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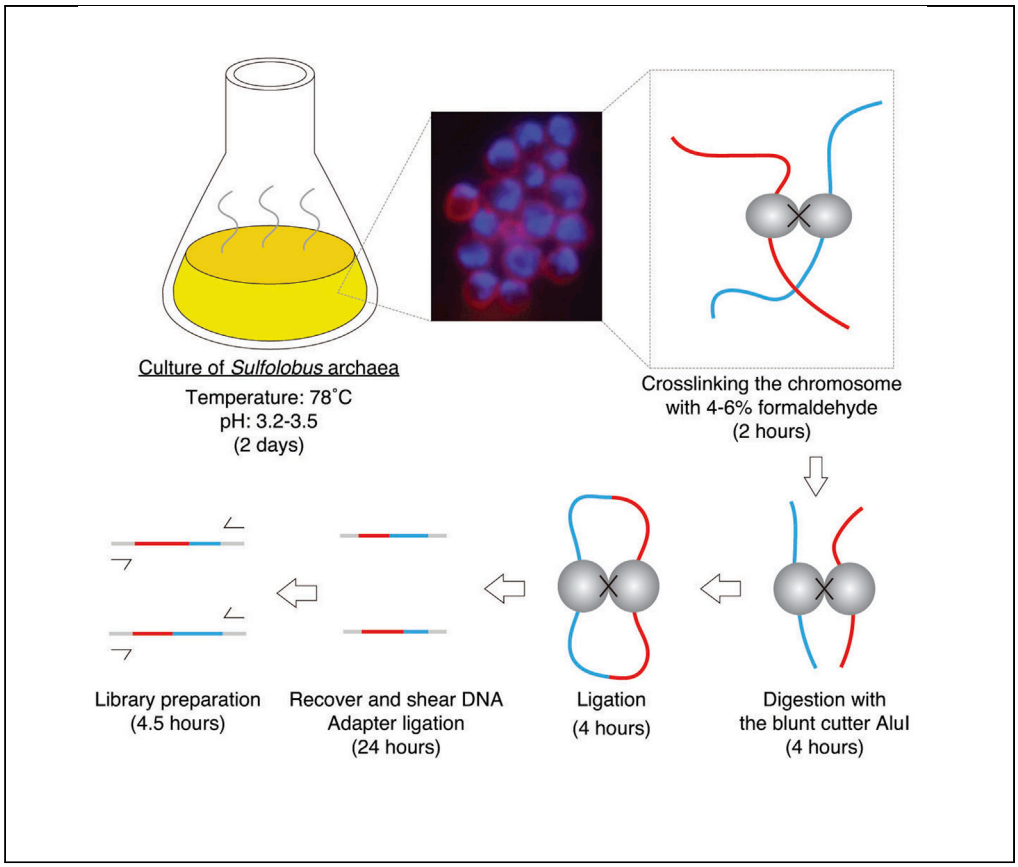
High-resolution analysis of chromosome conformation in hyperthermophilic archaea



Chromosome conformation capture (3C) techniques are emerging as promising approaches to study genome organization in Archaea, the least understood domain of life in terms of chromosome biology. Here, we describe a 3C technique combined with deep sequencing for the hyperthermophilic archaeal genus *Sulfolobus*. Instead of using restriction enzymes compatible with fill-in labeling, this protocol uses the 4-bp blunt cutter AluI to generate high-resolution (up to 2 kb) contact maps from *Sulfolobus* species.

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Highlights

High-resolution chromosome conformation capture of *Sulfolobus* archaea

Culture conditions for *Sulfolobus*

DNA digestion, ligation, and recovery procedures

Library preparation for next-generation sequencing

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High-resolution analysis of chromosome conformation in hyperthermophilic archaea

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SUMMARY

Chromosome conformation capture (3C) techniques are emerging as promising approaches to study genome organization in Archaea, the least understood domain of life in terms of chromosome biology. Here, we describe a 3C technique combined with deep sequencing for the hyperthermophilic archaeal genus *Sulfolobus*. Instead of using restriction enzymes compatible with fill-in labeling, this protocol uses the 4-bp blunt cutter AluI to generate high-resolution (up to 2 kb) contact maps from *Sulfolobus* species.

For complete details on the use and execution of this protocol, please refer to Takemata and Bell (2021).

BEFORE YOU BEGIN

For safety, wear gloves, safety glasses, and laboratory coat when handling reagents and chemicals. Also, it is essential that you read the appropriate Material Safety Data Sheets and the safety manuals provided by your institution.

Making medium for *Sulfolobus acidocaldarius*

Timing: 1 h

Prepare Brock's medium (Brock et al., 1972) as follows to cultivate *S. acidocaldarius*.

1. To 900 mL of Milli-Q water, add the following reagents (see materials and equipment for their recipes).
   a. 10 mL of 100 × Solution A
   b. 5 mL of 200 × Solution B
   c. 1 mL of 1,000 × Solution C
   d. 2 g of sucrose
   e. 1 g of tryptone
2. Adjust pH to 3.2 with 50% (v/v) H_2SO_4.
3. Adjust the volume to 1 L with Milli-Q water.
4. Filter sterilize the medium. We typically use 500 mL rapid-flow bottle top filters with a 0.2 μm aPES membrane, 75 mm diameter from Thermo Fisher. Do not autoclave. The medium can be stored for at least a year at 4°C.

