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Genome editing of *Sulfolobus acidocaldarius* using its endogenous CRISPR-Cas system

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Abstract

This protocol describes the use of the endogenous type I-D CRISPR-Cas system of *Sulfolobus acidocaldarius* for genome editing. The method is based on providing the spacer sequence required for target recognition on a plasmid, flanked by the cognate CRISPR repeats, together with a repair fragment carrying the desired mutation. After curing the plasmid, uracil auxotrophy is restored, allowing the selection marker to be re used for subsequent rounds of editing.

Related publication(s): Bost et al., 2023

Background

The availability of genetic tools and relatively easy cultivation protocols have made *Sulfolobus acidocaldarius* (*Saci*) a widely used organism for the study of Thermoproteota (Lewis et al., 2021; van Wolferen et al., 2022; Oren and Garrity, 2021).

In this protocol, we detail the method described by Bost et al. to introduce mutations in the genome using the *Saci* endogenous type I-D CRISPR system. In this method, a plasmid is introduced that carries a spacer flanked by the repeat sequences of cluster I, which is associated with the type I-D CRISPR-Cas system, under the control of a xylose-inducible promoter. This is transcribed an processed into a crRNA (CRISPR RNA) that is used by the host's own Cas proteins to target the gene of interest (GOI). Alongside the spacer sequence, a

repair fragment is provided. This repair fragment contains homology arms flanking the GOI that recombines with the genome to create the desired genotype. The new genotype does not contain the sequence recognized by the spacer, which is either deleted or disrupted.

This protocol shortens the time to obtain a mutant, compared to the pop-in/pop-out method(Wagner et al., 2012), because screening is already performed on the first selection plates and, if the constructs are well designed, almost all blue colonies carry the desired mutant.

For cloning of the spacer, a type IIS restriction enzyme (*Sap*I) is used. The spacers are designed as primer pairs containing an *Sap*I restriction site as well as repeats from the CRISPR cluster I. After annealing of the primers, restriction and ligation of both spacer and plasmid can be done in the same tube. After transformation into *E. coli* (DH5α, Top10, or similar), colonies containing the spacer can be selected by plating the cells on LB-agar plates containing X-gal, IPTG, and ampicillin.

The repair fragment can be provided as a purified linear PCR product, but it can also be cloned into the plasmid. There are two multiple cloning sites (MCS) in the plasmid that can be used for this purpose (Figure 1). In our experience, providing the linear fragment as a PCR product gave a higher yield of blue colonies. The *pyrE* gene is used as a selection marker in *Saci,* therefore, an uracil-auxotrophic strain must be used (e.g., MW001).

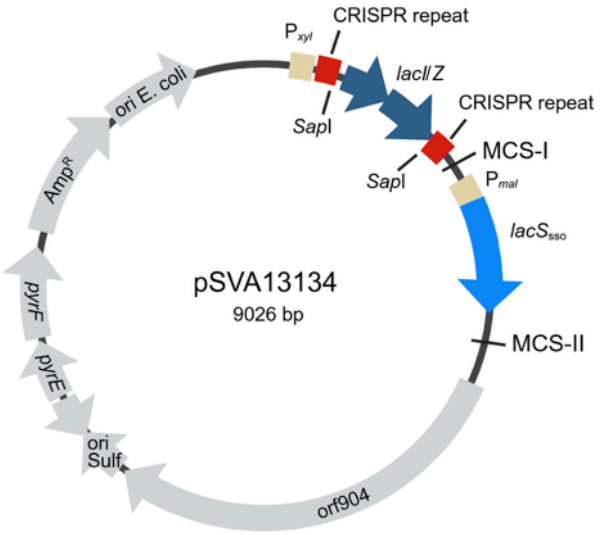


Figure 1. Schematic overview of the plasmid pSVA13134 used for genome editing of *S. acidocaldarius* with CRISPR type I-D. The plasmid contains the *lacI/Z* cassette, which is replaced by the spacer using the *Sap*I sites and facilitates selection in *E. coli.* The introduced spacer is placed under the control of a xylose-inducible promoter. The two multiple cloning sites (MCS) can be used to introduce the repair fragment. The $lacS_{SSO}$ allows the selection of transformants via blue/white screening in *Saci*. Selection markers are ampicillin and uracil, in *E. coli* and *Saci*, respectively.

