

Expression and Purification of the Cas10-Csm Complex from Staphylococci

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[Abstract] CRISPR-Cas (Clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins) is a class of prokaryotic immune systems that degrade foreign nucleic acids in a sequence-specific manner. These systems rely upon ribonucleoprotein complexes composed of Cas nucleases and small CRISPR RNAs (crRNAs). *Staphylococcus epidermidis* and *Staphylococcus aureus* are bacterial residents on human skin that are also leading causes of antibiotic resistant infections (Lowy, 1998; National Nosocomial Infections Surveillance, 2004; Otto, 2009). Many staphylococci possess Type III-A CRISPR-Cas systems (Marraffini and Sontheimer, 2008; Cao *et al.*, 2016), which have been shown to prevent plasmid transfer and protect against viral predators (Goldberg *et al.*, 2014; Hatoum-Aslan *et al.*, 2014; Samai *et al.*, 2015) in these organisms. Thus, gaining a mechanistic understanding of these systems in the native staphylococcal background can lead to important insights into the factors that impact the evolution and survival of these pathogens. Type III-A CRISPR-Cas systems encode a five-subunit effector complex called Cas10-Csm (Hatoum-Aslan *et al.*, 2013). Here, we describe a protocol for the expression and purification of Cas10-Csm from its native *S. epidermidis* background or a heterologous *S. aureus* background. The method consists of a two-step purification protocol involving Ni²⁺-affinity chromatography and a DNA affinity biotin pull-down, which together yield a pure preparation of the Cas10-Csm complex. This approach has been used previously to analyze the effects of mutations on Cas10-Csm complex integrity (Hatoum-Aslan *et al.*, 2014), crRNA formation (Hatoum-Aslan *et al.*, 2013), and to detect binding partners that directly interact with the core Cas10-Csm complex (Walker *et al.*, 2016). Importantly, this approach can be easily adapted for use in other *Staphylococcus* species to probe and understand their native Type III-A CRISPR-Cas systems.

Keywords: CRISPR-Cas Type III-A, Cas10-Csm, Staphylococci, Protein purification, Protein Expression, DNA affinity chromatography, Biotin pull-down

[Background] *Staphylococcus epidermidis* and *Staphylococcus aureus* are prevalent skin-dwelling bacteria that have a range of opposing impacts. While *S. aureus* asymptotically colonizes ~30% of the population (Conlan *et al.*, 2012), this organism is a leading cause of skin and soft tissue infections (Stryjewski and Chambers, 2008; Grice and Segre, 2011). In contrast, *S. epidermidis* is generally considered beneficial, and promotes human health by 1) preventing *S. aureus* colonization (Iwase *et al.*, 2010), 2) producing antimicrobial peptides that target skin pathogens (Cogen *et al.*, 2010), and 3) stimulating the human immune system to facilitate pathogen defense (Lai *et al.*, 2010; Naik *et al.*, 2015). However, when allowed to breach the skin barrier, this species can also cause antibiotic resistant

infections, particularly on indwelling medical devices (Otto, 2009; Harris and Richards, 2006). Furthermore, pathogenic staphylococci that are resistant to all known antibiotics have recently emerged in both hospital and community settings (Furuya and Lowy, 2006) and have become a major threat to global public health. Horizontal gene transfer (HGT), or the exchange of genetic information between related bacterial species, is a major route by which these organisms acquire virulence factors and multi-drug resistance. Therefore, it is of utmost importance to understand the factors that impact and regulate HGT in these organisms.

CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated proteins) is a class of bacterial immune systems that degrade invading nucleic acids and prevent all modes of HGT (Marraffini, 2015). CRISPR loci consist of short sequences derived from past invaders, known as spacers, which are integrated between repeat sequences of similar length (~30-40 nucleotides). These repeat-spacer arrays encode small CRISPR RNAs (crRNAs) that associate with Cas proteins, forming a ribonucleoprotein complex that destroys foreign DNA and/or RNA in a sequence-dependent manner. Many staphylococci possess Type III-A CRISPR-Cas systems (Marraffini and Sontheimer, 2008; Golding *et al.*, 2012; Cao *et al.*, 2016). The Type III-A system in *S. epidermidis* RP62a, a wild-type human isolate (Christensen *et al.*, 1987), encodes a multi-subunit complex called Cas10-Csm, composed of Cas10, Csm2, Csm3, Csm4, Csm5 and a crRNA (Hatoum-Aslan *et al.*, 2013). This system has been shown to prevent the conjugative transfer of antibiotic resistance genes (Marraffini and Sontheimer, 2008; Hatoum-Aslan *et al.*, 2014) and phage infection (Goldberg *et al.*, 2014; Maniv *et al.*, 2016), thus providing a natural barrier for HGT, and a model for Type III CRISPR-Cas systems in staphylococci.

The overexpression and purification of recombinant CRISPR-associated proteins from *Escherichia coli* (both the Cas10-Csm complex and individual subunits) followed by *in vitro* biochemical assays have revealed important insights into their functions (Hatoum-Aslan *et al.*, 2013; Samai *et al.*, 2015; Walker *et al.*, 2016). However, such assays fail to 1) recover information about protein function and stability in the native cellular environment, and 2) identify biologically relevant binding partners that are not a part of the core Cas10-Csm complex. Indeed, purification of Cas10-Csm from its native *S. epidermidis* background has yielded additional insights into the genetic requirements for complex stability and function, crRNA processing, and non-Cas binding partners that might play a role in the CRISPR-Cas pathway (Hatoum-Aslan *et al.*, 2013 and 2014; Walker *et al.*, 2016). Here, we provide a detailed protocol for the purification of Cas10-Csm from *S. epidermidis* or *S. aureus* strains bearing the Type III-A CRISPR-Cas system on a plasmid. The protocol involves two affinity-purification steps that can be carried out over the course of five days (Figure 1). Importantly, this protocol can be easily adapted to study Cas10-Csm complexes in other *Staphylococcus* species, thus providing an essential tool to probe and understand these important immune systems.