Making medium for *Sulfolobus islandicus*

Timing: 1 h

5. To 800 mL of Milli-Q water, add the following reagents (see materials and equipment for the recipes of 10 × Base Salts and 100 × Vitamins).
   a. 100 mL of 10 × Base Salts
   b. 10 mL of 100 × Vitamins
   c. 1 g of tryptone
   d. 2 g of sucrose
   e. 0.5 g of Bacto Yeast Extract
6. Adjust pH to 3.5 with 50% (v/v) H_2SO_4.
7. Adjust the volume to 1 L with Milli-Q water.
8. Filter sterilize the medium. It can be stored for at least a year at 4°C.

Pre-cultivation of *Sulfolobus* cells

Timing: 2–3 days

9. Inoculate cells of *S. acidocaldarius* or *S. islandicus* in an appropriate medium from your freezer stock. We typically prepare a 10-mL culture in a 25 cm^2 plastic cell culture flask (see Key Resource Table for more detail). If you grow an uracil auxotrophic strain, add uracil solution to final concentrations of 0.01 mg/mL and 0.02 mg/mL for *S. acidocaldarius* and *S. islandicus* respectively.
10. Cultivate the cells at 78°C with agitation (110 rpm) until they reach mid-log to stationary phase (2–3 days). We typically grow *Sulfolobus* cells in a water bath with a cover and stand the plastic flask upright in it. The culture can be kept at 21°C for a couple of weeks with little loss of viability.

⚠ CRITICAL: The culture is hot and needs to be handled with care.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Sulfolobus acidocaldarius DSM639	Lab stock (the strain can be obtained from https://www.dsmz.de/)	n/a
Sulfolobus islandicus REY15A	Lab stock – originally obtained as a kind gift from Dr. Qunxin She, Shandong University	n/a
Chemicals, peptides, and recombinant proteins		
Tryptone	Thermo Fisher	BP1421
Bacto Yeast Extract	Gibco	212750
37% Formaldehyde solution	Macron	5016-02
Phosphate-buffered saline (PBS) tablets	Oxoid	BR0014G
Proteinase K	Gold Biotechnology	P-480-1
10 × NEBuffer 2	New England Biolabs	B7002S
Triton X-100	Acros Organics	327371000
10 U/ μL AluI	New England Biolabs	R0137L
10 × T4 DNA Ligase Reaction Buffer	New England Biolabs	B0202S
400 U/ μL T4 DNA Ligase	New England Biolabs	M0202L
Phenol:chloroform:isoamyl alcohol	Sigma-Aldrich	77618-100ML

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
20 mg/mL Glycogen	USB	16445
RNase A	Sigma-Aldrich	R4875-500MG
Buffer EB	QIAGEN	19086
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
AMPure XP beads	Beckman Coulter	A63880
Critical commercial assays		
NEBNext Ultra DNA Library Prep Kit for Illumina	New England Biolabs	E7370S https://international.neb.com/products/e7370-nebnext-ultra-dna-library-prep-kit-for-illumina#Product%20Information
NEBNext Multiplex Oligos for Illumina	New England Biolabs	E7335S https://international.neb.com/products/e7335-nebnext-multiplex-oligos-for-illumina-index-primers-set-1#Product%20Information
Other		
1.5-mL Safe-Lock tubes	Eppendorf	0030 120.086
1.5-mL DNA LoBind tube	Eppendorf	0030108051
Bottle top filters	Thermo Fisher	595-4520
Tissue culture flasks	Corning	430168
Bioruptor	Diagenode	UCD-300
DynaMag™-2 Magnet	Thermo Fisher	12321D
Agilent 4150 TapeStation System	Agilent Technologies	G2992AA

MATERIALS AND EQUIPMENT

100 × Solution A		
Reagent	Final concentration	Amount
Milli-Q water	n/a	X mL
$(NH_4)_2SO_4$	0.98 M	130 g
$MgSO_4 \bullet 7H_2O$	0.1 M	25 g
$FeCl_3 \bullet 6H_2O$	7 mM	2 g
50% (v/v) H_2SO_4	0.15% (v/v)	3 mL
Total	**n/a**	**1 L**

Note: Filter sterilize and store the solution at 4°C. It is stable for at least a year.

200 × Solution B		
Reagent	Final concentration	Amount
Milli-Q water	n/a	X mL
$MnCl_2 \bullet 4H_2O$	2.8 mM	566 mg
$ZnSO_4 \bullet 7H_2O$	270 μM	78.3 mg
$CuCl_2 \bullet 2H_2O$	74.5 μM	12.7 mg
$VOSO_4 \bullet 5H_2O$	36 μM	9.32 mg
$CoSO_4 \bullet 7H_2O$	12.9 μM	3.63 mg
$Na_2B_4O_7 \bullet 10H_2O$	447 μM	170 mg
$Na_2MoO_4 \bullet 2H_2O$	29 μM	7.05 mg
50% (v/v) H_2SO_4	0.25% (v/v)	5 mL
Total	**n/a**	**1 L**

Note: Filter sterilize and store the solution at 4°C. It is stable for months.

1,000 × Solution C		
Reagent	Final concentration	Amount
Milli-Q water	n/a	X mL
$CaCl_2$●$2H_2O$	476 mM	14 g
Total	**n/a**	**200 mL**

Note: Autoclave and store the solution at 21°C. It is stable for at least a year.