Materials

Product name	Brand/Manufacturer	Catalogue number	Notes
E. coli DH5α, Top10 or similar			For plasmid cloning
E. coli ER1821 (pM.EsaBC4I)	New England Biolabs		For plasmid methylation before transformation in *Saci*

Product name	Brand/Manufacturer	Catalogue number	Notes
LB + ampicillin			Plates and liquid media. For cloning
LB + ampicillin and kanamycin			Liquid media
Basal Brock medium			See Recipes
20% N-Z-amine			Prepare a 20% solution. NZ-amine dissolves more easily when warmed. Sterilize by autoclaving.
20% Sucrose or Dextrin			Prepare a 20% solution Sterilize by autoclaving
Uracil			Prepare 5 mg/mL stock solution and store it aliquoted at -20°C. Heat the solution to dissolve the uracil more easily. Sterilize by filtration.
5-Fluoroorotic acid (5-FOA)	Thermo Fisher Scientific		Prepare 100 mg/mL stock solution in DMSO and store it aliquoted at -20°C
Sucrose 20 mM			Sterilize by autoclaving. Store at 4°C
Recovery Solution			Basal Brock medium, non-adjusted pH + 0.1% NZ-amine
1 M $MgCl_2$			Sterilize by autoclaving
0.5 M $CaCl_2$			Sterilize by autoclaving
1.2% Gelrite	Carl Roth		Prepare 200 mL. Autoclave. Can be stored at room temperature
X-gal solution	Carl Roth		Prepare a 25 mg/mL solution in DMF (Dimethylformamide) and store it at -20°C. Light sensitive.
Brock:glycerol (1:1)			50% glycerol in basal Brock media, not pH adjusted. Sterilize by filtration
1st selection plates			See Recipes
2nd selection plates			See Recipes
0.2 M NaOH			For cell lysis. Keep tightly closed. As NaOH tends to absorb CO_2, lysis may be inefficient old solutions are used. Replace the solution if it turns whiteish or the PCR is not working properly
0.2 M Tris-HCl pH 7.8			For cell lysis, pH neutralization

Equipment

Equipment name	Brand/ Manufacturer	Catalogue number	Notes
Electroporator			Must allow adjustment of parameters such as voltage, resistance, capacitance, gap size
Electroporation cuvettes			1 mm gap
Shaking incubator at 75°C			
Incubator at 75°C			
Thermocycler			For cloning
Thermoblock at 37°C/ 42°C/75°C with shaking option			For recovery and heat shock transformation of cells
Plastic boxes			To maintain humidity. They should close tightly
Erlenmeyer flasks			For cell growth. We use 50 mL culture in 100 mL flasks. Cover them with thick aluminum foil to avoid evaporation
Eppendorf tubes			For aliquoting cells and cell lysis
Pipettes and tips			For liquid handling
PCR tubes			
Ambar spray bottles			To spray the X-gal on the plates
Centrifuge with cooling			

Software and bioinformatics tools

Software/tool/script	Company/Developer	Version	Web address	Github repository
Cloning software	E.g., Clone manager, Snapgene, Benchling, etc.			

Protocol

A. Design and cloning of plasmid

1. Design of the spacer

a) The spacer is ordered as primer pairs ready to clone into the backbone plasmid pSVA13134.

b) Select a protospacer in the gene/region you want to edit. The protospacer needs to be 37 nt long and harbor a PAM sequence (CCN, TCN or GTN) at the 5' end. It does not matter if the PAM sequence is in the coding or non-coding strand, but it must be read from 5´ to 3´.

c) The forward primer corresponds to the strand of the protospacer where the PAM is located; the reverse primer is therefore its reverse complementary. Add *Sap*I sites (and additional nucleotides) and the repeats of the cluster you will be using, in this case cluster 1 (see figure 2). The final primer forward will be: 5' TATGCTCTTCAAACxxxxGTAAGAAGAGCAAT 3', where the *Sap*I site is in blue, the repeats in red, and xxxx represents the sequence of your spacer.

