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Preparation of Cas9 Protein

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Protocol status: Working
We use this protocol and it's working

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Abstract

This is a protocol from the **Doudna Lab** for preparation of Cas9 protein.

Attachments

PDF

Cas9_purification_Br...

135KB

Guidelines

The protocol workflow is as follows:

1. Expression of Cas9 in Rosetta2 E. coli

- Transformation (**Steps 1-5**)
- Starter culture (**Steps 6-8**)
- Expression (**Steps 9-21**)

2. Purification of Cas9

- Sonication (**Steps 22-29**)
- HisTrap (**Steps 30-40**)
- TEV (**Steps 41-45**)
- Heparin (**Steps 46-53**)
- BPTrap/Superdex 200 (**Steps 54-65**)

Materials

MATERIALS

Please see before starting in Guidelines section for materials.

Safety warnings

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

Before start

Materials

	TY media	1 L
	Tryptone	10 g
	Yeast extract	6 g
	NaCl	10 g
	Dissolve in 400 mL Rinse flasks thoroughly with MQ water Add 600 mL MQ water, media Autoclave (45', no dry time)	

Buffers

	Lysis	1L, pH7.5
	20mM HEPES	20 ml of 1M
	1M KCl	74.55 g
	10% glycerol	100 ml
	5mM TCEP	1.43 g
	10mM imidazole	680 mg

	Elution	500mL, pH7.5
	20mM HEPES	10 ml of 1M
	0.1M KCl	50 ml of 1M
	10% glycerol	50 ml
	5mM TCEP	717 mg
	300mM imidazole	10.2 g

	Ion Exchange buffer A	1L, pH7.5 (low salt)
	20mM HEPES	20 ml of 1M
	300mM KCl	300 ml of 1M
	10% glycerol	100 ml
	1mM TCEP	287 mg

	Ion Exchange buffer B	1L, pH7.5 (high salt)
	20mM HEPES	20 ml of 1M
	1M KCl	74.55 g
	10% glycerol	100 ml
	1mM TCEP	287 ml

	Gel Filtration	1L, pH7.5
	20mM HEPES	20 ml of 1M
	150mM KCl	150 ml of 1M
	10% glycerol	100 ml
	1mM TCEP	287 mg

Expression of Cas9 in Rosetta2 E. coli - Transformation

1 Mix 1 µl of 10 ng/uL DNA + 10 µl Rosetta 2 cells (pRAR-rare codons).
- 1 µL 10 ng/uL DNA
- 10 µL Rosetta 2 cells

2 Heat shock: 10′ ice, 45″ 42°C, 5′ ice.
- 00:10:00 ice
- 00:00:45 42°C
- 00:05:00 ice

3 Recover: Add 300 µl LB, 50′ 37°C on shaker.
- 300 µL LB
- 37 °C Shaker
- 00:50:00 Shaker

4 Spin down (6000 xg, 1′), and remove 150 µl LB.
- 00:01:00 Spin down

5 Plate 100 µl, 30 µl on 2 AMP plates.

Expression of Cas9 in Rosetta2 E. coli - Starter culture

6 Pick 6 colonies to add to 20 ml LB + Amp (Cf=100 ug/ml) starter culture.
- 20 mL LB + Amp (Cf=100 ug/mL) starter culture

7 Grow overnight.

8 Next day: Store XL1 blue 20% glycerol stocks in -80°C.
- -80 °C Storage

Expression of Cas9 in Rosetta2 E. coli - Expression

9 Add AMP to the flasks (Cf=100 ug/ml).

10 Add 20 ml starter culture in the morning,
20 mL starter culture

11 Grow to OD600=0.6 (150 rpm, ~4 hrs, 37°C).
37 °C Growing
04:00:00 Growing

12 Set shaker temp to 16°C.
16 °C Shaker temperature

13 *Save 1 ml for SDS-PAGE: **uninduced**.

14 Add 0.5 mM IPTG.

15 Grow overnight (150 rpm, at least 16 hrs, 16°C).
16 °C Growing
16:00:00 Growing

16 Harvest cells before lunch: Spin down (4000 rpm, 20′, 4°C).
4 °C Spin down
00:20:00 Spin down

Note

***Keep the cells cold from here.**

17 Completely resuspend pellets in 20 ml Lysis Buffer + 1mM PMSF per bottle.
20 mL Lysis Buffer + 1mM PMSF

18 Use additional 20 ml Lysis Buffer to wash all bottles.
20 mL Lysis Buffer

19 Transfer to 50 ml-tubes.

20 *Save 20 µl for SDS-PAGE: **cell pellet**

21 Freeze the cells at -80°C.
-80 °C Freezing cells

Purification of Cas9 - Sonication

22 Thaw cells in water.

23 Push beaker with a stir bar to the bottom of ice bucket.

24 Add ~50 ml Lysis Buffer and 1 protease inhibitor tablet/50 ml cell. Dissolve the tablets.
50 mL Lysis Buffer

25 Transfer the cells to the beaker.

26 Sonicate while stirring: 8′ total, 10″ on, 10″ off, level 4. Lysate should turn fluid and thin. Repeat 8 min cycle, if necessary.
00:08:00 total
00:00:10 on/off

27 Transfer to Oak Ridge tubes (30 ml/tube). *Balance within +/- 0.01g.

28 Pellet cell debris (18,000 rpm, 30′, 4°C). Prepare HisTrap purification.

HisTrap
CV=5mL, FR=2mL/min
Buffer A: Lysis buffer, Buffer B: Elution buffer

4 °C Pellet cell debris
00:30:00 Pellet cell debris

29 *Save 20 µl supernatant for SDS-PAGE: **cell lysate**

Purification of Cas9 - HisTrap

30 Filter sample.

Note

HisTrap
CV=5ml, FR=2ml/min
Buffer A: Lysis buffer, Buffer B: Elution buffer

31 Set-up Akta:

1. Turn on UV lamp.

2. Wash Akta with water: pump wash, and flow 50% A, 50% B.

3. Pump wash.

4. Flow 50% A, 50% B.

5. Flow 100% A, until steady baseline. Set baseline to zero.

32 Apply sample with pump, in the cold room:

1. Fill tubing with water, connect column.

2. Wash: 5CV water.

3. Equilibrate: 5CV lysis buffer.

4. Apply sample to the column. Collect flow-through: Ni-sample loading.
Re-apply flow-through to the column, if necessary.

