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Expression and purification of recombinant MM4 reverse transcriptase (RT)

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Diana A Tapia-Sidas

ABSTRACT

Reverse transcriptases (RTs) are RNA-dependent DNA polymerases able of synthesizing DNA (complementary DNA or cDNA) from an RNA template. RTs are especially useful in RNA-based nucleic acid detection techniques. Due to its high catalytic activity and fidelity, one of the most widely used RTs in diagnostics and molecular biology is the RT from the Moloney Murine Leukemia Virus (MMLV). However, RT-MMLV is thermally unstable, so previous studies have produced a RT variant called RT-MM4 carrying mutations of positive charges in four amino acids (E286R/E302K/L435R/D524A). This protocol describes the optimized expression process, as well as the FPLC purification of RT-MM4 for use in isothermal amplification techniques, such as end-point colorimetric or real-time fluorometric RT-LAMP.

GUIDELINES

During the process of protein purification maintain all samples that contain the protein of interest in a cold environment to avoid protein degradation.

MATERIALS

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PROTOCOL integer ID:

64229

Keywords: Moloney Murine Leukemia Virus, thermostable reverse transcriptase, protein expression, protein purification, MM4 RT

Reagents:

- Chaperone Plasmid Set **Takara Bio USA, Inc. Catalog #3340**
- Chemically Competent E. coli One Shot™ BL21(DE3) Invitrogen Thermo Fisher Catalog #C600003
- [M] 100 ng/µL pET-MM4-RT plasmid Step 3.1
- Stock [M] 30 mg/mL chloramphenicol
- Stock [м] 100 mg/mL carbenicillin
- Stock [м] 1 Molarity (М) IPTG
- Stock [м] 0.5 Molarity (М) EDTA
- -Stock [м] 1 Molarity (М) DTT
- Stock [м] 10 % (v/v) Triton X-100
- △ 250 µL of SOC medium Step 1.4
- △ 8 mL of TFBI solution Step 2.4
- △ 2.5 mL of TFBII solution Step 2.5
- Cryotubes with 4 500 µL of 30% v/v glycerol (sterilized)
- LB agar plates
- Tubes with 🚨 3 mL Luria-Bertani (LB) medium
- Tubes with 🗸 5 mL Luria-Bertani (LB) medium
- Tubes with <u>A 3 mL Terrific Broth (LB) medium</u>
- Flasks with 🔼 20 mL LB medium
- Flasks with 🗸 50 mL LB medium
- Flasks with <u>A 50 mL TB medium</u>
- Flask with 🔼 100 mL LB medium
- Flasks with 🗸 1 L LB medium
- △ 500 mL Lysis Buffer B (LB-B) Step 5.5/8.4
- △ 500 mL Elution Buffer-BI (EB-BI) Step 10.5
- A 1 L Desalting Buffer-B (DB-B) Step 11.2
- △ 500 mL Elution Buffer-BII (EB-BII) Step 12.5
- △ 500 mL Storage Buffer-B (SB-B) Step 13.1
- X HisTrap HP 5mL Cytiva Catalog #17524801
- HiPrep 26/10 Desalting Column Cytiva Catalog #17508701
- 🔯 HiTrap SP XL 5mL Cytiva Catalog #17516101

X Quick Start™ Bradford 1x Dye Reagent BioRad Sciences Catalog #5000205

Precision Plus Protein™ Unstained Protein Standards **Bio-rad**Laboratories Catalog #1610363

- Tricine-SDS-PAGE electrophoresis solutions (Step 6.2)
- 8% polyacrylamide gels for Tricine-SDS-PAGE (Step 6.2)

Equipments:

- Thermomixer

Equipment	
Thermomixer® R	NAME
Dry block heating and cooling shaker, 120 V, 60 Hz, 1/cs	TYPE
Eppendorf	BRAND
T3317	SKU

- Orbital shaker

Equipment	
MaxQ™ HP Incubated Tabletop Orbital Shaker	NAME
MaxQ™ HP, 120 V 60 Hz, 6,5 A o 230 V 50/60 Hz, 3,2 A	TYPE
Thermo Scientific	BRAND
SHKE420HP	SKU
https://www.thermofisher.com/order/catalog/product/SHKE420HP	LINK