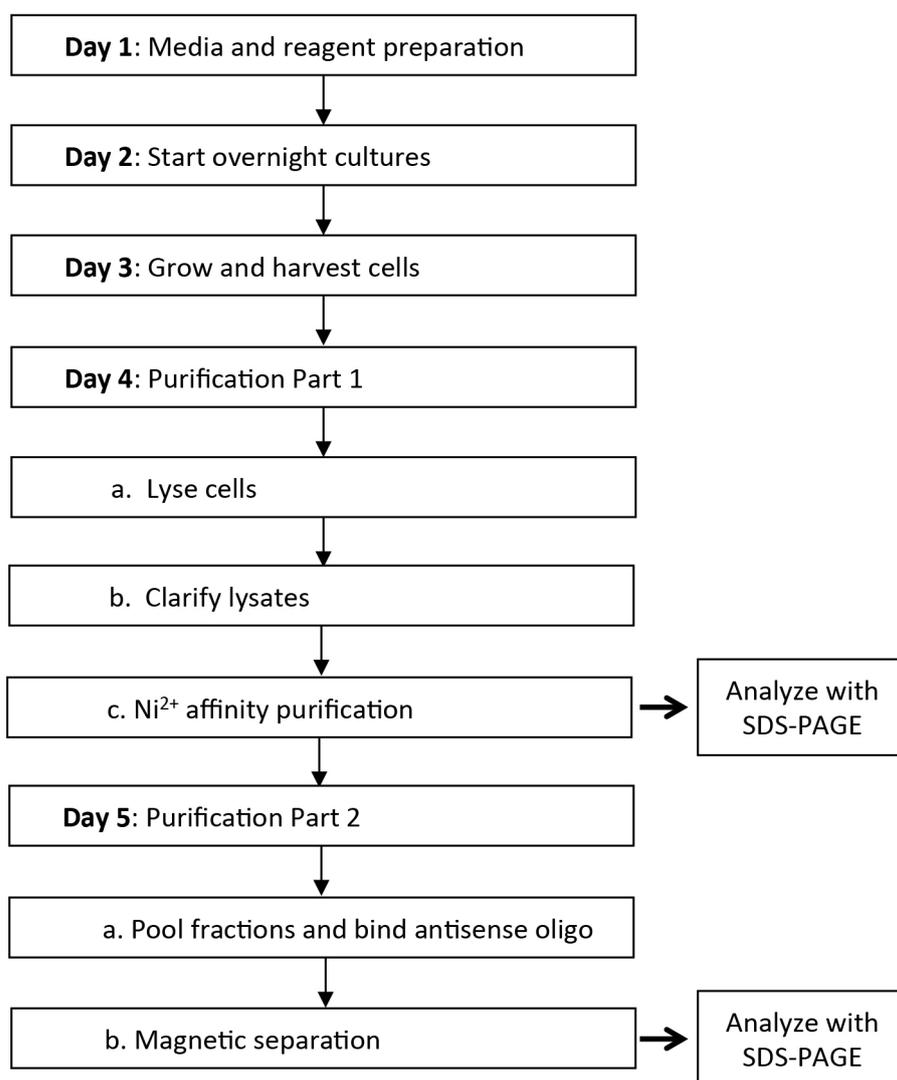


Figure 1. Timeline of activities for expression and purification of the Cas10-Csm complex from staphylococci

Materials and Reagents

Note: Equivalent materials and reagents may be used as substitutes.

1. Centrifuge tubes (50 ml) (VWR, catalog number: 21008-242)
2. Pipet tips with filter (0.1-10 μ l) (VWR, catalog number: 89368-972)
3. Pipet tips with filter (1-200 μ l) (VWR, catalog number: 89003-056)
4. Pipet tips with filter (100-1,000 μ l) (VWR, catalog number: 89003-060)
5. PES membrane vacuum filter (0.22 μ m) (VWR, catalog number: 10040-468)
6. Centrifuge tubes (15 ml) (VWR, catalog number: 21008-216)
7. Microcentrifuge tubes (VWR, catalog number: 87003-294)
8. Cellulose syringe filter (0.22 μ m) (VWR, catalog number: 28145-477)
9. Petri dishes (100 x 15 mm) (VWR, catalog number: 25384-088)

