – Aldrich
Tween-20	P9416	Sigma Aldrich
Nonidet P40 Substitute	74385	Sigma Aldrich
EDTA	ED2SS	Sigma Aldrich

MATERIALS TEXT

Buffer A, pH 8.0

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

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

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

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

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

[M]**0.1 % volume** Tween-20

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

Buffer B, pH 8.0

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

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

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

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

[M]**0.1 % volume** Tween-20

[M]**10 % volume** Glycerol

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

Buffer C , pH 8.0

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

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

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

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

[M]**0.1 % volume** Tween-20

[M]**10 % volume** Glycerol

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

Buffer HA, pH 8.0

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

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

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

Buffer HB, pH 8.0

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

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

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

DAY 1 – Plasmid transformation 1d

1 Transform 100 ng of plasmid containing codon-optimized M-MLV or Mashup into *E. coli* BL21(DE3) competent cells 2h using either heat shock or electroporation.

2 Spread transformed cells in LB Agar plates supplemented with [M]**0.05 mg/ml Kan** for **Mashup**, or [M]**0.1 mg/ml Amp** for **M-MLV** . 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** for **Mashup** or [M]**0.1 mg/ml Amp** for **M-MLV**. 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** of LB media supplemented with [M]**0.05 mg/ml Kan** for **Mashup** or [M]**0.1 mg/ml Amp** for **M-MLV** (1% inoculation). Grow at **37 °C** shaking at 200 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 Milimolar (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 Milimolar (mM)** PMSF and [M]**0.2 mg/ml** lysozyme. Incubate for **00:30:00** at **Room temperature** shaking at 80 rpm. 1h

7 Sonicate on ice for **00:04:00** using cycles of **00:00:01** ON and **00:00:06** OFF at 40% amplitude. Then, 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 30m

8 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.

9 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

10 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

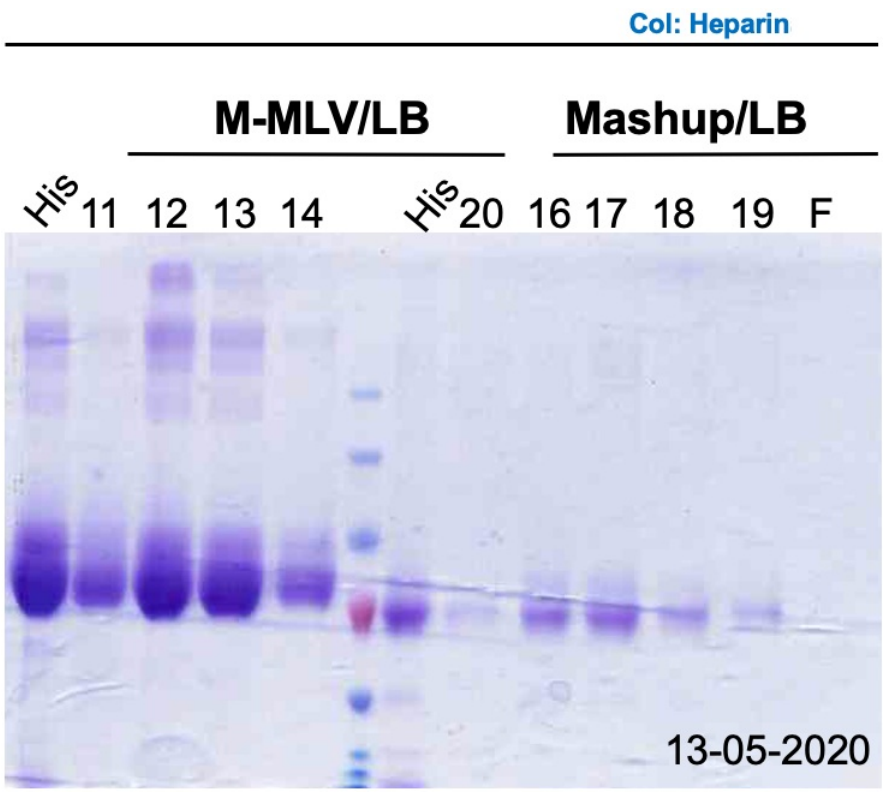
Dilute the pooled fractions 3X in 50 mM Tris-HCl pH 8.0, such that the final concentration of NaCl is 100 mM. Then, load onto a **1 ml HiTrap Heparin column** previously equilibrated with 10 c.v. (here, 10 mL) **buffer HA.**
Then, elute the protein using a 10 c.v. linear gradient against **buffer HB**, collecting the eluted fractions every **1 mL** in 1.5 mL tubes. The protein will elute at high concentrations between 200 and 400 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).

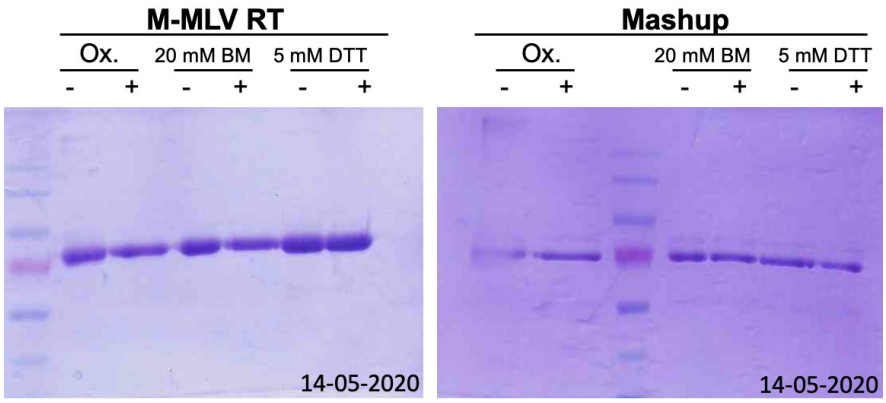
11 Again, determine your protein-containing fractions using the Bradford assay. Pool your fractions and determine its protein concentration using the same method and collect a **10 µl** sample for SDS-PAGE. 10m

For storage, supplement your pooled fraction with **0.2 % volume** Nonidet P-40, **0.2 Milimolar (mM)** EDTA and **10 Milimolar (mM)** DTT. Then, dilute the sample in an equivalent volume of 100% glycerol to achieve the final storage conditions: **25 mM Tris-HCl pH 8.0, ~150 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 5 mM DTT, 50% glycerol.**

With this protocol, our usual final protein concentrations for storage are between **0.1 mg/ml** and **0.3 mg/ml** . The protein yield was **4 mg per liter** of cell culture for **M-MLV** and **1 mg per liter** of cell culture for **Mashup**.



The unlabelled lane corresponds to the PageRuler protein ladder. His = Pooled fraction from HisTrap IMAC column (20 µl). Numbered lanes correspond to eluted fractions from Heparin column (10 µl). The high molecular weight contaminations in M-MLV are due to disulfide bonds between the free cysteins in the protein.



Effect of reducing agents on the elimination of species with high molecular weight. M-MLV and Mashup were incubated overnight in oxidizing (Ox) or reducing conditions using either BME or DTT as the reducing agent. For the SDS-PAGE, the loading buffer was prepared with (+) or without (-) reducing agents. Condition Ox/- shows some high molecular weight species, which are eliminated upon addition of reducing agents in the protein-containing solution (BME, DTT). For M-MLV, 5 µg were loaded on each lane, whereas 2 µg were loaded for Mashup.