Preparation of Cas9 Protein

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Abstract

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

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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)

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

Materials

✓ Please see before starting in Guidelines section for materials. by Contributed by users

Protocol

Expression of Cas9 in Rosetta2 E. coli - Transformation

Step 1.

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

AMOUNT
1 µl : 10 ng/uL DNA
AMOUNT
10 µl : Rosetta 2 cells

Expression of Cas9 in Rosetta2 E. coli - Transformation

Step 2.

Heat shock: 10’ ice, 45” 42°C, 5’ ice.

DURATION
00:10:00 : ice
DURATION
00:00:45 : 42°C

DURATION
00:05:00 : ice

Expression of Cas9 in Rosetta2 E. coli - Transformation

Step 3.

Recover: Add 300 µl LB, 50’ 37°C on shaker.

AMOUNT
300 µl : LB
TEMPERATURE
37 °C : Shaker
DURATION
00:50:00 : Shaker

Expression of Cas9 in Rosetta2 E. coli - Transformation

Step 4.

Spin down (6000 xg, 1’), and remove 150 µl LB.

DURATION
00:01:00 : Spin down

Expression of Cas9 in Rosetta2 E. coli - Transformation

Step 5.

Plate 100 µl, 30 µl on 2 AMP plates.

Expression of Cas9 in Rosetta2 E. coli - Starter culture

Step 6.

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

AMOUNT
20 ml : LB + Amp (Cf=100 ug/mL) starter culture

Expression of Cas9 in Rosetta2 E. coli - Starter culture

Step 7.

Grow overnight.

Expression of Cas9 in Rosetta2 E. coli - Starter culture

Step 8.

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

TEMPERATURE
-80 °C : Storage

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 9.

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

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 10.

Add 20 ml starter culture in the morning,

AMOUNT

20 ml : starter culture

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 11.

Grow to OD600=0.6 (150 rpm, 4 hrs, 37°C).

TEMPERATURE

37 °C : Growing

DURATION

04:00:00 : Growing

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 12.

Set shaker temp to 16°C.

TEMPERATURE

16 °C : Shaker temperature

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 13.

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

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 14.

Add 0.5 mM IPTG.

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 15.

Grow overnight (150 rpm, at least 16 hrs, 16°C).

TEMPERATURE

16 °C : Growing

DURATION

16:00:00 : Growing

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 16.

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

TEMPERATURE

4 °C : Spin down

DURATION

00:20:00 : Spin down

NOTES

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

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 17.

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

AMOUNT

20 ml : Lysis Buffer + 1mM PMSF

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 18.

Use additional 20 ml Lysis Buffer to wash all bottles.

AMOUNT

20 ml : Lysis Buffer

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 19.

Transfer to 50 ml-tubes.

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 20.

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

Expression of Cas9 in Rosetta2 E. coli - Expression

Step 21.

Freeze the cells at -80°C.

TEMPERATURE

-80 °C : Freezing cells

Purification of Cas9 - Sonication

Step 22.

Thaw cells in water.

Purification of Cas9 - Sonication

Step 23.

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

Purification of Cas9 - Sonication

Step 24.

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

AMOUNT

50 ml : Lysis Buffer

Purification of Cas9 - Sonication

Step 25.
Transfer the cells to the beaker.

Purification of Cas9 - Sonication

Step 26.

Sonicate while stirring: 8’ total, 10” on, 10” off, level 4. Lysate should turn fluid and thin. Repeat 8 min cycle, if necessary.

DURATION
00:08:00 : total
DURATION
00:00:10 : on/off

Purification of Cas9 - Sonication

Step 27.

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

Purification of Cas9 - Sonication

Step 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

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

Purification of Cas9 - Sonication

Step 29.

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

Purification of Cas9 - HisTrap

Step 30.

Filter sample.

NOTES

HisTrap

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

Purification of Cas9 - HisTrap

Step 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.