10. Spectrophotometer cuvettes (VWR, catalog number: 97000-586)
11. Syringe (10 ml) (BD, catalog number: 309604)
12. Syringe (3 ml) (BD, catalog number: 309657)
13. Centrifugation polypropylene bottles (400 ml) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 75007585)
14. Disposable gravity flow columns for protein purification (Geno Technology, G-Biosciences, catalog number: 786-169)
15. *S. epidermidis* LM1680 expressing *pcrispr/Csm2^{6HN}* (Hatoum-Aslan *et al.*, 2013) (see Note 1)
16. *S. aureus* RN4220 expressing *pcrispr/Csm2^{6HN}* (Hatoum-Aslan *et al.*, 2013) (see Note 1)
17. HisPur Ni-NTA resin (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 88222)
18. Sera-Mag™ magnetic streptavidin coated beads (GE Healthcare, catalog number: 30152105011150)
19. SDS PAGE Gel 0.75 MM 'Snap-A-Gels™ Mini Tris Glycine Precast Gels, Jule' (VWR, catalog number: 66025-389)
20. Color protein standard ladder (New England Biolabs, catalog number: P7712S)
21. BBL™ brain heart infusion (BHI) broth (BD, BBL™, catalog number: 211060)
22. Difco™ brain heart infusion (BHI) agar (BD, Difco™, catalog number: 241810)
23. Chloramphenicol (Alfa Aesar, catalog number: B20841)
24. 100% ethanol (Decon Labs, catalog number: V1016TP)
25. Neomycin sulfate (AMRESCO, catalog number: 0558-25G)
26. Magnesium chloride (MgCl₂) (AMRESCO, catalog number: J364)
27. Tris (AMRESCO, catalog number: 0497)
28. Hydrochloric acid (HCl) (VWR, BDH®, catalog number: BDH3030-2.5LPC)
29. Potassium chloride (KCl) (VWR, BDH®, catalog number: BDH9258-500G)
30. EDTA, disodium salt, dihydrate (EMD Millipore, OmniPur®, catalog number: 4050)
31. Sodium hydroxide (NaOH) (AMRESCO, catalog number: 0583)
32. Sodium dodecyl sulfate (SDS) (VWR, catalog number: 97064-862)
33. Bromophenol blue (VWR, catalog number: 97061-690)
34. Ambicin® L (Recombinant lysostaphin) (AMBI, catalog number: LSPN-50)
35. Sodium acetate anhydrous (NaOAc) (AMRESCO, catalog number: 0602)
36. Sodium phosphate monobasic (NaH₂PO₄) (AMRESCO, catalog number: 0571)
37. Sodium chloride (NaCl) (VWR, BDH®, catalog number: BDH9286)
38. Glycerol (AMRESCO, catalog number: M152)
39. Beta-mercaptoethanol (β-ME) (Geno Technology, G-Biosciences, catalog number: BC98)
40. Coomassie Blue G-250 (AMRESCO, catalog number: M140-10G)
41. Methanol (VWR, BDH®, catalog number: BDH20864.400)
42. Acetic acid (HAc) (VWR, BDH®, catalog number: BDH3096-2.5LPC)
43. Tris-glycine-SDS 10x buffer (AMRESCO, catalog number: 0783-5L)
44. Imidazole (Alfa Aesar, catalog number: A10221)

45. Pierce protease and phosphatase inhibitor mini tablets, EDTA-free (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 88669)
46. Triton X-100 surfactant (EMD Millipore, catalog number: TX1568)
47. Potassium hydroxide (KOH) (Alfa Aesar, catalog number: 13451)
48. Media and antibiotics (see Recipes)
 - a. Brain heart infusion (BHI) broth
 - b. Chloramphenicol stock (10 mg/ml)
 - c. Neomycin stock (15 mg/ml)
49. Stock solutions (see Recipes)
 - a. 1 M MgCl₂
 - b. 1 M Tris-HCl, pH 8.3
 - c. 1 M Tris-HCl, pH 6.8
 - d. 1 M KCl
 - e. 500 mM EDTA, pH 8.0
 - f. 10% sodium dodecyl sulfate (SDS)
 - g. 1% bromophenol blue
 - h. 2 mg/ml recombinant lysostaphin
 - i. 2x lysis buffer
 - j. 5x annealing buffer
 - k. 5x protein loading buffer
 - l. Coomassie Blue staining solution
 - m. Destaining solution
 - n. 1x Tris-glycine buffer
50. Buffers to prepare on Day 4 for purification—Part 1 (see Recipes)
 - a. Elution buffer
 - b. Resuspension buffer
 - c. Equilibration buffer
 - d. Wash 1 buffer
 - e. Wash 2 buffer

Equipment

1. Eppendorf Research® plus pipettes set (Eppendorf, catalog number: 2231000222), or equivalent pipettes set with a range of 1 µl to 1,000 µl
2. Media/storage bottles (250 ml) (VWR, catalog number: 10754-816), or equivalent
3. Erlenmeyer flask (2 L) (VWR, catalog number: 10545-844), or equivalent
4. Standard orbital shaker (VWR, model: 1000, catalog number: 89032-088), or equivalent shaker that can gently rotate for gel staining and destaining

5. New Brunswick I26 incubating shakers (Eppendorf, New Brunswick™, model: I26, catalog number: M1324-0008), or equivalent shaking incubator that can maintain 37 °C and 180 rpm
6. Heraeus Multifuge X1R centrifuge series (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Multifuge™ X1R, catalog number: 75004251), or equivalent refrigerated centrifuge with a rotor capable of holding 400 ml bottles and applying 13,000 x g of centrifugal force
7. General purpose water baths (VWR, model: VWR General Purpose Water Baths, catalog number: 89501-460), or equivalent capable of maintaining 37 °C
8. Sonifier® S-450 Analog sonicator (Emerson, Branson, model: S-450, catalog number: 101-063-198), or equivalent ultrasonic homogenizer with operating frequency of 20 kHz and tip diameter of 3.2 mm
9. 30 ml beaker (Corning, PYREX®, catalog number: 1000-30) or equivalent
10. Digital dry block heaters (VWR, catalog number: 12621-088), or equivalent block heater capable of heating up to 95 °C
11. Magnetic bead separation rack (Thermo Scientific, catalog number: MR02), or equivalent rack capable of collecting magnetic beads
12. Autoclave (Getinge), or equivalent autoclaving instrument capable of heating up to 121 °C while applying 15 atm pressure
13. Graduated cylinder (100 ml) (VWR, catalog number: 65000-006), or equivalent
14. Graduated cylinder (1 L) (VWR, catalog number: 65000-012), or equivalent
15. Media/storage bottles (1 L) (VWR, catalog number: 10754-820), or equivalent
16. Media/storage bottles (100 ml) (VWR, catalog number: 10754-814), or equivalent
17. Media/storage bottles (500 ml) (VWR, catalog number: 10754-818), or equivalent
18. Ultrospec™ 10 cell density meter (GE Healthcare, catalog number: 80-2116-30), or equivalent spectrophotometer that can measure the density of cells in suspension at 600 nm
19. Mini-PROTEAN tetra cell (Bio-Rad Laboratories, model: Mini-PROTEAN Tetra Cell, catalog number: 1658004EDU), or equivalent vertical electrophoresis system
20. pH meter Accumet™ AB15 plus basic (Fisher Scientific, model: Fisher Scientific™ accumet™ AB15+ Basic and BioBasic™, catalog number: 13-636-AB15P), or equivalent pH meter with a range of 0.000-10.000