10 × Base Salts		
Reagent	Final concentration	Amount
Milli-Q water	n/a	X mL
$(NH_4)_2SO_4$	227 mM	30 g
K_2SO_4	28 mM	5 g
KCl	13 mM	1 g
Glycine	93 mM	7 g
$MnCl_2$●$4H_2O$	40 μM	8 mg
$Na_2B_4O_7$●$10H_2O$	55 μM	21 mg
$ZnSO_4$●$7H_2O$	3.8 μM	1.1 mg
$CuSO_4$●$5H_2O$	1 μM	0.25 mg
Na_2MoO_4●$2H_2O$	619 nM	0.15 mg
$VOSO_4$●$5H_2O$	592 nM	0.15 mg
$CoSO_4$●$7H_2O$	177 nM	0.05 mg
$NiSO_4$●$6H_2O$	190 nM	0.05 mg
1% (w/v) FeSO4●7H2O solution	0.002%	2 mL
$Ca(NO_3)_2$●$4H_2O$	3 mM	708 mg
Total	**n/a**	**1 L**

Note: Dissolve FeSO4●7H2O in 0.5 M HCl to make 1% solution. Adjust pH of 10 × Base Salts to 3 with 50% (v/v) H_2SO_4. Autoclave and store 10 × Base Salts at 21°C. It is stable for at least a year.

100 × Vitamins		
Reagent	Final concentration	Amount
Milli-Q water	n/a	X mL
p-Aminobenzoic acid	36 μM	5 mg
Biotin	8 μM	2 mg
DL-Calcium pantothenate	10 μM	5 mg
Cyanocobalmine	73 nM	0.1 mg
Folic acid	4.5 μM	2 mg
Nicotinic acid	406 μM	5 mg
Pyridoxine-HCl	49 μM	10 mg
Riboflavin	13 μM	5 mg
Thiamine-HCl	15 μM	5 mg
Lipoic acid	24 μM	5 mg
Total	**n/a**	**1 L**

Note: Filter sterilize and store the solution in a dark room at 4°C. It is stable for months.

Uracil solution	
44 mM uracil	Dissolve 50 mg uracil in 10 mL Milli-Q water

Note: Filter sterilize and store the solution at 4°C. Warm the solution before use to dissolve a precipitate formed during storage. It is stable for months.

1× PBS	
1× PBS	Dissolve 10 PBS tablets in 1 liter Milli-Q water

Note: Autoclave and store the solution at 21°C. To wash fixed cells, store an aliquot of it at 4°C. It is stable for months.

Fixation Buffer	
5 or 7.5% Formaldehyde	10.8 or 16.2 mL 37% formaldehyde solution fill up to 80 mL with 1xPBS.

Note: Prepare immediately before use.

2.5 M glycine	
2.5 M Glycine	93.8 g Glycine, add 500 mL ddH_2O to 500 mL

Note: Autoclave and store the solution at 21°C. It is stable for months.

1× PBS/1 mM EDTA	
1× PBS/1 mM EDTA	Combine 49.9 mL 1× PBS with 0.1 mL 0.5 M EDTA (pH 8)

Note: Store at 4°C. It is stable for months.

10% Triton X-100	
10% Triton X-100	Add 1g Triton X-100 to 9 mL Milli-Q water

Note: Filter sterilize and store the solution at 21°C. Triton X-100 is light-sensitive, keep the 10% solution in the dark and prepare a fresh working solution every few months.

0.1 × TE (pH 8)	
0.1 × TE (pH 8)	Combine 200 µL of 1 M Tris-HCl (pH 8) with 40 µL of 0.5 M EDTA (pH 8), make up to 200 mL with Milli-Q water

Note: Autoclave and store the solution at 21°C. Solution is stable for several months.

⚠ CRITICAL: Formaldehyde is toxic. Handle it carefully while wearing appropriate protective equipment (gloves, safety glasses, laboratory coat, etc.) on and according to your

institution's safety guidelines. It is also essential that you read the appropriate Material Safety Data Sheet.

⚠ CRITICAL: Phenol:chloroform:isoamyl alcohol is toxic. Handle it carefully with protective equipment (gloves, safety glasses, laboratory coat, etc.) on and according to your institution's safety guidelines. It is also essential that you read the appropriate Material Safety Data Sheets.

Alternatives: Other equipment with similar functions can be used (Covaris Ultrasonicator for DNA shearing and Agilent 2100 Bioanalyzer for quality check of DNA libraries, for example).

STEP-BY-STEP METHOD DETAILS

Fixation of *Sulfolobus* cells

Timing: 2 days

Cultivate and fix *Sulfolobus* cells to preserve DNA-DNA contacts *in vivo*.

Day 1

Timing: 10 min

1. Inoculate the pre-culture of cells in 50 mL of an appropriate medium.
2. Cultivate the cells at 78°C with agitation (110 rpm) overnight until the culture reaches an appropriate growth phase for your experiment (mid-log phase, stationary phase, etc.). The anticipated doubling time during exponential growth will be ~3 h for the *S. acidocaldarius* strain DSM639 and ~4.5 h for the *S. islandicus* strain E233S.

⚠ CRITICAL: The culture is hot and needs to be handled with care.