- Floor model orbital shaker

Equipment	
MaxQ™ HP Incubated and Refrigerated Console Shakers	NAME
MaxQ™ 481 HP, 230 V, 50 Hz	TYPE
Thermo Scientific	BRAND
SHKE481HP	SKU
https://www.thermofisher.com/order/catalog/product/SHKE481HP	LINK

- Centrifuge

Equipment	
Sorvall™ Legend™ XT/XF Centrifuge Series	NAME
Thermo Scientific	BRAND
75004541	SKU
https://www.thermofisher.com/order/catalog/product/75004541	LINK

- Ultrasonic Processor 130W

Equipment	
Ultrasonic Processor	NAME
130-Watt Ultrasonic Processor	TYPE
Cole-Parmer	BRAND
ML-04714-52	SKU
https://www.coleparmer.com/p/cole-parmer-130-watt-ultrasonic-processors/44347	LINK

- Ultrasonic Processor 750W

T50-Watt Ultrasonic Processor CPX750 TYPE Cole-Parmer ML-04711-60 https://www.coleparmer.com/p/cole-parmer-500-and-750-watt-ultrasonic-processors/16401

-Nanodrop

Equipment	
NanoDrop™ One UV-Vis Spectrophotometer	NAME
spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU
https://www.thermofisher.com/order/catalog/product/ND-ONE-W	LINK
Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 SPECIFICATIONS nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit; Voltage: 12 V (DC); Wavelength Range: 190–850 nm	

- FPLC system

Equipment	
ÄKTA pure	NAME
Protein purification system	TYPE
Cytiva	BRAND
29046665	SKU
https://www.cytivalifesciences.com/en/us/support/products/akta-pure- 150-l-29046665	LINK

- Spectrophotometer UV/Vis
- Incubator (37°C)
- Water bath (60°C)
- Ultra Low-Temperature Freezer (-80°C)
- Freezer -20°C
- Refrigerator (4°C)
- Analytical balance

Other:

- Ice bath
- Microcentrifuge tubes
- Sterile 0.45 µm membrane filter
- 150 mL Superloop (Cytiva)
- Dialysis membrane
- Ultrafiltration tube (Amicon Ultra-15)



- Image Lab 6.1 Software (Bio-Rad)

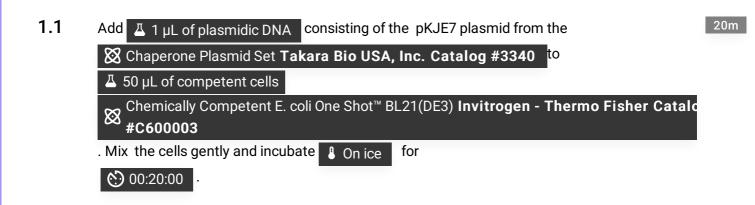
BEFORE START INSTRUCTIONS

Ensure to have all the necessary materials and reagents already cleaned, sterilized and filter (in case of the purification solutions).

Preparation of RT expression cells

2d 12h 20m 53s

1 Transformation of chemically competent BL21 (DE3) cells with pKJE7 plasmid.



1.2 Transfer the cells to a heat block at 42 °C and incubate for 00:00:53

53s

5m

1.4 **45** 225 rpm, 37°C, 01:00:00

1h

Note

SOC medium composition

A	В
Tryptone	2%
Yeast extract	0.5%
NaCl	10 mM
KCI	2.5 mM
MgCl2	10 mM
MgSO4	10 mM
Glucose	20 mM

Adjust to pH 7 and sterilize by filtration.

1.5 Plate A 25 µL of transformed cells culture onto LB agar with the corresponding selective agent. Incubate the plates Overnight at 8 37 °C

18h

Note

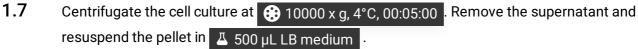
The pKJE7 plasmid requires [M] 30 μ g/mL chloramphenicol as selective agent.