Procedure

- A. Day 1. Preparation of media, reagents, and single colonies
 1. Prepare 100 ml of brain heart infusion (BHI) broth in a 250-ml bottle (for starting overnight cultures), and 1 L of BHI broth in a 2 L Erlenmeyer flask (for large-scale propagation and protein purification) (see Recipe 1).
 2. Prepare antibiotics listed in Recipe 1.
 3. Prepare all the stock solutions needed for the experiment (see Recipe 2).

4. Streak out a freezer stock of *S. epidermidis* LM1680 bearing *pcrispr/Csm2*^{6HN} (or *S. aureus* RN4220 bearing *pcrispr/Csm2*^{6HN}) on a BHI agar plate containing 10 µg/ml chloramphenicol. If *S. epidermidis* is used, supplement media with 15 µg/ml neomycin (see Note 1).
- B. Day 2. Overnight culture preparation
1. Transfer 10 ml of BHI broth into a 50-ml conical tube.
 2. Add 10 µl of 10 mg/ml chloramphenicol to select for *pcrispr/Csm2*^{6HN}. If *S. epidermidis* LM1680 is being used, add additionally 10 µl of 15 mg/ml neomycin. If other strains and plasmids are being used, supplement media with antibiotics as appropriate (see Note 1).
 3. With a sterile pipette tip, pick a single colony of *S. epidermidis* LM1680 bearing *pcrispr/Csm2*^{6HN} (or *S. aureus* RN4220 bearing *pcrispr/Csm2*^{6HN}).
 4. Re-suspend the colony into the 10 ml of autoclaved media containing appropriate antibiotic(s).
 5. Close loosely the 50-ml conical tube containing the autoclaved media, antibiotics, and colony of interest.
 6. Incubate the bacterial culture at 37 °C for 16-20 h in a shaking incubator set to 180 rpm.
- C. Day 3. Large-scale propagation of cells overexpressing the Cas10-Csm complex
1. Into the 1 L of BHI prepared in the 2 L Erlenmeyer flask, add 1 ml of 10 mg/ml chloramphenicol. If *S. epidermidis* LM1680 is being used, add additionally 1 ml of 15 mg/ml neomycin.
 2. Inoculate the 1 L of BHI plus antibiotic(s) with the entire 10 ml of overnight culture prepared on Day 2.
Note: The OD600 nm for overnight S. epidermidis and S. aureus cultures is approximately 5-6.
 3. Measure the initial OD600 nm of diluted culture with a cell density meter to facilitate estimation of time required to reach the final OD600 nm (see below).
 4. Incubate the bacterial culture at 37 °C in a shaking incubator set to 180 rpm.
 5. Let the bacterial culture grow until OD600 nm = 2.00 (approximately 7-9 h if starting with a fresh overnight culture and initial OD600 nm = 0.05).
 6. Distribute the 1 L of liquid culture into four 400-ml polypropylene bottles.
 7. Centrifuge cells at 5,000 x g, 4 °C for 10 min.
 8. Discard supernatant and resuspend each pellet on ice with 10 ml of ice-cold distilled H₂O (dH₂O).
 9. Combine cell suspensions from all pellets into a clean 50 ml conical tube (on ice) and centrifuge at 5,000 x g, at 4 °C for 10 min.
 10. Discard supernatant and store pelleted cells in -80 °C (for optimal purification up to a week later) or proceed directly to purification.
- D. Day 4. Purification–Part 1
1. Thaw the cell pellet on ice for 1 h.
 2. Prepare all buffers described in Recipe 3.

3. Resuspend thawed pellet in 9 ml of ice-cold dH₂O.
4. Add 200 μ l of 1 M MgCl₂ and 125 μ l of freshly thawed lysostaphin (2 mg/ml stock).
5. Incubate the cell suspension for 1 h in a 37 °C water bath. Invert the tube several times after 30 min of incubation to homogenize the suspension.
6. Add 10 ml of resuspension buffer to the lysed cells and invert tube several times. The cell lysate will become very viscous (see Figure 2).

