His10-MBP-Cas9 purification

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Abstract

This is a protocol from the Doudna Lab for His10-MBP-Cas9 purification.

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Guidelines

This protocol takes two days to execute.

Before start

DAY 1 materials

Lysis Buffer	**200 ml**
50 mM Tris-Cl, pH 7.5	10 ml 4M
500 mM NaCl	25 ml 4M
1 mM TCEP	400 µl 0.5M
5% glycerol	10 ml 100%
0.5mM PMSF	250 µl 400 mM
protease inhibitors (Roche)	4 tablets

Wash Buffer	**500 ml**
50 mM Tris-Cl, pH 7.5	25 ml 1M
500 mM NaCl	62.5 ml 4M
1 mM TCEP	1 ml
10 mM imidazole	1.67 ml 3M
5% glycerol	25 ml 100%

Elution Buffer	**200 ml**
50 mM Tris-Cl, pH 7.5	10 ml 1M
500 mM NaCl	25 ml 4M
1 mM TCEP	0.4 ml 0.5M
300 mM imidazole	20 ml 3M
5% glycerol	10 ml 100%

DAY 2 materials

Cas9 IEX Buffer A	**1500 ml**
20 mM Tris-Cl, pH 7.5	30 ml 1M
125 mM KCl	93.75 ml 2M
1 mM TCEP	3 ml 0.5M
5% glycerol	75ml 100%

Cas9 IEX Buffer B	**500 ml**
20 mM Tris-Cl, pH 7.5	20 ml 1M
1 M KCl	250 ml 2M
1 mM TCEP	1 ml 0.5M
5% glycerol	25 ml 100%

Cas9 Gel Filtration Buffer	**1000 ml**
20 mM Tris-Cl, pH 7.5	20 ml 1M
200 mM KCl	100 ml 2M
1 mM TCEP	2 ml 0.5M
5% glycerol	50 ml 100%

Materials

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

Protocol

DAY 1

Step 1.

Harvest cells from large-scale overnight cultures by spinning down in 1 L centrifuge bottles: 20 minutes at 4000 rpm, 4 °C.

TEMPERATURE
4 °C : Spinning down
DURATION
00:20:00 : Spinning down

NOTES

One of the two centrifuges can only go up to max speed of 3400 rpm.

DAY 1

Step 2.

Pour off supernatant and either freeze cell pellet (in centrifuge bottle) at -80 °C until purification, or proceed to step #3.

TEMPERATURE
-80 °C : Freezing cell pellet

NOTES

Better to not keep pellets in centrifuge bottles too long, since this will deplete the lab supply.

DAY 1

Step 3.

Resuspend cell pellets in lysis buffer. Use 10-15 ml per 1 L of culture. Multiply accordingly based on the number of flasks you grew. Either use resuspended cells and proceed directly to step #4, or transfer to 50 ml Falcon tubes, flash freeze in liquid nitrogen, and store at -80 °C until purification.

Save 20 µL for SDS-PAGE analysis: “cell pellet”

TEMPERATURE
-80 °C : Storage

DAY 1

Step 4.

Lyse cells by sonication. Do not do this in glass containers (they can shatter).

- Sonicate at power setting 6.0: 10 seconds on, 20 seconds off, total run time of 5 minutes. Cells must be on ice during this step, as the temperature will increase during sonication and the cells must stay cold.

DURATION
00:05:00 : total
DURATION
00:00:10 : on
DURATION
00:00:20 : off

NOTES

Either sonicate directly in 50 ml Falcon tubes, or Oakridge tubes, or for large volumes, in plastic beakers. For small cultures I use the sharp tipped needle on the sonicator, but usually (for resuspended pellets deriving from >2 L of culture) I use the blunt-ended tip on the sonicator. Make sure it is screwed on tightly. Before setting up sonication, wash the tip in H2O and EtOH, and dry before use with Kimwipe.

DAY 1

Step 5.

Clarify cell lysate by centrifugation at 15,000 rpm for 30 minutes, 4 °C.

During the 30 minute spin, equilibrate Ni-NTA resin in wash buffer.

(1) To equilibrate, transfer resin into 50 ml Falcon tube, and add mQ H_2O to about the 50ml mark. Invert a few times to fully resuspend the resin. Centrifuge at 2400 rpm (no higher!) for 3 minutes, and gently pour off supernatant into the sink immediately after spin is over. These pellets are quite fragile, and will begin to dissipate in the supernatant rather quickly. Avoid accidentally pouring off your Ni-NTA resin.

(2) Repeat (1) by adding more mQ H_2O, spinning, and pouring off supernatant.

(3) Repeat (2) 2-3 times, but using wash buffer instead.

TEMPERATURE
4 °C : Centrifugation

DURATION
00:03:00 : Centrifugation II
DURATION
00:30:00 : Centrifugation I

NOTES

It is critical that tubes are precisely balanced for these rotors spinning at these high speeds. Use the digital balance and balance to within +/- 0.01 g.