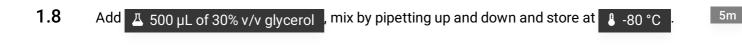
1.6 Select a single colony of transformed cells and inoculate in

🗸 3 mL Luria-Bertani (LB) medium supplemented with the selective antibiotic. Incubate

18h



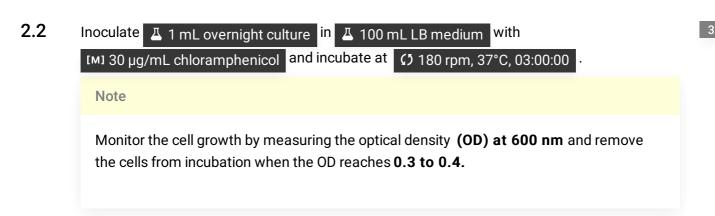




- 2 Preparation of chemically competent BL21 (DE3) cells harboring pKJE7 plasmid.
- Take BL21(DE3) cells harboring pKJE7 plasmid from a frozen glycerol stock using a bacterial inoculating loop and inoculate

 Δ 3 mL LB liquid medium with

 [M] 30 μg/mL of chloramphenicol . Incubate Overnight at (5 180 rpm, 37°C).



- 2.3 Chill the cell culture on ice for 00:10:00 and centrifugate the cells at 4000 x g, 4°C, 00:10:00

TFBI medium composition

A	В
Potassium acetate	30 mM
Rubidium chloride	100 mM
Calcium chloride	10 mM
Manganese chloride	50 mM
Glycerol	15% v/v

Adjust to pH 5.8 with 1M acetic acid and sterilize by filtration.

2.5

Gently resuspend the cell pellet in 🚨 2.5 mL of TFBII solution pre-coolded and incubate

On ice



Note

TFBII medium composition

A	В
MOPS	10 mM
Rubidium chloride	10 mM
Calcium chloride	75 mM
Glycerol	15% v/v

Adjust to pH $6.5\,\mathrm{with}\,1\mathrm{M}$ sodium hydroxide and sterilize by filtration.

2.6

30m



3 Transformation of chemically competent BL21 (DE3)/pKJE7 cells with the pET-MM4-RT plasmid, the expression vector for the quadruple mutant (E286R/E302K/L435R/D524A, designated as MM4) of the reverse transcriptase (RT) from Moloney Murine Leukemia Virus (MMLV).

CITATION

Yasukawa K, Mizuno M, Konishi A, Inouye K (2010). Increase in thermal stability of Moloney murine leukaemia virus reverse transcriptase by site-directed mutagenesis.. Journal of biotechnology. LINK

https://doi.org/10.1016/j.jbiotec.2010.09.961

3.1 Add 🗸 1 µL of plasmidic DNA of [M] 100 ng/µL pET-MM4-RT expression vector to Δ 50 μL of competent cells BL21 (DE3)/pKJE7. For the transformation procedure **Ξ5** go to step #1

Note

The pET-MM4-RT plasmid requires [M] 100 µg/mL carbenicillin as selective agent and the pKJE7 plasmid requires [M] 30 µg/mL chloramphenicol . Use LB medium supplemented with both antibiotics as selective media.

Small-scale screening cultures

1d 14h 16m 45s

- 4 Preparation of bacterial cultures for RT expression.
- 4.1 Inoculate \perp 5 μ L glycerol stock of BL21(DE3)/pKJE7/pET-MM4-RT or BL21(DE3)/pKJE7 cells in 🗸 5 mL culture medium (LB or TB) supplemented with selection agent(s). Incubate 👏 Overnight at **45** 200 rpm, 37°C

18h

For each treatment, inoculate \bot 500 µL overnight culture in \bot 50 mL culture medium with [M] 100 µg/mL carbenicillin . Use LB or TB according to the medium use for the overnight culture. Incubate \bigcirc 200 rpm, 37°C, 03:00:00 .

Note

Monitor the cell growth by measuring the optical density **(OD) at 600 nm** and remove the cells from incubation when the OD reaches **0.6**.