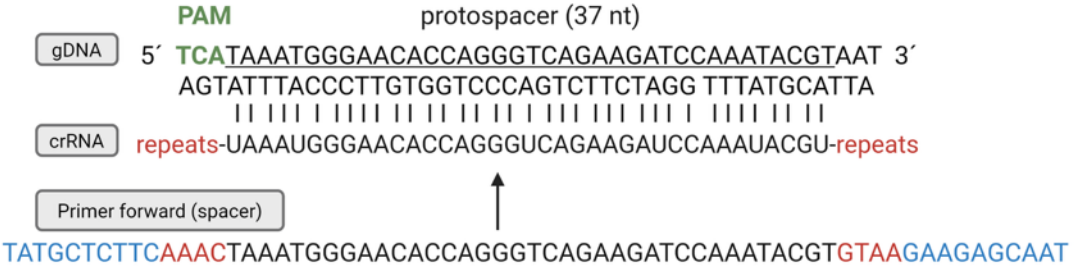


Figure 2. Representative diagram for the design of spacers. The PAM is located in the 5´->3´ direction, in this case, in the upper gDNA strand. The next 37 nucleotides following the PAM are the protospacer. The forward primer corresponds to the protospacer sequence, flanked by the cluster I repeat (red) and the *Sap*I restriction site (blue). Once cloned in the plasmid (figure 1), it will be transcribed to crRNA that will recognize the lower strand.

2. Cloning of the spacer

a) Anneal the primers in a thermocycler, mixing the following in a PCR tube: 1 µL of each primer (100 µM stock) and 8 µL molecular grade water. The program is as follows: 98°C 10 s and 50°C 10 s, then hold at 12°C. Proceed to the next step without purifying. The reaction can be saved at -20°C.

b) Set up the restriction reaction as follows: in a tube, mix 1 µL spacer (annealed primers from previous step), 2 µL backbone plasmid pSVA13134 (conc. around 100-150 ng/µL), 1 µL 10X restriction buffer (e.g. CutSmart, NEB), 0.75 µL *Sap*I and 5 µL molecular-grade water.

c) Incubate the restriction reaction 1 h at 37°C, followed by heat inactivation at 65°C for 20 min.

d) After the tubes has cooled down, add 1.25 µL 10 mM ATP and 1.25 µL T4 ligase.

e) Ligate at room temperature (RT) for 1-2 h, or overnight (ON) at 4°C.

f) Transform via heat-shock into *E. coli* Top 10, DH5α or similar.

g) Plate on LB-ampicillin plates containing X-gal and IPTG. Incubate ON at 37°C.

h) The next day, pick the white colonies and grow them in liquid media with ampicillin ON.

i) Purify the plasmid and verify it by sequencing.

3. Design of the repair fragment (as in the Pop-in/pop-out method)

a) According to your desired modification, design the repair fragment: this should be at least 1000 bp long. E.g., for gene deletion, take 500 bp upstream and 500 bp downstream of your gene of interest. Take into account possible overlaps with the start and stop codons or promoters of other

genes. The repair fragment should make your protospacer disappear or alter it close to the PAM sequence. If you are introducing a tag or a point mutation, you can also change the codons of the gene in the protospacer region, so that the amino acids remain the same, but the DNA sequence is not recognized by CRISPR anymore.

b) Design primers to clone the repair fragment. E.g., for gene deletion, one set of primers for the upstream (US Fw and Rv) and another set for the downstream (DS Fw and Rv) regions. The US Rv and the DS Fw should have overlapping regions of at least 20 bp. The US Fw and DS Rv should contain restriction enzyme sites to clone the fragment into the plasmid. The fragment can also be designed to be cloned with *in vivo* assembly, Gibson assembly, etc. Cloning can be done in *E. coli* Top10 or similar, before or after the cloning of the spacer.

4. Plasmid methylation
   a) Once the plasmid has been correctly cloned and confirmed by sequencing, transform it into *E. coli* ER1821 for methilation.
   b) Grow the cells directly in liquid media with ampicillin and kanamycin

B. Competent *Saci* cells preparation
   a) Grow *S. acidocaldarius* uracil-auxotroph strain in Brock media with appropriated supplementation until exponential OD_{600} is reached (0.3-0.6).
   b) Re inoculate a calculated amount of culture into fresh Brock medium, so that the OD_{600} the next day is 0.2-0.3 at the start of the experiment.
   c) The next day, once the cells reached OD_{600} between 0.2-0.3, put the cells on ice to cool down. Cool down a centrifuge as well. Keep the cells cold all the time during the protocol.
   d) Centrifuge the cells for 20 min at 2500 xg and 4°C.
   e) Resuspend the cells in 30 mL of ice-cold 20 mM Sucrose.
   f) Centrifuge the cells for 20 min at 2500 xg and 4°C.
   g) Repeat steps e) and f) 1-2 times.
   h) Resuspend the cells in 1 mL 20 mM Sucrose.
   i) Centrifuge for 5 min at 2500 xg at 4°C.
   j) Resuspend the cells in a volume of 20 mM Sucrose so that the theoretical OD600 is 20.
   k) Aliquot the cells in 50 µL fractions into 1.5 mL eppendorf tubes.
   l) Use immediately or store them at -80°C. Do not use liquid nitrogen.