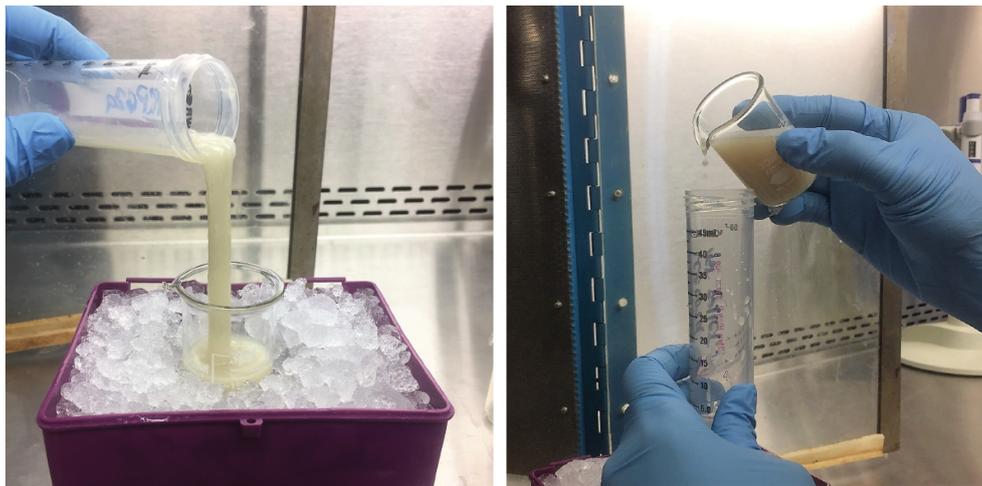


Figure 2. Viscosity of cell lysate before and after sonication of *S. epidermidis* LM1680 cells expressing *pcrispr/Csm2*^{6HN}. Before sonication (left), the cell lysate is milky white with the consistency of mucus. After sonication (right), the cell lysate becomes tinted brown with a more watery consistency.

Important Note: All subsequent steps should be performed on ice or at 4 °C.

7. Adjust the Sonifier S-450 to power level 6.0 and duty cycle percent to 6.

Note: These settings correspond to a 60 W power output with a fixed repetition rate of 1 pulse per second. A duty cycle of '6' indicates that power will be delivered 60% of each 1 sec pulse (with a rest 40% of each second).

8. Pour the lysate into a 30-ml beaker set in an ice bucket. The cell lysate should occupy $\sim\frac{2}{3}$ of the beaker's volume such that lysate within has at least 1 inch depth.
9. Immerse the sonicator tip into the lysate at a depth of $\sim\frac{1}{2}$ - $\frac{2}{3}$ inch such that there remains $\sim\frac{1}{2}$ an inch of clearance from the bottom of the beaker.

Important Note: The tip should not touch the sides or bottom of the beaker.

10. Sonicate the lysate on ice for 30 sec at a time with one minute rest period in between. This cycle should be repeated three times or until the lysate has a more watery consistency. Often, the lysate will also acquire a brown tint (see Figure 2).
11. Pour the lysate back into a 50-ml conical tube and centrifuge for 20 min, 4 °C, at 10,000 x g. The clarified lysate after centrifugation often retains the brownish tinge (see Figure 3).

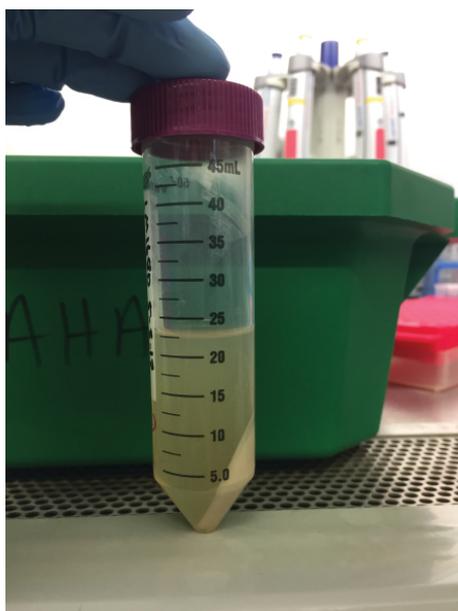


Figure 3. Appearance of sonicated cell lysate following the first centrifugation. After the cell debris is pelleted, the clarified lysate retains a yellow-brown tinge.

12. Decant the supernatant into a fresh 50-ml conical tube and centrifuge for 20 min, 4 °C, at 13,000 x g.

Note: The cell pellet may be saved and checked later on SDS-PAGE for the presence of the Cas10-Csm complex to assess its solubility.

13. During the two spins, prepare/pack the column (to be done in the cold room or inside a deli refrigerator):
 - a. Pipet 1 ml of slurry Ni-NTA resin into a disposable gravity flow column and allow the liquid to flow through.
 - b. Equilibrate the Ni-NTA resin by passing 10 ml of equilibration buffer through the column at 4 °C.
 - c. Cap the column once most of the buffer has passed through and ~2-3 mm of buffer remains atop the resin.

14. After the final spin (step D12), pass the lysate through a 0.22 µm PES membrane vacuum filter and catch the filtrate into a sterile bottle.

15. Pass the filtered lysate through the packed Ni-NTA column (prepared in step D13) and collect the flow through in a 50-ml conical tube labeled 'lysate flow-through'.

Note: The lysate flow-through can be checked later on SDS-PAGE for the presence of the Cas10-Csm complex in case the complex was suspected to have passed through the column without binding.