4.3 Once the culture reaches an OD600 of 0.6, incubate the cell cultures on ice for 00:20:00 before adding the inducer (IPTG).

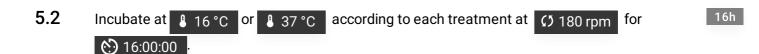
20m

- 5 Small-scale RT expression under different induction conditions.
- 5.1 Induce the expression of the RT under different conditions. Each treatment should be evaluated in triplicate. For example:

Strain	[IPTG]	Temperature	Medium
BL21(DE3)/pKJE7*	0.5 mM	16°C	LB
BL21(DE3)/pKJE7*	0.5 mM	37°C	LB
BL21(DE3)/pKJE7*	0.5 mM	16°C	ТВ
BL21(DE3)/pKJE7/pET-MM4- RT	0 mM	16°C	LB
BL21(DE3)/pKJE7/pET-MM4- RT	0.1 mM	16°C	LB
BL21(DE3)/pKJE7/pET-MM4-RT	0.5 mM	16°C	LB
BL21(DE3)/pKJE7/pET-MM4- RT	1.0 mM	16°C	LB
BL21(DE3)/pKJE7/pET-MM4-RT	0 mM	37°C	LB
BL21(DE3)/pKJE7/pET-MM4- RT	0.5 mM	37°C	LB

Strain	[IPTG]	Temperature	Medium
BL21(DE3)/pKJE7/pET-MM4-RT	0.5 mM	16°C	ТВ

^{*} BL21(DE3)pKJE7 strain is used as negative expression control.



- 5.3 Centrifugate the cell cultures at 6000 x g, 4°C, 00:10:00. Discard the supernatant, remove all the liquid and leave the cell pellet as dry as posible.
- **5.4** Weigh the centrifugation tube with the cell pellet (total weight).

Weigh the empty tube prior centrifugation and subtract it to the total weight to calculate the weight of the cell pellet and hence the biomass produced.

5.5 Resuspend the cell pellet in A 5 mL lysis buffer B (LB-B) (pre-cooled).

Lysis buffer B composition (LB-B)

A	В
NaH2PO4/Na2HPO4 pH7.8	50 mM
NaCl	300 mM
DTT	2.5 mM
Imidazole	10 mM
Glycerol	5% v/v
PMSF	3 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.8. Store at 4°C.

Disrupt cells by ultrasonication at an amplitude of 40%. Apply five cycles of and 00:00:30 off.

Note

Place the tubes On ice while processing.

Equipment	
Ultrasonic Processor	NAME
130-Watt Ultrasonic Processor	TYPE
Cole-Parmer	BRAND
ML-04714-52	SKU
https://www.coleparmer.com/p/cole-parmer-130-watt-ultrasonic-proc	essors/44347 LINK

Centrifugate at $6000 \times g$, 4° C, 00:15:00. Recover the supernatant (soluble protein fraction) and discard the pellet.

15m

- 6 Analysis of RT expression.
- **6.1** Measure total protein concentration by measuring absorbance at 280 nm in a NanoDrop spectrophotometer.

3m

Equipment NanoDrop™ One UV-Vis Spectrophotometer NAME spectrophotometer TYPE Thermo Scientific BRAND ND-ONE-W SKU https://www.thermofisher.com/order/catalog/product/ND-ONE-W LINK Sample Volume (Metric): Minimum 1μL; Spectral Bandwidth: ≤1.8 nm
(FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit;
Voltage: 12 V (DC); Wavelength Range: 190−850 nm

CITATION

Hermann Schägger (2006). Tricine-SDS-PAGE. Nature Protocols.

LINK

10.1038/nprot.2006.4

6.3 Select the best conditions for protein expression according to the results analysis (biomass, total protein production and electrophoretic profile).