C. Transformation in *S. acidocaldarius* (please note that there are additional steps compared with the standard transformation protocol)
   a) Thaw 50 µL competent cells on ice. Cool down an electroporation cuvette. Start heating the thermoblock to 75°C.
   b) Add 200 ng of methylated plasmid and, if necessary, 400 ng of linear repair fragment. Mix gently and transfer the suspension to the electroporation cuvette.

c) Prepare the recovery solution (see recipes)
d) Electroporate 2000 V, 600 Ohm, 25 µF, 1mm.
e) Add 400 µL of recovery solution directly to the cuvette and pass the suspension to a 1.5 mL Eppendorf tube.
f) Recover placing the tubes in the thermoblock for 30 min at 300 rpm and 75°C.
g) Prepare tubes with 5 mL of Brock media supplemented with 0.2% dextrin, 0.1% NZ-amine and 0.2% xylose.
h) After recovery, inoculate 50 µL of the cell suspension into the 5 mL of medium and incubate at 75°C and 120 rpm for 2-3 days.
i) After at least 2 days, plate 100-150 µL of the cell culture on a first selection plate.
j) Place the plate in a plastic box with wet paper towels at the bottom to prevent evaporation
k) Incubate at 75°C for 6-7 days. Make sure the paper towels remain wet.
l) When colonies are visible, take out the plate and prepare a fresh X-gal + 0.2% dextrin solution (mix 1:4 parts, respectively) in an amber spray bottle.
m) Spray the plates in the sink or the hood. This step does not need to be sterile. The leftover mix can be stored in the fridge for some days but be aware if there is a change of color.
n) Incubate for ≥1 h at 75°C in a humid plastic box.
o) Pick the blue colonies and prepare a master on first selection plates. Let them grow for 2-3 days at 75°C in a humidity chamber.

D. Screening of mutants
a) Prepare Eppendorf tubes with 20 µL of 0.2M NaOH
b) Number your colonies on the master plate
c) With a sterile toothpick, take some cells from one colony on the master plate and resuspend them in the NaOH. Vortex. Repeat with several colonies and number the tubes according to the colonies on the master plate.
d) Add 80 µL of 0.2 M Tris pH 7.8 to each tube. Vortex.
e) Using the outside flanking primers you used to clone the repair fragment, prepare a master mix for the number of samples you have + a negative control + 2 positive controls (gDNA and plasmid, respectively). Use 1 µL of the lysed as template for the screening PCR.
f) IIf you have candidate mutants, repeat the PCR from the lysate with primers that bind in the genome, outside the repair fragment. Sequence the PCR product to confirm.
g) If you are doing point mutations, you can avoid the screening PCR. Do a PCR with the primers binding in the genome, outside of the repair fragment, and send it for sequencing.

E. Plasmid curing
a) Plasmid curing can be done in liquid media or on second selection plates; we prefer to do it on plates.

b) Inoculate cells of the detected mutant from the master plate, into 5 mL of Brock media supplemented with sugar and NZ-amine, and let them grow for 1-2 days (until growth is visible).

c) Prepare dilutions of the culture in brock media (1/300, 1/500, etc.) and plate 100 – 150 µL of the dilution on fresh second selection plates.

d) Incubate the plates for 5-6 days at 75°C in a humidity box until colonies are visible.

e) Pick colonies and inoculate them on liquid media to prepare glycerol stocks.

f) Check for plasmid loss by growing the cells in medium without uracil; growth should not be visible. X-gal can additionally be used to distinguish real colonies from background on plates.

F. Glycerol stock preparation

a) Once the mutant has been confirmed, grow it from the plate in 5 mL Brock medium supplemented with 0.1% N-Z amine, 0.2% dextrin and 10 µL/mL uracil.

b) Transfer an appropriate aliquot to 50 mL Brock medium supplemented as above.

c) When OD_{600} reaches 0.3-0.6, centrifuge the cells at low speed at RT.

d) Resuspend the cells in 1/10 of the original volume in Brock:glycerol::1:1 (e. g. if 10 mL was centrifuged, resuspend in 1 mL glycerol Brock mix)

e) Make aliquots (Usually 50 or 100 µL) and store at –80°C until use.