Day 2

Timing: 2 h

3. Fix cells as follows. It is important to take a cell culture and mix it with formaldehyde as quickly as possible. Also, make sure to incubate the mixture at 25°C. Variation in the temperature during fixation could lead to variable fixation efficiency (see troubleshooting problem 1 for more detail).
   a. Take 20 mL of the culture while keeping the flask in the water bath. Quickly mix it with 80 mL of ambient temperature Fixation Buffer (approximately 20°C). Use Fixation Buffer containing 5% formaldehyde for *S. acidocaldarius* (the final concentration is 4% after mixing) and 7.5% for *S. islandicus* (the final concentration is 6% after mixing).
   b. Incubate the mixture for 30 min at 25°C with agitation (110 rpm).
   c. Add 11.2 mL of 2.5 M glycine to quench the crosslinking reaction.
   d. Incubate the mixture for 10 min at 21°C.
4. Wash the fixed cells as follows.
   a. Dispense the mixture into two 50-mL tubes and centrifuge them for 30 min at 3,120 × *g*, 4°C.
   b. Carefully remove the supernatant but leave ~2 mL of it in each tube to avoid loss of cells.
   c. Resuspend the cells in the left supernatant and dispense the suspension into as many 1.5-mL tubes as you need.
   d. Spin down for 2 min at 21,000 × g, 4°C.
   e. Remove the supernatant and resuspend the pellets together in a total of 1 mL ice-cold 1× PBS.
   f. Spin down for 2 min at 21,000 × g, 4°C and remove the supernatant.
   g. Resuspend the pellet in 1 mL of ice-cold 1× PBS.

h. Repeat the sub-step f.

5. Store the pellet at −80°C.

Note: The fixed pellet can be kept at least for a month.

Note: In the protocol described here for 3C analyses, cells are fixed with higher concentrations of formaldehyde than used for Hi-C analyses of *Sulfolobus* cells (Takemata et al., 2019; Takemata and Bell, 2021). These fixation conditions have been optimized by us to to allow capture of contacts between AluI (4-bp cutter) fragments, which are much smaller than HindIII (6-bp cutter) fragments generated in the Hi-C protocol.

DNA digestion followed by proximity ligation

Timing: 11 h

This section describes how to digest crosslinked cellular DNA with AluI. Generated fragments are used for proximity ligation without biotin labeling.

6. Resuspend a cell pellet in 320 μL of 1× PBS/1 mM EDTA (for *S. acidocaldarius*) or 1 × NEBuffer 2 (for *S. islandicus*).
7. Mix 20 μL of the suspension with 780 μL of 1× PBS to measure OD_{600}.
8. Dilute the remainder of the cell suspension to an OD_{600} of 4 (roughly corresponding to a 2- or 3-fold dilution of the original suspension). For the dilution, use the same buffer as used in step 6. Use 400 μL of the diluted cell suspension for subsequent steps.
9. If you are using *S. acidocaldarius*, treat cells with proteinase K as follows to partially disrupt the cell wall. Skip this step when using *S. islandicus*.
   a. Dilute proteinase K solution to 2 mg/mL in 1× PBS/1 mM EDTA. Add 4.8 μL of the diluted proteinase K solution and incubate the sample for 20 min at 37°C with agitation (600 rpm).
   b. Immediately spin down for 5 min at 21,000 × *g*, 4°C. Remove the supernatant.
   c. For wash, resuspend the pellet with 1 mL of ice-cold 1× PBS/1 mM EDTA and spin down for 5 min at 21,000 × *g*, 4°C. Remove the supernatant.
   d. Repeat sub-step c three times for a total of 4 washing steps.
   e. Resuspend the cells with 1 mL of ice-cold 1 × NEBuffer 2.
10. Spin down for 5 min at 21,000 × *g*, 4°C to remove the supernatant.
11. Resuspend the pellet in 50 μL of 1 × NEBuffer 2.
12. The total volume of the cell suspension will be variable due to residual supernatant not removed in step 10. For accuracy, transfer 50 μL of the suspension to a new 1.5-mL tube.
13. To permeabilize the cells and remove non-crosslinked DNA-binding proteins for subsequent digestion, add 5.55 μL of 10% SDS for a final concentration of 1%. Incubate the mixture for 15 min at 65°C with agitation (600 rpm).
14. Immediately cool down the tube on ice for exactly 90 s.

Note: If the tube is left on ice too long, a precipitate could form and affect the downstream steps.

15. Spin down condensate briefly and mix by pipetting.
16. Assemble the following reactions in 1.5-mL tubes. For Sample D, add AluI last and mix the other components well to make sure that the reaction is properly buffered and SDS is quenched with Triton X-100 before adding the enzyme.

Sample U (undigested control)		
Reagent	Final concentration	Amount
Cell lysate	n/a	12.5 µL
10 × NEBuffer 2	n/a	1.6 µL
1 × NEBuffer 2	n/a	20.9 µL
10% Triton X-100	2%	10 µL
Milli-Q water	n/a	5 µL
Total	**n/a**	**50** µL

17. Incubate the samples for 3.5 h at 37°C with agitation (600 rpm).
18. Prepare two new 1.5-mL Eppendorf Safe-Lock Tubes and dispense 50 µL of Sample D to each of them. Label the two tubes as "Sample L."