Purification of Cas9 - HisTrap

Step 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.

Purification of Cas9 - HisTrap

Step 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.

Purification of Cas9 - HisTrap

Step 34.

Wash2: 3CV lysis buffer + 0.1% Triton X.

Collect flow-through: **Ni-wash2**.

*Save 20 µl for SDS-PAGE.

Purification of Cas9 - HisTrap

Step 35.

Purification using Akta:

First, attach column to Akta (FR=0.9 ml/min).

Purification of Cas9 - HisTrap

Step 36.

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

Purification of Cas9 - HisTrap

Step 37.

Elute: 25CV elution buffer, 100% gradient.

Purification of Cas9 - HisTrap

Step 38.

Collect fractions (Fraction size=2.5 ml).

Purification of Cas9 - HisTrap

Step 39.

Clean-up:

1. Wash: 5CV water

2. Store: 5CV 20% ethanol

3. Detach column.

4. Wash Akta with water.

Purification of Cas9 - HisTrap

Step 40.

Pool appropriate fractions. Rinse collection tubes with elution buffer.

*Save 10 µl for SDS-PAGE: **Ni-elution**

Purification of Cas9 - TEV

Step 41.

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

Purification of Cas9 - TEV

Step 42.

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

AMOUNT

1.25 mg : TEV

Purification of Cas9 - TEV

Step 43.

Incubate on shaker, at 4°C overnight.

TEMPERATURE

4 °C : Incubation on shaker

DURATION

16:00:00 : overnight incubation

Purification of Cas9 - TEV

Step 44.

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

Purification of Cas9 - TEV

Step 45.

Add 25 ml of buffer A to sample.

AMOUNT
25 ml : Buffer A

Purification of Cas9 - Heparin

Step 46.

Heparin

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

Purification of Cas9 - Heparin

Step 47.

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

Purification of Cas9 - Heparin

Step 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.

Purification of Cas9 - Heparin

Step 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).

Purification of Cas9 - Heparin

Step 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.

Purification of Cas9 - Heparin

Step 51.

Pool appropriate fractions. Rinse collection tubes with buffer B.

*Save 10 µl for SDS-PAGE: **Hep-elution**

Purification of Cas9 - Heparin

Step 52.

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

Purification of Cas9 - Heparin

Step 53.

Leave concentrator on ice, at 4°C overnight.

TEMPERATURE

4 °C : Concentrator on ice

Purification of Cas9 - MBPTrap/Superdex 200

Step 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.

Purification of Cas9 - MBPTrap/Superdex 200
Step 55.

Concentrate sample to 500 µl. Rinse concentrator with 500 µl gel filtration buffer.

AMOUNT
500 µl : Gel filtration buffer

Purification of Cas9 - MBPTrap/Superdex 200
Step 56.

Spin down sample (11000 rpm, 4’, 4°C). Transfer supernatant to a new 1.5 ml-tube.

TEMPERATURE
4 °C : Spin down
DURATION
00:04:00 : Spin down

Purification of Cas9 - MBPTrap/Superdex 200
Step 57.

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

Purification of Cas9 - MBPTrap/Superdex 200
Step 58.

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

Purification of Cas9 - MBPTrap/Superdex 200
Step 59.

[LOAD] Inject sample to the loop with syringe.

Purification of Cas9 - MBPTrap/Superdex 200
Step 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).

Purification of Cas9 - MBPTrap/Superdex 200

Step 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.

Purification of Cas9 - MBPTrap/Superdex 200

Step 62.

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

Purification of Cas9 - MBPTrap/Superdex 200

Step 63.

Concentrate to desired concentration (40 uM).

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

Purification of Cas9 - MBPTrap/Superdex 200

Step 64.

Aliquot 20 µl each into 1.5 ml-tubes.

Purification of Cas9 - MBPTrap/Superdex 200

Step 65.

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

TEMPERATURE

-80 °C : Storage

Warnings

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