Cas9 Expression and Purification Protocol

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Abstract

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Protocol

Expression Day

Step 1.

Start starter culture (containing amp & chlor) from a glycerol stock. If there's no glycerol stock, transform and plate Rosetta cells and pick colonies. Reserve shaker as needed

Make Terrific Broth and autoclave as needed. The media should contain MgCl2 and glycerol

Expression Day 2

Step 2.

- Preheat shaker to 37°C
- Add ampicillin (100 µg/L) and chloramphenicol (35 µg/mL) to the media. Remove small sample of media to use as blank later.
- Measure O.D.600 of starter culture (may have to dilute 1:10 to get reading ≤1.0). Add enough to each flask so that O.D.600 of flask ≈ 0.1 (5 ml).
- Shake (250 rpm) for 4 hrs until A600 ≈ 1.0 – check regularly starting at 2 hours.
- Add IPTG to cultures to 0.5 mM ; change shaker temp to 16°C; leave cultures in cold room while shaker cools down.
- Shake (250 rpm) at 16°C overnight (16 h).

Expression Day

Step 3.

- Pre-chill high-speed centrifuge to 4°C.
- Collect cultures and pour into 1 L jars.
- Spin using JLA-9.1000 rotor at 4000×G for 15′
- Prepare appropriate volume of Lysis Buffer (60 ml per 1 L culture grown) and add 1 M TCEP (pH 7.5) stock to 1 mM.
- Thoroughly resuspend pellets in Lysis Buffer and combine.

- • Freeze. (No flash-freeze necessary.)

Purification Day 1

Step 4.

- Thaw 6 × 1 L cell pellets. Pre-chill high speed centrifuge with rotor JA -20. Prepare 100 mL Lysis Buffer, 100 mL Wash Buffer, and 50 mL Elution Buffer, all containing 1 mM TCEP.

Purification Day 1

Step 5.

- Add PMSF to 1 mM. Crush & dissolve one EDTA-free Complete protease inhibitor tablet per liter in small quantity of lysis buffer and add to thawed, resuspended pellet solution.

ANNOTATIONS

Shaohsuan Wen 04 May 2018

I would like to know the recipes of buffers

Purification Day 1

Step 6.

Using a 60 mL syringe fitted with a 21 gauge (green) needle, pass the solution into a new container.

Purification Day 1

Step 7.

Homogenize: Flush system with water, and then with lysis buffer. Pass sample through at no pressure, then slowly increase air pressure. Pass through twice at pressure. For instructions on homogenizer use, see Ariana, Nick, Frederick, or Luke.

Purification Day 1

Step 8.

Spin down homogenate in balanced Oakridge tubes (≤33 mls/tube). Spin at 18,000 × g for 30′. Start the step below.

Purification Day 1

Step 9.

During the above spin, set up peristaltic pump (P-1). Run water through pump to remove air. Attach 2 × 5 mL HisTrap (Fast Flow) columns in tandem (use wet connections throughout). Flow 25 mL Lysis Buffer to equilibrate columns. For ≤ 6L of prep, set up a second peristalitic pump and load additional HisTrap columns in parallel.

Purification Day 1

Step 10.

After the spin is complete, combine supernatants and keep the container on ice. Take 20 µL sample for gel, and run the sample through column at 3 ml/min (“4” on the dial). Collect flowthrough in clean

vessel in case binding fails.

Purification Day 1

Step 11.

Run through 100 ml (10CV- scale all of these up if changing CV) of lysis buffer. Collect sample from flowthrough for SDS-PAGE

Run through 100 mL of wash buffer at room temperature. take sample from flowthrough

Run through 100 ml of lysis buffer. Take sample from the output for SDS-PAGE. Label 10 × 15 mL conical tubes E1–E10.

Purification Day 1

Step 12.

Elute. Begin flowing 50 mL of elution buffer, and collect 5 mL in tube E1. Stop the pump and wait for 5-10′. Resume the flow and collect nine more 5 mL fractions in tubes E2–10. Save the fractions on ice and take a sample from each for SDS-PAGE

Purification Day 1

Step 13.