Sample D (digestion reaction)		
Reagent	Final concentration	Amount
Cell lysate	n/a	37.5 µL
10 × NEBuffer 2	n/a	4.8 µL
1 × NEBuffer 2	n/a	62.7 µL
10% Triton X-100	2%	30 µL
10 U/ µL AluI	1 U/ µL	15 µL
Total	**n/a**	**150** µL

19. Add reagents as follows to quench the digestion.

To Sample U		
Reagent	Final concentration	Amount
(Sample U)	(n/a)	(50 µL)
1 × NEBuffer 2	n/a	50 µL
10% SDS	n/a	10 µL
0.5 M EDTA (pH 8)	22 mM	5 µL
Total	**n/a**	**115** µL

To Sample D		
Reagent	Final concentration	Amount
(Sample D)	(n/a)	(50 µL)
1 × NEBuffer 2	n/a	50 µL
10% SDS	n/a	10 µL
0.5 M EDTA (pH 8)	22 mM	5 µL
Total	**n/a**	**115** µL

To each Sample L		
Reagent	Final concentration	Amount
(Sample L)	(n/a)	(50 µL)
10% SDS	n/a	5.56 µL
Total	**n/a**	**55.6** µL

20. Incubate the samples for 30 min at 37°C with agitation (600 rpm).
21. Add the following reagents to each Sample L. To make sure that the reaction is properly buffered and SDS is quenched with Triton X-100 before adding T4 DNA ligase, mix the other components well first and then add the enzyme.

Reagent	Final concentration	Amount
(Sample L)	(n/a)	(55.6 μL)
Milli-Q water	n/a	734 μL
10 × T4 DNA Ligase Reaction Buffer	1×	100 μL
10% Triton X-100	n/a	100 μL
400 U/μL T4 DNA Ligase	4 U/μL	10 μL
Total	**n/a**	**1,000 μL**

22. Incubate all samples for 4 h at 16°C with agitation (600 rpm). Mix the Samples L by inverting the tubes every 30 min.

Cross-link reversal

Timing: > 12 h (overnight)

Remove crosslinks for subsequent DNA purification.

23. Add the following reagents to each Sample L.

Reagent	Final concentration	Amount
(Sample L)	(n/a)	(1,000 μL)
10% SDS	n/a	100 μL
0.5 M EDTA (pH 8)	n/a	50 μL
Total	**n/a**	**1,150 μL**

24. Add 5 μL of 20 mg/mL proteinase K to each of all samples.
25. Incubate the samples for 6 h at 65°C and then for > 6 h at 37°C. Agitate the samples at 600 rpm during the incubation. Overheating of the samples could lead to DNA degradation by endogenous nucleases (see troubleshooting problem 3 for more detail).

DNA purification 1

Timing: 8 h

This section describes how to purify DNA and confirm successful DNA digestion and ligation.

26. Combine and dispense the two Samples L into four 1.5-mL tubes (~560 μL /tube).
27. Add 1 volume of phenol:chloroform:isoamyl alcohol to each tube and mix by hand. Spin down for 10 min at 10,000 × *g*, 21°C. Phenol:chloroform:isoamyl is toxic and must be handled with care as noted above.
28. Transfer aqueous phase in each tube (80 μL for Sample U and Sample D and 510 μL for Sample L) to a new 1.5-mL tube. Be careful not to disturb the interphase.

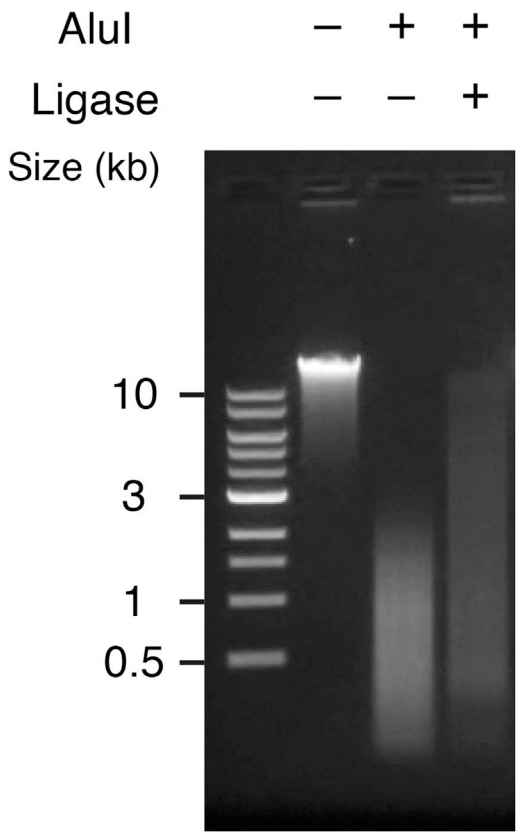

Figure 1. Quality check of 3C DNA by gel electrophoresis

3C DNA obtained from *S. acidocaldarius* was run on a 0.7 % agarose gel together with control samples. DNA marker: 1 kb DNA Ladder (New England Biolabs N3232S).