Data analysis

Plasmid and mutant sequences should be analyzed with the appropriated software.

For sequencing the strains, design primers outside of the flanking regions used in the plasmid to make sure the mutation is inserted at the correct position and to avoid false positives.

Recipes

- Basal Brock medium

Prepare separately:

a) Brock I (1000x)

70 g/L $CaCl_2$ x2H_2O. Autoclave.

b) Trace element solution (2000x)

In 800 mL water, dissolve 9.0 g $Na_2B_4O_7$ x10H_2O by adding H_2SO_4 dropwise. Final pH is not important. Subsequently, add 0.44 g $ZnSO_4$ x7H_2O, 0.10 g $CuCl_2$ x2H_2O, 0.06 g Na_2MoO_4x2H_2O, 0.06 g $VOSO_4$ x2H_2O, 0.02 g $CoSO_4$ x7H_2O, and 3.60 g $MnCl_2$x4H_2O, respectively. Fill up to 1 L with water. Autoclave. Store at 4°C.

c) Brock II/III (100x)

130 g/L $(NH_4)_2SO_4$, 25 g/L $MgSO_4$ x7H_2O, 28 g/L KH_2PO_4 in water. Add 50 mL trace element solution and 2.25 mL H_2SO_4 (50%). Autoclave.

d) Iron solution

20g/L $FeCl_3$ in water. Sterilize by filtration. Store in the dark.

Basal Brock:

Final volume Component	1 L	50 mL
Brock I	1 mL	50 µL
Brock II/III	10 mL	500 µL
Iron solution	1 mL	50 µL

Adjust pH to 3-3.5 with H_2SO_4 (1:1) and store at room temperature.

For supplementation, add NZ-amine to a final concentration of 0.1% and sugar (sucrose or dextrin) to 0.2%. Uracil is required for auxotroph at 10 µL/mL. Dissolve completaly at 75°C before adding it to the medium.

- Recovery solution

Add to 50 mL of water:

- 50 µL Brock I
- 500µL Brock II/III
- 50 µL iron solution
- 0.1% NZ-amine

Do not adjuste pH. Sterilize by filtration.
It can be stored on the bench for several months.

- First selection plates

Add to 200 mL of water:

- 400 µL Brock I
- 4 mL Brock II/III
- 400 µL iron
- 4 mL $MgCl_2$ 0.5 M
- 2.4 mL $CaCl_2$ 1 M
- 4 mL dextrin 20%
- 2 mL NZ amine 20%

- For second selection plates, add additionally:
- 400 µL 5-FOA 100 mg/mL
- 800 µL uracil 5 mg/mL (dissolve at 75°C before adding)

Warm the mixture in the 75°C incubator (do not microwave). Melt the 1.2% gelrite by boiling in the microwave and mix both in equal proportions. Pour thick plates immediately and let them solidify.
First selection plates can be stored in the fridge but must be warm up prior to use. For second selection plates, make them fresh. You can use old second selection plates for master plates.

Additional Notes

- If *ApaI/NdeI* sites are used to clone the repair fragments, the same fragment can be cloned directly into pSVA431 (pop-in/pop-out plasmid).
- While making competent cells, keep the cells cold at all time.
- Do not use liquid nitrogen to freeze Sulfolobus cells.
- Always make sure that plates are at least at room temperature before plating the cells. You can briefly warm them up in the incubator.
- Do not store *Saci* cells (liquid or plates) in the fridge
- Always use media at least at RT, never cold.

Trial and error

- The incubation at 75°C with shaking for 2-3 days before plating the cells is important to obtain blue colonies. If well designed, normally all blue colonies are mutants.
- Second selection plate should be fresh (maximum 2-3 days old) for the plasmid curing. For master plates, old plates can be used.
- In our experience, TCA PAM works the best, but CCA and GTA can be used if needed.
- If you experience problems growing *Saci*, check the water quality. Also, the dextrin can cause problems, in that case, it can be replaced by sucrose.
- Plasmid curing in liquid involves passing the cells 2-3 generations in liquid Brock supplemented with sugar, NZ-amine, uracil and 5-FOA in the same concentration as in the plates.

Competing interests

The authors declare that they have no conflict of interest.

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