Spec each sample from tubes E1–E10 and record the results. Pool all fractions with an estimated concentration >1 mg/mL. Take a sample for SDS-PAGE. Calculate rough estimate of yield in mgs (assuming 1 O.D.280 = 1 mg/ml). Add one 200 µl aliquot of 2 mg/ml TEV protease per 40 mg of eluted material (1 mg TEV per 100 mg Cas9). Incubate overnight (16 h) at 4°C. For especially large volumes, gently mix during this step.

Purification Day 2

Step 14.

Use pump P-1 to run 0.2 M NaOH over 2 × 5 mL HiTrap Heparin HP columns connected in tandem for 30′ to remove endotoxin. Wash with 10 mL H2O as rinse.

Purification Day 2

Step 15.

Take sample of TEV-digested material. Pre-chill centrifuge to 4°C.

Purification Day 2

Step 16.

Concentrate sample (Amicon Ultra, 100 kDa MWCO) at 4°C, 4000 × g until the total volume is 2 ml (per 6 L prep - if prep is ≥6 L, perform additional buffer exchange via dilution w/ **A** to reduce [salt] before loading).

Purification Day 2

Step 17.

Add TCEP to 1 mM for Ion Exchange Buffers **A** and **B**. Make 3 × 50ml aliquots of **A**. Run 50 mL **A** over heparin columns to equilibrate

Purification Day 2

Step 18.

Once protein is concentrated to 2 mL, take out 2 µl sample for SDS-PAGE and add the rest to 50 mL **A**. also take a sample of sample of the flow-through. Run the sample over heparin columns & post-wash w/ 50 mL **A** (can perform “no wash” FPLC program if this step is performed; can also let the standard FPLC program wash with **A**).

Purification Day 2

Step 19.

Meanwhile, set up FPLC. Put line A1 into buffer A and B1 into buffer B (250 mL each). Run setup A B program

Purification Day 2

Step 20.

Once the sample is loaded onto the columns, bring it to the FPLC. Put 2 96-well blocks into the fraction collector & let it sense their location. Follow the FPLC protocol for ion exchange to connect the column & prepare the system. Run Heparin_5ml-up-flow program (90′).

Purification Day 2

Step 21.

After the run: remove the blocks, decide on peak, take samples of every other fraction (and any unexpected peaks) to run on SDS-PAGE. Combine chosen fractions and refrigerate overnight

Purification Day 3

Step 22.

• NaOH wash Superdex 200 column as needed (0.2 M, 30′ contact time) and equilibrate into Gel Filtration Buffer (500 mL in bottle, double check waste container). Equilibration takes about 2 hrs.

Purification Day 3

Step 23.

Concentrate chosen fractions down to ≤2 mL per 6 L prep; when removing sample from Amicon concentrator, rinse filter w/ GF buffer.

Purification Day 3

Step 24.

Pre-chill microcentrifuge. Spin down the ≤2 mL sample in eppendorf tube(s) at max speed for 5′ to pellet any precipitate. Take 1 µl sample from for SDS page analysis from the bottom of the concentrator.

Purification Day 3

Step 25.

Load sample into 3ml syringe using low gauge needle. Bring the sample to the FPLC.

Purification Day 3

Step 26.

Run the manual load program, put one 96-well block into fraction collector. Flush import valve by injecting a few mL of buffer. Then screw in your syringe and manually load sample, injecting at a rate of 1 mL/min (6 seconds per 100 µL). Next start the run (2 h). It’s OK to stop after peak is fully eluted (at 80 mL)

Purification Day 3

Step 27.

Remove block from fraction collector. Take samples of peak fractions (especially upstream edge of main peak) and anything else interesting for SDS-PAGE. Spec to determine concentration

Purification Day 3

Step 28.

Run gel of all samples taken so far. Based on the results, pool pure fraction

Purification Day 3

Step 29.

Purification Day 3

Step 30.

Aliquot, label with name/date/concentration/amount, and flash-freeze in liquid nitrogen. Consider setting aside dedicated aliquots for endotoxin testing, activity assays, etc.