29. (For Samples L only) Repeat 28. Take 450 μL of aqueous phase from each tube and combine them for a total of 1,800 μL. Dispense the combined extract into three 1.5-mL tubes.
30. Add 0.1 volume of 3 M NaOAc (pH 5.2) to each tube.
31. For Samples U and D, add 2 μL of 20 mg/mL glycogen to each tube. For Sample L, add 2.7 μL of 20 mg/mL glycogen to each tube.
32. Add 1 volume of isopropanol and leave the samples for 1 h at −20°C.
33. Spin down for 1 h at 21,000 × *g*, 4°C.
34. Remove the supernatant and rinse the pellet in 500 μL of 70% ethanol.
35. Spin down for 5 min at 21,000 × *g*, 4°C.
36. Remove the supernatant and spin down briefly to remove residual ethanol.
37. Leave the tubes open for 10 min at 21°C to air-dry the pellets.
38. For Samples U and D, dissolve the pellet in 20 μL of TE buffer containing 0.1 mg/mL RNase A. For Sample L, dissolve the three pellets together in 40 μL of TE buffer containing 0.1 mg/mL RNase A.
39. Incubate the samples for 30 min at 37°C.
40. Run 5 μL of each sample on a 0.7% agarose gel for quality control. In Sample U (undigested control), you will see a band above 10 kb in size (Figure 1). The band will be slightly smeared, but it is acceptable. If digestion with AluI is successful, DNA in Sample D (digested, but not ligated) should be seen as a smear below 3 kb. In Sample L (digested and then ligated), you will see a broader smear that starts from approximately the same position as the DNA band in Sample U. This shift toward the larger DNA size is a sign of successful proximity ligation. See troubleshooting problems 2 and 3 for potential problems and solutions.

Pause point: You can stop the experiment by storing the remainder of Sample L at −20°C.

DNA purification 2 and DNA shearing

Timing: 4 h

This section includes second DNA purification and DNA shearing in preparation for library construction.

41. Adjust the volume to 100 μL by adding Buffer EB.
42. Add 1 volume of phenol:chloroform:isoamyl and mix by vortexing.
43. Spin down for 10 min at 10,000 × *g*, 21°C.
44. Transfer 80 μL of the aqueous phase to a new 1.5-mL tube.
45. Add back 80 μL of Buffer EB to the tube containing phenol:chloroform:isoamyl. Mix by vortexing and spin down for 10 min at 10,000 × *g*, room temperature. Combine 80 μL of the aqueous phase with the extract previously taken for a total of 160 μL.
46. Add 0.1 volume of 3 M NaOAc (pH 5.2).
47. Add 2 volume of 100% ethanol. Leave the sample on ice for 1 h.
48. Spin down for 30 min at 21,000 × *g*, 4°C.
49. Remove the supernatant and rinse the pellet in 500 μL of 70% ethanol.
50. Spin down for 5 min at 21,000 × *g*, 4°C.
51. Remove the supernatant and spin down briefly to remove residual ethanol.
52. Leave the tubes open for 10 min at 21°C to air-dry the pellets.
53. Dissolve the pellet in 90 μL of Buffer EB. Transfer the solution to a 0.6-mL tube and chill it on ice for 10 min.
54. Shear the DNA into fragments of 300–400 bp on average using a Bioruptor and the following parameters. Power: Low, ON time: 30 s, OFF time: 30 s, Cycles: 40.

Note: We generate DNA fragments in this step that are slightly larger than those desired in the same step for Hi-C of *Sulfolobus* (Takemata et al., 2019; Takemata and Bell, 2021) Over-shearing of DNA generates fragments that do not contain ligation junctions and thus are unusable for generation of contact maps. These fragments can be easily removed in the biotin pull-down step in Hi-C but not in this protocol, in which the biotin enrichment procedures are omitted.

Pause point: You can stop the experiment by storing the sheared DNA at −20°C.

Library construction

Timing: 4.5 h

This section describes how to prepare a 3C-seq library using NEBNext Ultra DNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina. The protocol essentially follows the manufacturer's instructions (https://www.neb.com/protocols/2014/05/22/protocol-for-use-with-nebnext-ultra-dna-library-prep-kit-for-illumina-e7370).

Optional: Measure the DNA concentration of your sample using Qubit dsDNA HS Assay Kit before starting the library preparation.

55. Perform NEBNext End Prep as follows.
    a. Mix the following reagents in a PCR tube.

Reagent	Final concentration	Volume
Sheared DNA	n/a	55.5 μL
10× End Repair Reaction Buffer (provided in the library prep kit with a green cap)	1×	6.5 μL
End Prep Enzyme Mix (provided in the library prep kit with a green cap)	n/a	3 μL
Total	**n/a**	**65 μL**

b. Incubate the reaction in a thermocycler with the heated lid on as follows: 30 min at 20°C, 30 min at 65°C, and then hold at 4°C.

56. Perform Adapter Ligation as follows.
    a. Mix the following reagents in a 1.5-mL DNA LoBind Tube. Blunt/TA Ligase Master Mix is very viscous and should be pipetted carefully (see troubleshooting problem 5 for more detail).

Reagent	Final concentration	Volume
End Prep reaction	n/a	65 μL
15 μM NEBNext Adaptor for Illumina (provided in the multiplex oligo kit with a red cap)	0.45 μM	2.5 μL
Ligation Enhancer (provided in the library prep kit with a red cap)	n/a	1 μL
Blunt/TA Ligase Master Mix (provided in the library prep kit with a red cap)	n/a	15 μL
Total	**n/a**	**83.5 μL**

Note: Do not premix the Ligation Master Mix, Ligation Enhancer, and adaptor before use.

Note: If you measure the DNA concentration of your sample using Qubit dsDNA HS Assay Kit and find that your input DNA is < 100 ng, dilute NEBNext Adaptor for Illumina according to the manufacturer's instructions (https://www.neb.com/protocols/2014/05/22/protocol-for-use-with-nebnext-ultra-dna-library-prep-kit-for-illumina-e7370). We usually get > 100 ng of input DNA and therefore skip the dilution step.

    b. Incubate the sample for 15 min at 20°C.
    c. Add 3 μL of USER Enzyme (provided in the multiplex oligo kit with a red cap).
    d. Incubate the sample for 15 min at 37°C.