33 Purification:
In the cold room:
First wash: 15CV lysis buffer. Collect flow-through: Ni-wash1.
Collect flow-through: **Ni-wash1**. *Save 20µl for SDS-PAGE.

34 Wash2: 3CV lysis buffer + 0.1% Triton X.
Collect flow-through: **Ni-wash2**.

*Save 20 µl for SDS-PAGE.

35 Purification using Akta:
First, attach column to Akta (FR=0.9 ml/min).

36 Wash: 5CV lysis buffer, until steady UV baseline.

37 Elute: 25CV elution buffer, 100% gradient.

38 Collect fractions (Fraction size=2.5 ml).

39 Clean-up:

1. Wash: 5CV water

2. Store: 5CV 20% ethanol

3. Detach column.

4. Wash Akta with water.

40 Pool appropriate fractions. Rinse collection tubes with elution buffer.
*Save 10 µl for SDS-PAGE: **Ni-elution**

Purification of Cas9 - TEV

41 Concentrate with 30K Amicon to ~3 ml volume. Collect flow-though: Ni-Amicon FT.

42 Add ~1.25 mg TEV/3 ml sample, and mix with pipet.
1.25 mg TEV

43 Incubate on shaker, at 4°C overnight.
4 °C Incubation on shaker
16:00:00 overnight incubation

44 Next day: *Save 10 µl for SDS-PAGE: **postTEV**

45 Add 25 ml of buffer A to sample.
25 mL Buffer A

Purification of Cas9 - Heparin

46 **Heparin**
CV=5 mL, FR=1 mL/min
Buffer A: IEX low salt buffer, Buffer B: IEX high salt buffer

47 Set-up Akta (FR=2 ml/min).

48 Apply sample with pump, in the cold room:

1. Equilibrate: 10CV buffer A.

2. Apply sample to the column. Collect flow-through: Hep-sample loading.

49 Purification:

1. Attach column to Akta (FR=0.9 ml/min).

2. Wash: 15CV buffer A, until steady baseline.

3. Collect flow-through: **Hep-wash**. *Save 20 µl for SDS-PAGE.

4. Elute: 20CV buffer B, 100% gradient.

5. Collect fractions (Fraction size=2 ml).

50 Clean-up (FR=2ml/min):

1. Wash: buffer B, until steady conductivity baseline for over 30 ml.

2. Store: 5CV 20% ethanol.

3. Detach column.

4. Wash Akta with water.

51 Pool appropriate fractions. Rinse collection tubes with buffer B.
*Save 10 µl for SDS-PAGE: **Hep-elution**

52 Concentrate sample to ~1 ml. Collect flow-through: Hep-Amicon FT.

53 Leave concentrator on ice, at 4°C overnight.
4 °C Concentrator on ice

Purification of Cas9 - MBPTrap/Superdex 200

54 **MBPTrap/Superdex 200**

CV=120 mL, FR=0.5-1 mL/min
Buffer A: Gel filtration buffer

Previous day:
1. Set-up Akta (FR=2 mL/min):
2. Prepare columns:
a. MBPTrap wash: 5CV water, 5CV gel filtration buffer (FR=2 mL/min), in the cold room.
b. Attach MBPTrap and Superdex200 to Akta.
c. Equilibrate overnight: 2CV gel filtration buffer (FR=0.3 mL/min).
*Set end timer volume=240 mL, max pressure alarm=0.25 MPa.

55 Concentrate sample to ~500 µl. Rinse concentrator with 500 µl gel filtration buffer.
500 µL Gel filtration buffer

56 Spin down sample (11000 rpm, 4′, 4°C). Transfer supernatant to a new 1.5 ml-tube.
4 °C Spin down
00:04:00 Spin down

57 To inject sample, first, clean injection port, sample loop with water, gel filtration buffer.

58 Now, draw up sample with 1 ml-syringe, and replace the syringe on the injection port.

59 [LOAD] Inject sample to the loop with syringe.

60 Purification

1. [INJECT] Apply sample to the column (~3 ml).

2. [LOAD] Flow ~1CV gel filtration buffer. Cas9 elutes at 65-85 ml.

3. Collect fractions (Fraction size=2 ml).

61 Clean-up (FR=2 ml/min):
1. Detach MBPTrap.
2. Wash MBPTrap: 10CV 10mM maltose + gel filtration buffer, in the cold room.
3. Store MBPTrap: 5CV 20% ethanol.
4. Wash Superdex200: 2CV water (Long-term storage in 20% ethanol).
5. Detach Superdex200.
6. Wash Akta with water.

62 Pool appropriate fractions. Rinse collection tubes. *Save 5 µl for SDS-PAGE: **Cas9**

63 Concentrate to desired concentration (~40 uM).

*Cas9	MW=158,441.4 g/mol Molar absorption coefficient=120,575 L/mol·cm

64 Aliquot 20 µl each into 1.5 ml-tubes.

65 Snap-freeze in LN2, and store at -80°C.

-80 °C Storage