Large-scale production of RT

1d 14h 24m 20s

- 7 Expression of recombinant RT.
- 7.1 Inoculate Δ 10 μL glycerol stock of BL21(DE3)/pKJE7/pET-MM4-RT expression cells in Δ 20 mL LB medium supplemented witt [M] 100 μg/mL carbenicillin and

[м] 30 µg/mL chloramphenicol . Incubate 👏 Overnight at

ና5 180 rpm, 37°C -

Inoculate $\[\]$ 10 mL overnight culture in $\[\]$ 1 L LB medium with [M] 100 µg/mL carbenicillin and [M] 30 µg/mL chloramphenicol . Incubate $\[\]$ 200-220 rpm, 37°C, 03:00:00 .

3h

7.3 Place the inoculum on ice for 00:30:00 and then add [M] 0.5 millimolar (mM) IPTG for induction.

Do not add any additional inducers. For expression of the chaperones contained in pKJE7 plasmid, the basal expression is enough to promote correct RT enzyme folding.

7.4 Incubate at \$\(\) 180 rpm, 16°C, 16:00:00 for recombinant protein expression.

16h

- 8 Soluble protein fraction recovery
- 8.1 Centrifugate at 6000 x g, 4°C, 00:12:00 to harvest cells. Discard the supernatant ensuring to remove all the liquid and leave the cell pellet as dry as posible.

12...

8.2 Weigh the centrifugation tube with the cell pellet (total weight).

3m

Note

Weigh the empty tube prior centrifugation and subtract it to the total weight to calculate the weight of the cell pellet and hence the biomass produced.

- Resuspend the cell pellet in <u>A 50 mL lysis buffer B (LB-B)</u> (pre-cooled). If neccesary, defroze the cell pellet in an ice bath before adding the lysis buffer.

Lysis buffer B composition (LB-B)

A	В
NaH2PO4/Na2HPO4 pH7.8	50 mM
NaCl	300 mM
DTT	2.5 mM
Imidazole	10 mM
Glycerol	5% v/v
PMSF	3 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.8. Store at 4°C.

Disrupt cells by ultrasonication with an ultrasonic processor at an amplitude of 40% applying 4m 20s 8.5 pulses of 00:00:10 of ultrasonication and 00:00:00:10 of pause during 00:04:00

Note



8.6 Centrifugate at 11000 x g, 4°C, 00:30:00 Recover the supernatant (soluble protein fraction) and discard the pellet.

30m

Note

Place the supernatant in an ice bath or store at 4°C until use.

Purification of recombinant RT by FPLC

9 Sample preparation.

Note

Keep all protein samples on ice during the purification process to avoid protein degradation.

9.1 Filter the supernatant (soluble protein fraction) through a 0.45 µm membrane.

- 9.2 Load the soluble protein fraction onto a 150 mL Superloop (Cytiva). Store at use.
- 10 Immobilized metal affinity chromatography (Ni²⁺-IMAC).
- 10.1 Connect a HisTrap HP 5mL Cytiva Catalog #17524801 to a FPLC system.



- **10.2** Equilibrate the column with 8 column volumes (CV) of lysis buffer B (LB-B) at a flow of 2.5 mL/min.
- 10.3 Connect the Superloop charged with the protein fraction and load the sample onto the column at a flow of 2.5 mL/min.
- 10.4 Wash the column with 10 CV of LB-B at a flow of 2.5 mL/min

10.5 Wash the column with 10 CV of 2% elution buffer-BI (EB-BI) at a flow of 2.5 mL/min.

Note

Elution buffer-BI composition (EB-BI).

A	В
NaH2PO4/Na2HPO4 pH7.8	50 mM
NaCl	300 mM
DTT	2.5 mM
Imidazole	500 mM
Glycerol	5% v/v
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.8. Store at 4°C.

- 10.6 Elute the proteins by passing 5 CV of 100% EB-BI through the column using a flow of 2.5 mL/min.
- 10.7 Immediatly after elution add [M] 2 millimolar (mM) EDTA and [M] 2.5 millimolar (mM) DTT to the eluted fractions.
- 10.9 Pool all elution fractions carrying the recombinant RT protein. Store at 4 °C until use

11 Desalting step.

- 11.1 Connect a System. We HiPrep 26/10 Desalting Column Cytiva Catalog #17508701 to the FPLC system.
- 11.2 Wash the column with 2.5 CV of Mili-Q water. Then, equilibrate the column with 2 CV of desalting buffer-B (DB-B). For both steps use a flow of 10 mL/min.