57. Perform the "Size Selection of Adapter Ligated DNA" step as follows to purify DNA of 400–500 bp (insert + adapter).

Note: As mentioned in the sonication step before the library construction, this 3C-seq protocol aims to shear ligated DNA into larger sizes than those desired in the Hi-C protocol for *Sulfolobus* (Takemata et al., 2019; also see Takemata and Bell, 2021). Due to this difference, the size of DNA purified in step 57 is also larger than the size range in the same step for Hi-C of *Sulfolobus* (~320 bp).

    a. Add 13.5 μL of Milli-Q water to the reaction for a total of 100 μL.
    b. Resuspend AMPure XP Beads by vortexing.

Note: Warm AMPure XP Beads to 21°C before use.

    c. Mix the reaction with 40 μL of resuspended AMPure XP Beads.

d. Incubate the mixture for 5 min at room temperature.
e. Place the tube on a DynaMag™-2 Magnet to separate the beads. Transfer the supernatant (containing DNA) to a new 1.5-mL DNA LoBind Tube.
f. Mix the supernatant with 20 μL of resuspended AMPure XP Beads.
g. Incubate the mixture for 5 min at 21°C.
h. Place the tube on a DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
i. For wash, add 200 μL of 80% ethanol to the tube containing the beads. Incubate the tube for 30 sec at 21°C and then remove the ethanol. Keep the tube on DynaMag™-2 Magnet during the wash.
j. Repeat sub-step i.
k. To air-dry the beads, leave the tube with the lid opened on the DynaMag™-2 Magnet for 5 min.
l. Elute the selected DNA from the beads by resuspending them in 17 μL of 0.1 × TE (pH 8). Incubate the suspension for 2 min at 21°C.
m. Place the tube on DynaMag™-2 Magnet to separate the beads. Transfer 15 μL of the supernatant to a PCR tube.

58. Perform the "PCR Enrichment of Adapter Ligated DNA" step as follows.
    a. Mix the following reagents.

Reagent	Final concentration	Volume
Bead suspension	n/a	15 μL
10 μM NEBNext Universal Primer for Illumina (provided in the multiplex oligo kit with a blue cap)	1 μM	5 μL
10 μM NEBNext Index Primer for Illumina (provided in the multiplex oligo kit with a blue cap)	1 μM	5 μL
NEBNext Q5 Hot Start HiFi PCR Master Mix (provided in the library prep kit with a blue cap)	n/a	25 μL
Total	**n/a**	**50 μL**

    b. Do PCR as follows.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	8 or 9 cycles
Annealing/ Extension	65°C	75 sec	
Final extension	65°C	5 min	1
Hold	15°C	Forever	

59. Perform Cleanup of the PCR Amplification products as follows.
    a. Transfer the reaction to a new 1.5-mL DNA LoBind Tube.
    b. Resuspend AMPure XP Beads by vortexing.

    Note: Warm AMPure XP Beads to 21°C before use.
    c. Mix the reaction with 45 μL of resuspended AMPure XP Beads.
    d. Incubate the mixture for 5 min at 21°C.
    e. Place the tube on a DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
    f. For a wash, add 200 μL of 80% ethanol to the tube containing the beads. Incubate the tube for 30 s at 21°C and then remove the ethanol. Keep the tube on the DynaMag™-2 Magnet during the wash.

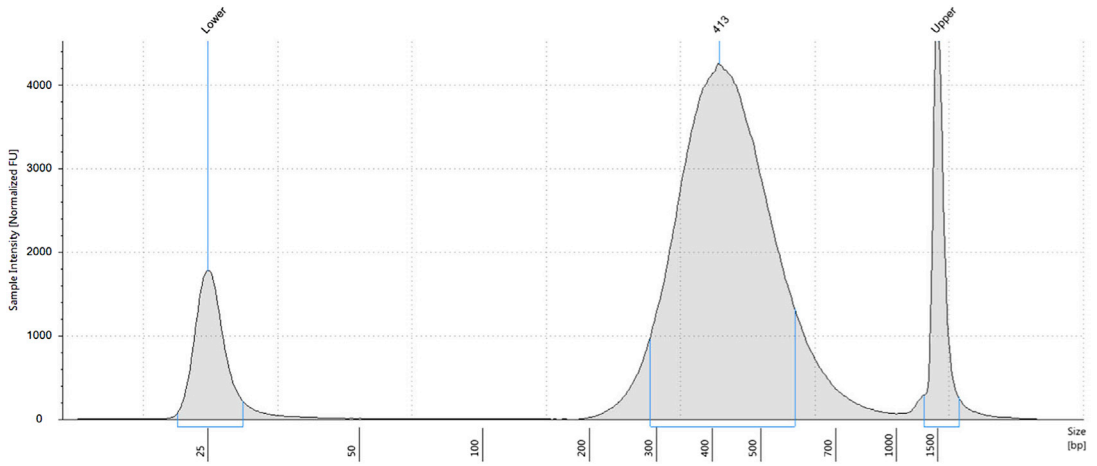

Figure 2. Size distribution of a typical 3C-seq DNA library from *S. acidocalarius*

A 3C-seq library was constructed using NEBNext Ultra DNA Library Prep Kit for Illumina and analyzed by Agilent 4150 TapeStation System and a D1000 ScreenTape. The peaks for the DNA library and upper and lower markers are indicated.

g. Repeat sub-step f.
h. To air-dry the beads, leave the tube with the lid opened on the DynaMag™-2 Magnet for 5 min.
i. Elute the DNA from the beads by resuspending them in 25 μL of 0.1 × TE (pH 8). Incubate the suspension for 2 min at 21°C.
j. Place the tube on the DynaMag™-2 Magnet to separate the beads. Transfer 22 μL of the supernatant to a new 1.5-mL DNA LoBind Tube.

