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Recombinant Protein Expression of MMLV-RT H+

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Works for me

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

Reclone.org (The Reagent Collaboration Network)

Tech. support email: protocols@reclone.org

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ABSTRACT

This protocol has been optimized for recombinant expression of molony virus based reverse transcriptases (RT). The plasmid used contains a reverse transcriptase gene which is derived from Moloney Murine Leukemia Virus. The enzyme when expressed recombinantly can synthesize a complementary DNA strand from single-stranded RNA, DNA, or an RNA:DNA hybrid. The enzyme contains point mutations to generate a highly processive, thermostable, and improved fidelity mutant reverse transcriptase. Details, including the sequence of this enzyme are published and can be found here PMID: 22691702. The enzyme contains an active RNase H domain and is highly thermostable.

The point mutations utilised here are currently filed under a provisional patent with Thermo Fisher Scientific. Commercial use of this enzyme must go through Thermo Fisher Scientific.

This expression protocol can also be implemented to express other MMLV based reverse transcriptases or diversely related retroviral RTs, provided the genes are in an expression construct with an N-terminal 6-10 His Tag and are expressed under a T7 promoter.

The plasmid used can be found on reclone.org

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Baranauskas A, Palikša S, Alzbutas G, et al. Generation and characterization of new highly thermostable and processive M-MuLV reverse transcriptase variants. *Protein Eng Des Sel.* 2012;25(10):657-668. doi:10.1093/protein/gzs034

MATERIALS

NAME	CATALOG #	VENDOR
Lysozyme	12671-19-1	Sigma Aldrich
Imidazole	I5513	Sigma
NaCl	53014	Sigma Aldrich
Triton X-100	93426	Sigma
Glycerol	G5516	Sigma Aldrich
DL-Dithiothreitol (DTT)	43815	Sigma Aldrich
Ethylenediaminetetraacetic acid	E9884	Sigma Aldrich
AEBSF Protease Inhibitor	78431	Thermo Fisher
Tris Hydrochloride (Tris-HCl)	RES3098T-B7	Sigma Aldrich

MATERIALS TEXT

Buffer B (pH 8.0): 500mL

- 20mM Tris/HCL = 10mL 1M Tris-HCL pH 8.0
- 300mM NaCl = 8.766g
- 0.5% Triton X-100 = 2.5mL
- 10% (v/v) glycerol = 50mL

- 25mM imidazole = 12.5mL 1M imidazole
- + supplemented with 1mg lysozyme/ml

Buffer C (pH 8.0): 250mL

- 20mM Tris/HCl = 5mL 1M Tris-HCL pH 8.0
- 300mM NaCl = 4.383g
- 0.5% Triton X-100 = 1.25mL
- 10% (v/v) glycerol = 25mL
- 80mM imidazole = 20mL 1M imidazole

Elution / Buffer D (pH 8.0): 250mL

- 20mM tris/HCl = 5mL 1M Tris-HCL pH 8.0
- 300mM NaCl = 4.383g
- 0.5% Triton X-100 = 1.25mL
- 10% (v/v) glycerol = 25mL
- 250mM imidazole = 62.5mL 1M imidazole

Storage / Buffer E (pH 8.3 @ 25°C): 250mL

- 50mM Tris-HCL = 12.5mL 1M Tris-HCL pH 8.0
- 100mM NaCl
- 1mM EDTA
- 5mM DTT
- 0.1% (v/v) Triton X-100 = 250µL
- 50% (v/v) glycerol = 125mL

For 1x lysis buffer

- 1mM AEBSF (add fresh)
- 1mg/ml lysozyme (add fresh)

Protein Expression

2d

- 1 Streak Carb^rCam^r LB plate supplemented with from frozen stock of **Rosetta DE3** . Grow plate overnight at 37°C. ^{1d}
- 2
 - Select single colony from overnight streak plate and inoculate 5mL of LB broth supplemented with Carb and Cam. ^{1d}
 - Grow overnight at 37°C shaking at 250rpm.
 - Prepare 500mL LB and sterilize by autoclave.
 - Allow sterile LB to come to room temperature overnight.
- 3
 - 1 mL of this culture was used to inoculate 500 mLs of LB in the morning. 500mL LB broth is then supplemented with Carb and Cam. ^{1d}
 - The large culture was incubated at 37°C in a shaker at 275 RPM.
 - OD₆₀₀ was monitored until it reached 0.8-1.0 and was induced with 0.5 mM IPTG.
 - Monitor OD₆₀₀ every 30-40 mins. As OD₆₀₀ approaches 0.5 monitor every 5-10 mins.
 - Induction occurred at 18°C for 20 - 24 hours.
- 4
 - Collect cells by centrifugation. Conditions as follows: 4°C, at 4,000 x g for 30min. ^{3h}
 - Cells were harvested by centrifugation, washed once with 1X Lysis Buffer (20 mM Imidazole) then resuspended in 5 mL of ice cold 1X Lysis Buffer per gram of wet pellet.
 - Lysozyme was added to a concentration of 1 mg/mL and lysed by sonication, with 15 cycles of 10 seconds on/30 seconds off in salted ice water.
 - Sonicate more if the cells are not completely disrupted (Lysis is complete when the cloudy cell suspension becomes translucent. Avoid protein denaturation by frothing).
 - Spin 30min 18000rpm 4°C. Separate soluble proteins (supernatant) from insoluble or inclusion bodies proteins (pellet). Use supernatant for next step. Keep sample of 40µl of supernatant for PAGE-SDS: **soluble proteins**
 - Resuspend pellet in 5ml column buffer and keep sample of 40µl for PAGE-SDS: **insoluble proteins, or unlysed cells**.

5 The following steps are performed at 4°C

- Clarified lysate was then loaded onto 0.5 mL of Ni Sepharose Fast Flow beads (use whatever you got) that had been equilibrated with 1X Lysis Buffer (20 mM Imidazole).
- Collected flow-through was re-loaded onto the column and ran a second time
- Column was washed with 10 column volumes of buffer C
- Eluted with 5mL Buffer D (500mM Imidazole)
- Each 1ml of the elution buffer is collected separately in 1.5ml tubes.
- Eluted fractions are measured for protein absorbance on a nanodrop, using the imidazole containing elution buffer as a blank reference sample.
- samples values are recorded. Protein absorbance reads will peak and wane to background levels. Once eluted fractions are at background levels
- The column was regenerated with 10 mL washes of water/1M NaOH/water/50 mM EDTA/water/100 mM NiCl₂/20% EtOH and stored with 20% EtOH in the fridge.

- 6 Amicon protein concentrators can be substituted with dialysis into 50% glycerol containing storage buffer. Amicon^{3h} protein concentration can cause proteins to crash out of solution if the solution is too concentrated, resulting in loss of protein yield. However they are much quicker than dialysis and do not require making large volumes of buffers containing 50% glycerol. Dialysis will minimize yield loss, but will not purify your protein from possible low molecular weight contaminants. Refer to ThermoFisher dialysis methods for implementing this approach.

Separate protein using an amicon with molecular weight cut off ~1/3 the molecular weight of the purified target protein. MMLV = 79.8kDa, Amicon filter cutoff 25kDa.

Exchange buffer with storage buffer at this point. Exchange with storage buffer containing 2× concentration of salts and no glycerol. Exchange buffer 3x, spin at 6000g 30-60 min each time, or until the

- 7 If using Amicon size separation protein can be stored in a storage buffer containing 50% glycerol. The exchange buffer contains 2× the concentration of salts and can be diluted 50% via the addition of 100% glycerol. From here aliquats can be made and stored at -20C