Note

Desalting buffer-B composition (DB-B).

A	В
HEPES pH 7.5	50 mM
NaCl	40 mM
EDTA	2 mM
DTT	5 mM
Glycerol	5% v/v
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- 11.3 Load the sample onto the column at a flow of 5 mL/min.
- 11.4 Wash the column with 2 CV of DB-B for protein elution at a flow of 10 mL/min.

- Load the pool of desalted fractions onto a 150 mL Superloop (Cytiva). Store at use.
- 12 Cation exchange chromatography (CEC).
- 12.1 Connect a 🔀 HiTrap SP XL 5mL Cytiva Catalog #17516101 to the FPLC system.
- **12.2** Equilibrate the column with 10 CV of DB-B at a flow of 2 mL/min.
- 12.3 Connect the Superloop charged with the protein fraction and load the sample onto the column at a flow of 2 mL/min.
- 12.4 Wash the column with 5 CV of DB-B at a flow of 2 mL/min.
- 12.5 Elute proteins by washing the column with a linear gradient of 10 CV of elution buffer-BII (EB-BII). Use a flow of 2 mL/min.

Elution buffer-BII composition (EB-BII).

А	В
HEPES pH 7.5	50 mM
NaCl	1 M
EDTA	2 mM
DTT	5 mM
Glycerol	5% v/v
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- 12.7 Pool all elution fractions carrying the recombinant RT protein. Store at 4 °C until use.
- 13 Purified RT enzyme concentration and formulation.
- 13.1 Load the purified RT enzyme pool onto a dialysis membrane (pre-hydrated). Place the membrane into a beaker with precooled storage buffer-B (SB-B) at a ratio 1:50 (v/v).

Storage buffer-B composition (SB-B).

A	В
Tris-HCl pH 7.5	50 mM
NaCl	150 mM
EDTA	0.1 mM
DTT	1 mM
Glycerol	50% v/v

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- Dialyze Overnight at 4 °C with slow agitation.
- Recover the dialized protein, load it onto an **Amicon Ultra-15ML 30 kDa cutoff centrifugal filter.** Concentrate until a concentration equal or higher than [M] 1 mg/mL

Equipment

NAME **Amicon Ultra-15**

PLTK Ultracel-PL membrane, 15 ML - 30 kDa cutoff

BRAND

TYPE

Millipore

SKU

UFC903024

 $https://www.merckmillipore.com/MX/es/product/Amicon-Ultra-15-Centrifugal-Filter-\ ^{LINK}$ Unit,MM_NF-UFC903024?ReferrerURL=https%3A%2F%2Fwww.bing.com%2F&bd=1

Note

Monitor protein concentration measuring absorbance at 280 nm using a NanoDrop spectrophotometer.

Equipment

NanoDrop™ One UV-Vis Spectrophotometer

NAME

spectrophotometer

TYPE

Thermo Scientific

BRAND

ND-ONE-W

SKU LINK

https://www.thermofisher.com/order/catalog/product/ND-ONE-W

SPECIFICATIONS

Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10,

64 bit; Voltage: 12 V (DC); Wavelength Range: 190-850 nm

- 13.4 Prepare aliquots of Δ 50 μL of concentrated RT enzyme
- 13.5 Add [M] 0.05 % (v/v) tergitol NP-40 to the enzyme aliquots and store at $$ -20 \,^{\circ}\text{C} $$.
- **13.6** Determine final protein concentration by measuring absorbance at 280 nm in a NanoDrop spectrophotometer.
- 13.8 Analyze the electrophoresis gel by densitometry using the **Image Lab 6.1 Software (Bio-Rad)**. Determine protein concentration for each RT enzyme aliquot analyzed using the protein ladder as weight standard.

The protein ladder

Precision Plus Protein™ Unstained Protein Standards **Bio-rad Laboratories Catalog** #1610363

includes three reference bands: The 50 KDa with 750 ng, the 20 KDa and 100 KDa bands with 150 ng each per each 10 μ L of the protein ladder mix.