60. Use an Agilent 4150 TapeStation System to check the size and concentration of library DNA. Please see troubleshooting problem 5 when the DNA size deviates from the expected one.
61. Perform paired-end sequencing using an Illumina sequencing platform. We routinely use NextSeq for sequencing of Hi-C libraries. We typically aim to obtain 80–100 million of total reads per library. Although the genomes of *S. acidocaldarius* and *S. islandicus* are AT-rich (63% and 65% respectively), we get good quality reads with a small amount of PhiX spike-in (0.5%).

EXPECTED OUTCOMES

A typical library is 10–40 ng/μL (50–200 nM) with a peak at ~400 bp (Figure 2). See troubleshooting problems 4 and 5 in case you do not obtain the expected outcomes.

We routinely map 3C-seq reads using HiC-Pro (Servant et al., 2015; Takemata and Bell, 2021). Table 1 shows typical read statistics generated by HiC-Pro. In our 3C-seq using *Sulfolobus*, roughly 25% of the total reads are categorized as valid ligation products, which can be used to generate a contact map.

LIMITATIONS

Since this protocol uses the blunt cutter AluI and does not include biotin pull-down of ligation products, the fraction of reads usable to generate a contact map is relatively small (~30%). It should also be noted that, when the genome of *S. islandicus* is binned at 2 kb, some bins will contain a very low number of reads because of the presence of repeated elements in the form of insertion sequences (ISs). These IS-containing loci will be hard to analyze with high resolution.

Table 1. Read statistics of a typical 3C-seq library from *S. acidocaldarius*

Total read pairs		101,464,197	
Reported pairs after mapping		97,393,355	(96.0%)
Invalid pairs	Dangling-end pairs	48,116,757	(47.4%)
	Religation pairs	20,539,284	(20.2%)
	Self circle	2,380,340	(2.3%)
	Singletons	0	(0.0%)
	Dumped pairs	1,411	(0.0%)
Valid pairs	Before removal of PCR duplicates	26,355,563	(26.0%)
	After removal of PCR duplicates	24,589,591	(24.2%)

Percentage relative to the number of the total read pairs is also shown.

TROUBLESHOOTING

Problem 1

Digestion/ligation efficiency of DNA is variable between replicates (steps 16–22).

Potential solution

This could be due to variable efficiency in fixation caused by the variability in the reaction temperature. Make sure to take a cell culture while keeping the flask in a hot water bath. Also, mix the culture with formaldehyde as quickly as possible and then incubate the mixture at 25°C.

Problem 2

Digestion efficiency of DNA is low (step 16)

Potential solution

- Triton X-100 may be too old to quench SDS. Prepare a fresh working solution.
- Make sure that the cell lysate is mixed well enough with Triton X-100 before AluI is added.

Problem 3

Ligation efficiency of DNA is low (step 22).

Potential solution

- If the undigested control DNA (Sample U) is highly smeared, DNA may be degraded during the fixation because of low pH. Check pH of the medium, PBS buffer, and formaldehyde solution used for the experiment. Although we do not adjust pH of PBS buffer, adjusting it to a higher pH could be helpful. Since long-term storage of formaldehyde causes it to oxidize to formic acid, replacing the formaldehyde solution with a fresh one could solve the problem.
- Alternatively, DNA may have been degraded by endogenous nucleases during crosslinking reversal. Thermostable endonucleases in hyperthermophilic *Sulfolobus* cells could be active even in the presence of SDS at 65°C. Make sure that the temperature of your incubator is properly controlled. Adding more proteinase K may help inactivate the nucleases.
- If you are working on *S. acidocaldarius*, overdigestion of its cell wall with proteinase K before proximity ligation may also allow the enzyme to degrade chromosomal proteins, leading to disruption of DNA-DNA contacts. In this case, the amount of proteinase K and/or the duration of incubation should be titrated.
- DNA ligase may be inactivated by SDS because Triton X-100 is too old to quench it. Prepare a fresh working solution.

Problem 4

The yield of a 3C-seq library is lower than expected (step 60).

Potential solution

Measure the concentration of your 3C DNA to make sure that you use an enough amount of starting material for library construction. We recommend quantifying the DNA concentration using Qubit dsDNA HS Assay Kit for accuracy. You can also simply re-amplify your library because our protocol usually generates only a small fraction of PCR duplicates.

Problem 5

The size distribution of library DNA is not as expected (step 60).

Potential solution

Make sure to warm AMPure XP Beads to 21°C before use. Also, check the volume of your reaction before adding the beads. It could deviate from the expected volume, for example, due to improper pipetting of viscous Blunt/TA Ligase Master Mix. The reaction volume could also decrease after heating for PCR.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen D. Bell (stedbell@iu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

N.T. and S.D.B. designed experiments and wrote the manuscript. N.T. performed all experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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