Recombinant expression and purification of codon-optimized M-MLV and Mashup V.2

Maira Rivera[1], Javiera Reyes[1], Paula Blazquez-Sanchez[1], Cesar A Ramirez-Sarmiento[1]
[1]Institute for Biological and Medical Engineering, Pontificia Universidad Católica de Chile

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1 Works for me
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Cesar A Ramirez-Sarmiento
Pontificia Universidad Catolica de Chile

ABSTRACT

This protocol has been optimized for the recombinant expression of codon-optizimed M-MLV and MashUp RT.

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.

M-MLV RT has been further functionally tested and works as expected. The sequence of the plasmid encoding the codon-optimized M-MLV RT enzyme can be found here.

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MATERIALS TEXT

MATERIALS

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

PMSF **Sigma**

Aldrich Catalog #P7626

Sodium phosphate dibasic **Sigma**

Aldrich Catalog #7558-79-4

DTT **Sigma**

Aldrich Catalog #D0632

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

Glycerol **Sigma**

Aldrich Catalog #G5516

Dextrose **Sigma –**

Aldrich Catalog #D9434

Tween-20 **Sigma**

Aldrich Catalog #P9416

Nonidet P40 Substitute **Sigma**

Aldrich Catalog # 74385

EDTA **Sigma**

Aldrich Catalog #ED2SS

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 HA, pH 8.0

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[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

Storage Conditions

[M]**25 Milimolar (mM)** Tris-HCl, pH 8.0

[M]**150 Milimolar (mM)** NaCl

[M]**0.1 Milimolar (mM)** EDTA

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

[M]**5 Milimolar (mM)** DTT

[M]**50 % volume** Glycerol

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 using either heat shock or electroporation. 2h

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 **250 rpm, 37°C** . 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 **250 rpm, 37°C** 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 **160 rpm, 18°C** . 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. 30m

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

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8 Sonicate on ice for **00:04:00** using cycles of **00:00:01** ON and **00:00:06** OFF at 40% amplitude (Qsonica Q125, 125W). 30m

9 Centrifuge the unclarified lysate **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

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

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 **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 2h

12 Dilute the pooled fractions 3-fold in **[M]50 Milimolar (mM)** **Tris-HCl pH 8.0**, such that the final concentration of NaCl is 100 mM. 5m

13 Load the diluted sample 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 **0.5 mL** in 1.5 mL tubes. 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**, using a syphon or tubing, and withdrawing solution from the **buffer HA** container to the column using a peristaltic pump or by gravity. The protein will elute at high concentrations between 200 and 400 mM NaCl. 1h

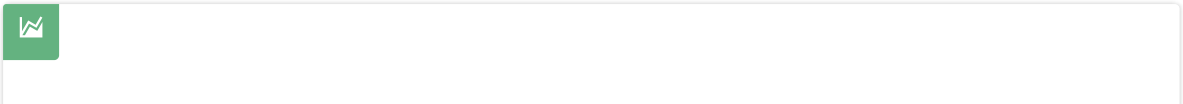
14 Determine your protein-containing fractions using the Bradford assay. Pool your fractions and determine its protein concentration using the same method. Also, collect a **10 µl** sample for SDS-PAGE. 10m

15 For storage, supplement your pooled fraction with **[M]0.2 % volume** Nonidet P-40, **[M]0.2 Milimolar (mM)** EDTA and **[M]10 Milimolar (mM)** DTT. Then, add glycerol up to **[M]100 % volume** to reach **Storage Conditions.** Do consider that a final protein concentration of **[M]0.3 mg/ml** is appropriate for subsequent experiments. 5m

16 Generate **200 µl** aliquots of the enzyme and store it at **-20 °C** until required. 30m

IMAC SDS-PAGE Result 10m

17



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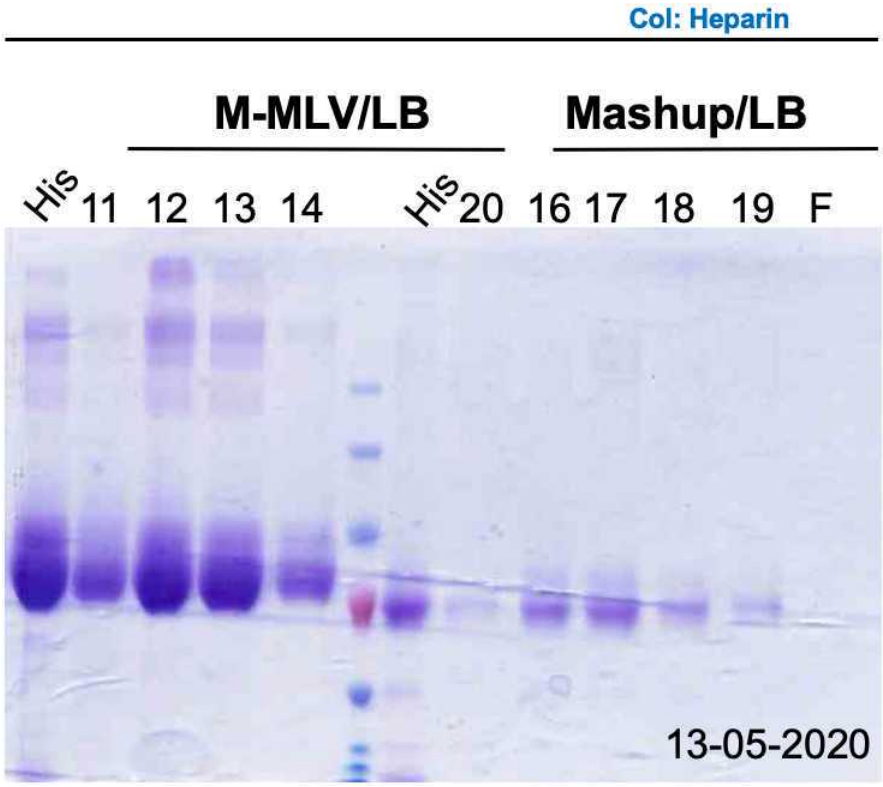
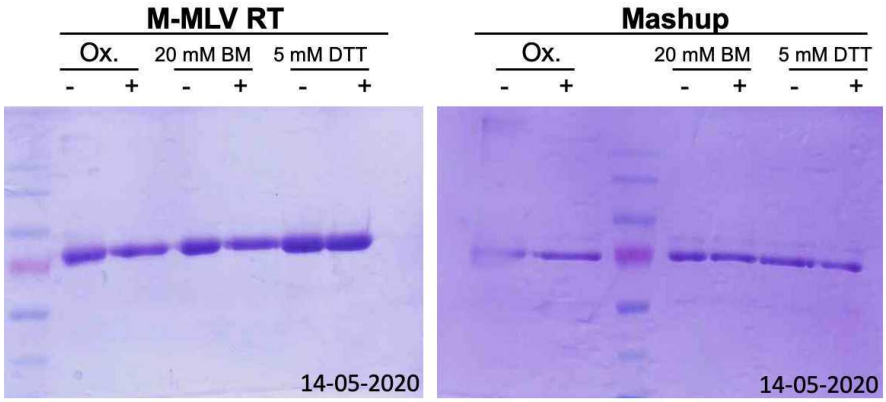


The unlabelled lane corresponds to the PageRuler protein ladder. His = Pooled fraction from HisTrap IMAC column (20 µl). Numbered lanes correspond to eluted fractions from Heparin column (10 µl). The high molecular weight contaminations in M-MLV are due to disulfide bonds between the free cysteins in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

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