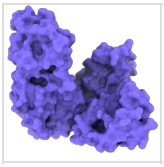

Version 2

Jul 14, 2020

Recombinant Protein Expression of MMLV-RT H+ (SkiBar H+ RT) V2 V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bijzkcp6

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ABSTRACT

About:
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 synthesizes a complementary DNA strand from single-stranded RNA, DNA, or an RNA:DNA hybrid.

Enzyme Engineering:
The enzyme contains point mutations to generate a highly processive, thermostable, and improved fidelity mutant reverse transcriptase. Details, incluiding the sequence of this enzyme are published and can be found in the references below and addgene link. The **enzyme contains an active RNAse H domain and is highly thermostable**. This MMLV construct contains the following mutations: D200N, L603W, T330P, L139P, E607K,D524G, E562Q, D583N and D653N. These appear to be the same set of mutations present in the **Thermo Fisher Maxima H- RT**, which is one of the best preforming RTs you can buy. The enzyme in our hands is extremely active. Moreover, while the MMLV RT is thermostable and will work up to 65°C it runs optimally at 42°C.

The point muitations utilized here are currently filed under a provisional patent with Thermo Fisher Scientific. Commcercial use of this enzyme must go though Thermo Fisher Scientific.

It is also possible to further engineer this enzyme with to increase thermostability and eliminate RNase H activity to generate an enzyme which closely mimicks Thermo Fisher Maxima **H+** RT.

Introducing mutations **(D524G, E562Q, D583N and D653N)** and/or **(D524, D583, E562, H204, V223, T306, F309)** are best suted eliminate RNase H activity completely, and it will increase the thermostability somewhat as well

Comments:
Reference 2 highlights several point mutations which can be introduced to eliminate Rnase H activity which may also enhance RT sensitivity. Thermo Product information brochure from 2013 suggest that this mutant or some combintation of other identified point mutations from reference 2 is Maxima RT, Maxima H-. The paper has also identified a few more useful point mutations which may further optimise MMLV-RT

This expression protocol can also implemented to express other MMLV based reverse transcriptases or diversely related retroviral RTs, provided the genes are in an expresison 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 and addgene

SkiBar H+ RT was synthesized as a gBlock from IDT and is codon optimized. Expressed in Rosetta DE3 his tag is on still but seems to work fine with it on.

Tgatcc motif at MCS is a result of issues cloning in with BamHI. Had to use a compatible sticky end from BclI-HF

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Protein Properties:
79.9 kDa
Isoelectric point (pI) : 7.77
Charge at pH 7.0: 9.52

Name:
The name **SkiBar H+ RT** is an abbreviation of the first two first authors where the MMLV mutations used in the construct were originally discovered (**Ski**rgaila & **Bar**anauskas).

References:
Baranauskas, Aurimas Paliksa, Sigitas Alzbutas, Gediminas Vaitkevicius, Mindaugas Lubiene, Judita Letukiene, Virginija
Burinskas, Sigitas Sasnauskas, Giedrius Skirgaila, Remigijus
Generation and characterization of new highly thermostable and processive M-MuLV reverse transcriptase variants
Protein Engineering, Design and Selection (2012)

Skirgaila, Remigijus Pudzaitis, Vaidas Paliksa, Sigitas Vaitkevicius, Mindaugas Janulaitis, Arvydas
Compartmentalization of destabilized enzyme-mRNA-ribosome complexes generated by ribosome display: A novel tool for the directed evolution of enzymes
Protein Engineering, Design and Selection (2013)

DOI
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GUIDELINES
The enzyme in our hands is extremely active. Moreover, while the MMLV RT is thermostable and will work up to 65°C, **it runs optimally at 42°C.**

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

NAME	CATALOG #	VENDOR
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/HLC = 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^{r}Cam^r LB plate supplemented with from frozen stock of **Rosetta DE3** . Grow plate overnight at 37°C. 1d

2 1d
- Select single colony from overnight streak plate and inoculate 5mL of LB broth supplemented with Carb and Cam. 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 1d
- 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.
- The large culture was incubated at 37°C in a shaker at 275 RPM.
- OD_{600} was monitored until it reached 0.8-1.0 and was induced with 0.5 mM IPTG.
- Monitor OD_{600} every 30-40 mins. As OD_{600} approaches 0.5 monitor evey 5-10 mins.

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- Induction occurred at 18°C for 20 - 24 hours.

4 3h
- Collect cells by centrifugation. Conditions as follows: 4°C, at 4,000 x g for 30min.
- Cells were harvested by centrifugation, washed once with 1X Lysis Buffer (20 mM Imidizole) 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**.

IMAC Purification 1h 30m

5 1h 30m
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 eleution buffer is collected separately in 1.5ml tubes.
- Eluted fractions are measured for protein absorbance on a nanodrop, using the imidizole 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 NiCl/20% EtOH and stored with 20% EtOH in the fridge.

Amicon Size Separation or Dialysis 3h

6 3h
Amicon protein concentrators can be substituted with dialysis into 50% glycerol containing storage buffer. Amicon 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 contaminates. Refer to ThemoFisher dialysis methods for implementing this approach.

Separate protein using an amicon with molecular weight cut off ~1/3 the molecular wight 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

Storage 10m

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% glycrol. From here aliquats can be mae and stored at -20C

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