16. Wash the column by passing 10 ml of wash 1 buffer through the column.

17. Collect wash 1 flow-through into a 15-ml conical tube.

Note: The wash 1 flow-through may be saved and checked later for the Cas10-Csm complex in

- case the complex was suspected to have passed through the column during the wash.
18. Wash the column a second time by passing 10 ml of wash 2 buffer through the column.
 19. Collect wash 2 flow-through into a 15-ml conical tube.
Note: The wash 2 flow-through may be saved and checked later for the Cas10-Csm complex in case the complex was suspected to have passed through the column during the wash.
 20. Elute proteins from the column with 5-7 aliquots of elution buffer (500 μ l for each fraction). As each aliquot flows through, capture it into a fresh, appropriately-labeled microcentrifuge tube.
 21. Into fresh labeled microcentrifuge tubes, combine 20 μ l of each fraction with 5 μ l of 5x protein loading buffer.
 22. Heat samples at 95 $^{\circ}$ C for 5 min on a heating block.
 23. Load samples alongside a protein standard into a 12% SDS-PAGE gel.
 24. Run the gel in Tris-glycine-SDS 1x buffer at 120 V for 1 h.
 25. Stain the gel with Coomassie Blue solution for 10 min and destain with destaining solution for up to 40 min.
 26. Image the gel under white light (Figure 4).
 27. Save the desired elutions at -20 $^{\circ}$ C (see Note 2).

Note: Avoid repeated freeze-thaw cycles of the complex as this leads to protein aggregation. If necessary, aliquot the desired elutions into smaller volumes (~100 μ l). Protein complexes that have not been repeatedly thawed can be stored in the elution buffer up to a year at -20 $^{\circ}$ C without noticeable degradation of protein or crRNAs.

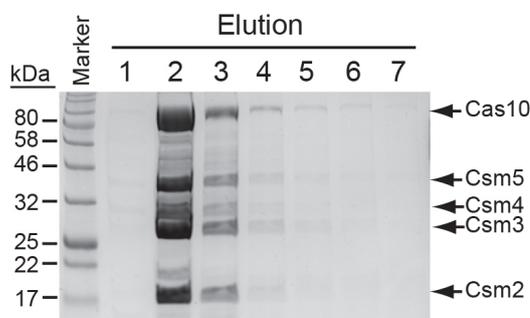


Figure 4. Elutions of the Cas10-Csm complex following purification from *S. epidermidis* LM1680 expressing *pcrispr/Csm2*^{6HN}. Shown are the seven fractions (E1-E7) collected from a nickel-agarose column and analyzed on a 12% SDS PAGE gel. Proteins were stained with Coomassie Blue G-250.

E. Day 5. Purification–Part 2

1. Pipette 10 μ l of Sera-MagTM magnetic streptavidin coated beads into a microcentrifuge tube.
Note: Mix the bead suspension well by pipetting up and down several times before use.
2. Resuspend the beads with 100 μ l of 1x annealing buffer (see Recipe 2).
Note: Bead re-suspension is achieved once the solution becomes a homogenous brown color

and no beads accumulation is seen at the side or bottom of the tube. When tubes are removed from the magnetic rack, the beads within can be easily resuspended.

3. Pellet the beads by placing the microcentrifuge tube into a magnetic separation rack and waiting for the beads to collect on the side of the tube.

Note: The collection process can vary from 30 sec up to 2 min depending on beads and rack used. The collection process will be completed once the supernatant becomes clear and the beads (brown color) aggregate on the side of the tube where the magnet is located. If the beads are broadly spread around the side of the tube, twist the microcentrifuge tube a few degrees clockwise, or counter-clockwise within the rack to pull all beads closer together.

4. Gently pipette out the supernatant without disturbing the beads.
5. Repeat the wash (steps E2-E4) three times.
6. Pool the most concentrated Cas10-Csm complex fractions (obtained in Day 4) and combine with 2 ng of a 5'-biotinylated oligonucleotide antisense to a crRNA (see Note 3).
7. Let the mixture incubate for 30 min at room temperature.
8. Remove the microcentrifuge tube that contains the beads away from the magnetic rack and add the Cas10-Csm complex/oligo mixture into the equilibrated streptavidin beads.
9. Resuspend beads until the mixture appears homogenous and let it anneal at room temperature for 30 min.
10. Collect beads to the side of the tube by placing the microcentrifuge tube back into the magnetic rack ~30 sec.
11. Gently pipette out the supernatant without disturbing the beads.
12. Remove tube from the magnetic rack and resuspend the beads in 100 μ l of 1x annealing buffer.
13. Collect beads to the side of the tube by placing the microcentrifuge tube back into the magnetic rack ~30 sec.
14. Gently pipette out the supernatant without disturbing the beads.
15. Repeat the wash (steps E12-E14) two more times.
16. Add 20 μ l of 1x annealing buffer to the beads and 5 μ l of 5x protein loading buffer.
17. Heat the samples for 5 min at 95 °C on a heating block.
18. Place the tube back into the magnetic rack to remove the beads from solution.
19. Load the supernatant alongside a protein standard into a 12% SDS-PAGE gel.
20. Run the gel in Tris-glycine-SDS 1x buffer at 120 V for 1 h.
21. Stain the gel with Coomassie Blue solution for 10 min and destain with destaining solution for up to 40 min.
22. Image the gel under white light (Figure 5).

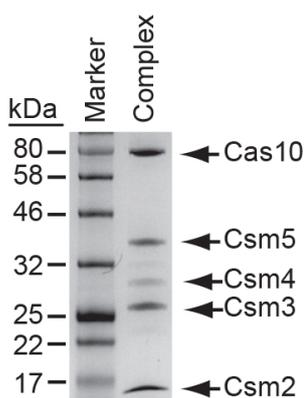


Figure 5. *S. epidermidis* Cas10-Csm complex collected from magnetic streptavidin beads following an affinity purification with a biotinylated oligonucleotide antisense to *spc1* crRNAs. Proteins were resolved on a 12% SDS PAGE gel and visualized with Coomassie Blue G250.