To equilibrate Ni-NTA resin in wash buffer, I generally use a column volume of ~5 ml resin for 2-4 L of culture, and then scale accordingly if growing less or more. (Note that the Ni-NTA stock bottles are a 50% (v/v) suspension in EtOH, and so for 5 ml column volume you need to use 10 ml of this suspension.

DAY 1

Step 6.

After the spin in step #5 concludes, **save 20 µl for SDS-PAGE analysis: “cell lysate”**

DAY 1

Step 7.

Now, transfer entire supernatant (by decanting or with a serological pipette... I prefer a pipette) to the Falcon tube containing your Ni-NTA resin equilibrated in wash buffer.

NOTES

If volume of supernatant is larger than will fit into one Falcon tube, you can split sample into 2 (or more), being sure that the Ni-NTA resin and your lysate is evenly mixed between each. Be careful to avoid disturbing the cell debris pellet when removing the supernatant.

DAY 1

Step 8.

Rock the Ni-NTA resin with cell lysate at 4°C, 30-60 minutes, in the Falcon tube.

TEMPERATURE
4 °C : Rocking Ni-NTA resin
DURATION
00:30:00 : Rocking Ni-NTA resin

DAY 1

Step 9.

Pour Ni-NTA resin with lysate into disposable plastic (or glass) column. If using a glass column, be sure to thoroughly wash with copious amounts of EtOH and water before use.

Save 20 µl of the flow-through for SDS-PAGE analysis: “flow-through, FT.” Aside from this,

the flow-through will not be saved and so can be collected in a waste container.

NOTES

During steps #9-12, also monitor the amount of protein in each fraction via the Bradford assay.

a) Bradford reagent undergoes a color change in the presence of protein. It can be used to do quantitative protein concentration measurements, or in this case, give us a quick colorimetric read-out for: if our washes succeeded than there should be no protein coming off the column during wash #5, and so no color change. Similarly, Bradford assay will tell us which of our elution fractions contain a substantial amount of our protein of interest (or any protein at all!)

b) Note that one could also use the A280nm reading to determine protein concentration, but it's more work at this stage then necessary.

DAY 1

Step 10.

Wash the resin with 5 column volumes.

Save 20 µl of the flow-through for SDS-PAGE analysis: "wash #1, W1." Aside from this, the washes will not be saved and can be collected in a waste container.

DAY 1

Step 11.

Repeat step #10 4 more times, for a total of 5 washes.

Save 20 µl of the flow-through for SDS-PAGE analysis: "W2, W3, etc." Aside from this, the washes will not be saved and can be collected in a waste container.

DAY 1

Step 12.

Elute by adding 1 column volume of elution buffer, 5 times. Be sure to collect these fractions which contain our protein of interest! Collect each elution fraction in a separate 15 ml Falcon tube.

Save 20 µl of each elution fraction for SDS-PAGE analysis: "elution #1, E1, E2, etc."

DAY 1

Step 13.

Based on Bradford, pool the elution fractions that contain protein. Measure the absorbance at 280 nm and record this, along with the volume, to give us the overall yield. Also, print out the absorbance spectrum for your notebook, since this has information on the purity of protein (or, if it is co-purifying with any nucleic acid).

DAY 1

Step 14.

If cleaving off the His_{10}-MBP fusion, add TEV protease to elution fractions.

NOTES

The TEV protease purified by Kaihong is typically 0.5 mg per tube (250 µl); use 1 tube for every 10–20 mg of purified protein.

DAY 1

Step 15.

After adding TEV protease to the eluate, dialyze overnight in Wash Buffer using a Slidealyzer cassette, MWCO = 10k or 30k. Make sure the buffer is pre-chilled to 4 °C.

DAY 2

Step 16.

Run TEV-cleaved Cas9 back over the Ni-NTA resin, being sure to equilibrate the resin in Wash Buffer first (>10 CV). Collect the flow-through. Add 1 column volume of wash buffer and collect with the flow-through.

DAY 2

Step 17.

Place TEV-cleaved Cas9 back into a Slidealyzer cassette, and dialyze into IEX Buffer A for **2– 4 hours**.

DURATION

02:00:00 : Dialysis

NOTES

It is critical that this dialysis not proceed longer, as Cas9 is not very soluble at low salt and can will precipitate with prolonged incubation. (The disadvantage of raising the salt concentration is that Cas9 will no longer bind the SP column efficiently.)

DAY 2

Step 18.

Purify Cas9 by ion exchange, using a HiTrap HP SP column. Protocol: 0–100% Buffer B over the course of 20 column volumes. Cas9 should elute at 26 %B. Concentrate to <2 ml.

DAY 2

Step 19.

Purify Cas9 by gel filtration, using a Superdex 200 16/60 column. Concentrate and snap freeze.

Warnings

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