Purification of the PE2 nCas9-RT protein

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1 Works for me Share

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ABSTRACT

This protocol describes the process of expressing and purifying the nicking Cas9-MMLV RT fusion protein for prime editing.

Protocol overview

A. Heat-shock Transformation
B. Protein Expression
C. Protein Purification

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MATERIALS TEXT

Item	Vendor	Catalog #
Tryptone	U.S. Biotech Sources, LLC	T01PD-500
Yeast extract	BD Bacto	288620 212750
NaCl	Fisher Scientific	S271
KCl	Macron Fine Chemicals	6858-06
MgCl2	Fisher Scientific	BP214
MgSO4	Fisher Scientific	M63
Glucose	Sigma	G8270
Kanamycin	Goldbio	K-120-SL25
Chloramphenicol	Goldbio	C-105-5
IPTG	Goldbio	I2481C
HEPES	Omnipur	5320
Imidazole	Sigma	12399
DTT	Goldbio	DTT100
PMSF	Sigma	P7626
Ni-NTA Superflow	QIAGEN	30410
HiTrap heparin HP	GE Healthcare	17040601
Spin-X UF 20 50 kDa MWCO	Corning	431488
DMSO	Fisher Scientific	BP231-100
Leupeptin	Millipore	634987
Pepstatin	Sigma	P5318
Chymostatin	Sigma	C7268
Aprotinin	Sigma	A6279
Antipain	Millipore	6C0417

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A. Heat-shock Transformation 2h 34m 45s

1 Thaw frozen competent cells **On ice** until just thawed.

2 Gently mix the thawed competent cells (Rosetta 2 (pLysS)) by flicking the tube.

3 Transfer 100 µl competent cells to a chilled culture tube.

4 Add 1 ng DNA plasmid to the cells.

5 Immediately return the tubes **On ice** for **00:30:00** 30m

6 Heat-shock the cells at **42 °C** for **00:00:45** . 45s

7 Immediately place the tube **On ice** for **00:02:00** . 2m

8 Add 900 µl of cold SOC medium to the tube and incubate for **01:00:00** at **37 °C** with shaking **175 rpm** 1h

8.1 SOC medium

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A	B
Tryptone	2%
Yeast extract	0.5 %
NaCl	10 mM
KCl	2.5 mM
MgCl2	10 mM
MgSO4	10 mM
Glucose	20 mM

9 Pellet the cells by centrifugation at **12000 x g, 00:02:00** 2m

10 Remove the supernatant and plate onto agar plates containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol.

11 Incubate the plate at **37 °C** for **Overnight** 1h

B. Protein Expression 16h 10m

12 Inoculate one colony into 50 ml LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Incubate **Overnight** on shaker **175 rpm** at **37 °C** . 16h

12.1 LB

A	B
Tryptone	2 %
Yeast extract	0.5 %
NaCl	10 mM

13 Transfer the overnight culture into 1 liter LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol to reach OD_{600} of 0.1.

14 Incubate at **37 °C** with shaking **175 rpm** to reach OD_{600} of 0.6.

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15 Add IPTG to a final concentration of 0.5 mM and grow for **16:00:00** at **18 °C** . 16h

16 Harvest the cells by centrifugation at **5000 x g, 4°C, 00:10:00** 10m

17 Re-suspend the cell pellet with PBS, spin down and snap-freeze in liquid nitrogen for later purification.

C. Protein Purification 35m

18 Assemble a table column and fill the column with Ni-NTA resin to create a bed volume of 5 ml

19 Wash the column with 100 ml H2O.

20 Equilibrate the column with 5 CVs Ni-NTA loading buffer.

20.1 Ni-NTA loading buffer

A	B
HEPES-KOH pH 7.6	25 mM
KCl	150 mM
Imidazole	20 mM
DTT	1 mM
PMSF	1 mM

21 Thaw the cell pellet **On ice** until just thawed.

22 Re-suspend cell pellet (from 1 liter) with 35 mL lysis buffer

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22.1 Lysis buffer

A	B
HEPES-KOH pH 7.6	25 mM
KCl	1 M
Imidazole	20 mM
DTT	1 mM
PMSF	1 mM
Protease Inhibitor Cocktail	×1

Protease Inhibitor Cocktail (in 70% DMSO; 1000x)

A	B
Leupeptin	0.5 mg/ml
Pepstatin	0.5 mg/ml
Chymostatin	0.5 mg/ml
Aprotinin	0.5 mg/ml
Antipain	0.5 mg/ml

35m

23 Sonicate for **00:05:00** (20 seconds on/off) and clarify by centrifugation at **25000 x g, 00:30:00**

24 Filter the supernatant through a 0.22 µm syringe filter.

25 Pour the supernatant into the table column in a single, continuous motion.

26 Wash the resin with 100 ml Ni-NTA loading buffer followed by 50 ml Ni-NTA wash buffer.

26.1 Ni-NTA wash buffer

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A	B
HEPES-KOH pH 7.6	25 mM
KCl	150 mM
Imidazole	40 mM
DTT	1 mM
PMSF	1 mM

27 Elute the protein in batch six times with 5 ml Ni-NTA elution buffer.

27.1 Ni-NTA elution buffer

A	B
HEPES-KOH pH 7.6	25 mM
KCl	150 mM
Imidazole	500 mM
DTT	1 mM
PMSF	1 mM

28 Analyze fractions by 7.5% SDS-PAGE and coomassie staining.

29 Collect relevant elution fractions, dilute into a low-salt buffer and filter through a 0.22 µm syringe filter

29.1 Low salt buffer

A	B
HEPES-KOH pH 7.6	25 mM
KCl	100 mM
DTT	1 mM
PMSF	1 mM

30 Load onto a 1 ml HiTrap heparin HP column pre-equilibrated in low-salt buffer.

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31 Elute the protein with a linear gradient of 100 mM to 1M KCl over 40 CVs.

32 Analyze fractions by 7.5% SDS-PAGE and coomassie staining.

33 Pool peak elution fractions and concentrate using a Spin-X UF 20 50 kDa MWCO to 8 mg/ml (determine protein concentration by UV at wavelength of 280 nm).

34 Make 3 µl protein sample aliquot and snap-freeze in liquid nitrogen.

35 Store protein at -80 °C.

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