Data analysis

This assay is used as a qualitative measure to assess Cas10-Csm complex integrity. The presence or absence of a particular protein subunit on the SDS-PAGE gel will indicate whether or not it is stably associated with the complex. Using this protocol, the presence or absence and lengths of crRNAs within the complex can be determined by extracting crRNAs directly from Cas10-Csm complex aliquots obtained from the 1st purification process, followed by 5'-end labeling with γ -³²P-ATP, and resolving these on an 8-12% polyacrylamide gel. Before a definitive determination of complex stability can be made, these assays must give consistent results over three independent replicates. For complexes that are being characterized for the first time, the identity of each subunit in the gel must be confirmed using Western blotting. These additional methods are described in (Hatoum-Aslan *et al.*, 2013).

Notes

1. This protocol describes the purification of Cas10-Csm complexes from *S. epidermidis* LM1680 or *S. aureus* RN4220 cells bearing *pcrispr/Csm2*^{H6N}, a plasmid that encodes the entire Type III-A CRISPR-Cas system from *S. epidermidis* RP62a with a 6x-His tag introduced into the N-terminus of *csm2* (Hatoum-Aslan *et al.*, 2013). *S. epidermidis* LM1680 is a derivative of *S. epidermidis* RP62a with a chromosomal deletion encompassing the CRISPR-Cas locus (Hatoum-Aslan *et al.*, 2013). *S. aureus* RN4220 is a *crispr*-strain (Nair *et al.*, 2011) that is used as a cloning intermediate. When re-introduced on *pcrispr/Csm2*^{H6N}, the CRISPR-cas system retains full functionality in both strains. *pcrispr/Csm2*^{H6N} requires selection in 10 μ g/ml of chloramphenicol. Additionally, 15 μ g/ml of neomycin is used to select specifically for *S. epidermidis* RP62a or LM1680. This protocol can also be adapted to overexpress the

CRISPR-Cas system on different plasmids, or in different strains, however, antibiotic selection would also have to be modified accordingly. If working with wild-type strains, overexpressing a plasmid encoding the CRISPR-Cas system with one subunit 6x-His tagged should, in theory, allow for Cas10-Csm complex purification even in the presence of the chromosomal copy of the system in the background.

2. Mass spectrometry analysis may be performed following the first purification to detect potential binding partners loosely associated with the complex. In addition, the presence or absence of crRNAs within complexes and their lengths can be determined by extracting RNAs from complexes following the first purification, end-labeling the RNAs, and resolving on a urea PAGE gel. If a cleaner prep is desired or to assess complex stability, the second purification described for day 5 should be performed.
3. The sequence of the biotinylated oligonucleotide used in this example is complementary to the first spacer (*spc1*) of the repeat-spacer array:

5'-Biotin-TEG-ACGAGAACACGUAUGCCGAAGUAUAUAAAUC

Recipes

1. Media and antibiotics
 - a. Brain heart infusion (BHI) broth
 - i. Dissolve 3.7 g per 100 ml dH₂O
 - ii. Autoclave at 121 °C for 30 min
 - iii. Store at room temperature
 - b. Chloramphenicol stock (10 mg/ml)
 - i. Dissolve 100 mg chloramphenicol into 10 ml of 100% ethanol
 - ii. Pass through a 0.22 µm syringe filter
 - iii. Store at 4 °C
 - c. Neomycin stock (15 mg/ml)
 - ii. Dissolve 150 mg neomycin into 10 ml dH₂O
 - iii. Pass through a 0.22 µm syringe filter
 - iv. Store at 4 °C
2. Stock solutions
 - a. 1 M MgCl₂
 - i. Place 9.521 g MgCl₂ into a 100-ml graduated cylinder
 - ii. Fill with dH₂O up to 100 ml
 - iii. Store in a bottle at room temperature
 - b. 1 M Tris-HCl, pH 8.3
 - i. Place 121.14 g Tris into a 1-L graduated cylinder
 - ii. Dissolve with 800 ml of dH₂O
 - iii. Adjust pH to 8.3 with 12 N HCl

- iv. Fill with dH₂O up to 1 L
- v. Store in a bottle at room temperature
- c. 1 M Tris-HCl, pH 6.8
 - i. Place 121.14 g Tris into a 1-L graduated cylinder
 - ii. Dissolve with 800 ml of dH₂O
 - iii. Adjust pH to 6.8 with 12 N HCl
 - iv. Fill with dH₂O up to 1 L
 - v. Store in a bottle at room temperature
- d. 1 M KCl
 - i. Place 7.455 g KCl into a 100-ml graduated cylinder
 - ii. Fill with dH₂O up to 100 ml
 - iii. Store in a bottle at room temperature
- e. 500 mM EDTA, pH 8.0
 - i. Place 93.06 g EDTA, disodium salt, dihydrate (MW = 372.24 g/mol) into a 500-ml beaker
 - ii. Dissolve as much as possible with 350 ml of dH₂O
 - iii. Adjust pH to 8.0 with 10 N NaOH while constantly stirring

Note: The EDTA will not dissolve completely unless its pH is at 8.0. However, the pH of EDTA continually drops as it gets dissolved. Therefore, constant addition of 10 N NaOH must be used to get the EDTA completely dissolved with a final pH of 8.0.
 - iv. Fill with dH₂O up to 500 ml
 - v. Store in a bottle at room temperature
- f. 10% sodium dodecyl sulfate (SDS)
 - i. Place 1 g of SDS into a 15-ml conical tube
 - ii. Dissolve with 6 ml of dH₂O
 - iii. Fill with dH₂O up to 10 ml
 - iv. Store at room temperature

Note: Since powdered SDS can cause serious eye and lung damage, a 20% solution may be purchased (VWR Cat. No. 97062-440) and diluted with dH₂O (1:1).
- g. 1% bromophenol blue
 - i. Place 0.1 g of bromophenol blue into a 15-ml conical tube
 - ii. Fill with dH₂O up to 10 ml
 - iii. Store at room temperature
- h. 2 mg/ml recombinant lysostaphin
 - i. Dissolve 50 mg lysostaphin in 25 ml NaOAc, pH 4.5
 - ii. Distribute 0.5 ml aliquots into microcentrifuge tubes
 - iii. Store at -80 °C
 - iv. Thaw just prior to use

- i. 2x lysis buffer
 - i. Place 11.998 g NaH_2PO_4 into a 1-L graduated cylinder ([final] = 100 mM)
 - ii. Place 35.064 g NaCl into the graduated cylinder ([final] = 600 mM)
 - iii. Fill to 800 ml with dH_2O
 - iv. Adjust pH to 8.0 with 10 N NaOH
 - v. Fill up to 1 L
 - vi. Store at room temperature
- j. 5x annealing buffer
 - i. Place 2.5 ml of 1 M Tris-HCl, pH 8.3 into a 100-ml bottle ([final] = 25 mM)
 - ii. Place 37.5 ml of 1 M KCl into the bottle ([final] = 375 mM)
 - iii. Place 1 ml of 500 mM EDTA, pH 8.0 into the bottle ([final] = 5 mM)
 - iv. Add 59 ml of dH_2O
 - v. Store bottle at room temperature
- k. 5x protein loading buffer
 - i. Place 0.5 ml of 1 M Tris-HCl, pH 6.8 into a 15-ml conical tube ([final] = 62.5 mM)
 - ii. Place 0.8 ml of 100% glycerol into the tube ([final] = 10%)
 - iii. Place 1.6 ml of 10% SDS solution into the tube ([final] = 2%)
 - iv. Place 0.4 ml of 14.3 M β -mercaptoethanol ([final] = 715 mM)
 - v. Add 0.4 ml of 1% bromophenol blue into the tube ([final] = 0.05%)
 - vi. Add 4.3 ml of dH_2O
 - vii. Distribute 1 ml aliquots into microcentrifuge tubes
 - viii. Store at $-20\text{ }^\circ\text{C}$
- l. Coomassie Blue staining solution
 - i. Place 1 g of Coomassie Blue G-250 into a 1-L graduated cylinder ([final] = 0.1%)
 - ii. Place 500 ml of methanol into the graduated cylinder ([final] = 50%)
 - iii. Place 100 ml of acetic acid into the graduated cylinder ([final] = 10%)
 - iv. Place 400 ml of dH_2O into the graduated cylinder ([final] = 40%)
 - v. Store in a bottle at room temperature
- m. Destaining solution
 - i. Place 500 ml of methanol into a 1-L graduated cylinder ([final] = 50%)
 - ii. Place 100 ml of acetic acid into the graduated cylinder ([final] = 10%)
 - iii. Place 400 ml of dH_2O into the graduated cylinder ([final] = 40%)
 - iv. Store in a bottle at room temperature
- n. 1x Tris-glycine-SDS buffer
 - i. Place 100 ml of 10x Tris-glycine-SDS buffer into a 1-L graduated cylinder
 - ii. Fill with dH_2O up to 1 L
 - iii. Store in a bottle at room temperature

3. Buffers to prepare on Day 4 for purification—Part 1

Note: All the following buffers should be freshly prepared. Keep buffers on ice. Prepare 10 ml of each buffer per cell pellet from 1 L of culture.

- a. Elution buffer (10 ml [Final volume])
 - i. Place 60 mg NaH₂PO₄ into a 50-ml conical tube ([final] = 50 mM)
 - ii. Add 175 mg NaCl ([final] = 300 mM)
 - iii. Add 170 mg imidazole ([final] = 250 mM)
 - iv. Add 1 ml glycerol ([final] = 10%)
 - v. Add 8 ml dH₂O
 - vi. Adjust pH to 8.0 with 10 N NaOH
 - vii. Bring final volume up to 10 ml with dH₂O
- b. Resuspension buffer (10 ml [Final volume])
 - i. Place 9.2 ml 2x lysis buffer into a 50-ml conical tube
 - ii. Add 800 µl elution buffer
 - iii. Add 1 cOmplete tablet protease inhibitor (free EDTA)
 - iv. Cap tube tightly and vortex until the tablet has completely dissolved (30 sec-1 min)
 - v. Add 10 µl Triton X-100 ([final] = 0.1%)
 - vi. Invert tube gently until the Triton X-100 has completely dissolved
- c. Equilibration buffer (10 ml [Final volume])
 - i. Place 5 ml 2x lysis buffer into a 50-ml conical tube
 - ii. Add 5 ml dH₂O
- d. Wash 1 buffer (10 ml [Final volume])
 - i. Place 5 ml 2x lysis buffer into a 50-ml conical tube
 - ii. Add 800 µl elution buffer
 - iii. Add 4.2 ml dH₂O
- e. Wash 2 buffer (10 ml [Final volume])
 - i. Place 5 ml 2x lysis buffer into a 50-ml conical tube
 - ii. Add 800 µl elution buffer
 - iii. Add 3.2 ml dH₂O
 - iv. Add 1 ml glycerol ([final] = 10%)

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