Chromosome organization in archaea has long been enigmatic due, in part, to the typically small cell size of archaea and the extremophilic nature of many of the model archaeal species studies, rendering live-cell imaging technically challenging. To circumvent these problems, we recently applied chromosome conformation capture combined with biotin enrichment and deep sequencing (Hi-C) to members of hyperthermophilic archaeal genus Sulfolobus. Our optimized Hi-C protocol described here permits delineation of how Sulfolobus species organize their chromosomes.
Protocol

Chromosome conformation capture assay combined with biotin enrichment for hyperthermophilic archaea

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SUMMARY
Chromosome organization in archaea has long been enigmatic due, in part, to the typically small cell size of archaea and the extremophilic nature of many of the model archaeal species studies, rendering live-cell imaging technically challenging. To circumvent these problems, we recently applied chromosome conformation capture combined with biotin enrichment and deep sequencing (Hi-C) to members of hyperthermophilic archaeal genus Sulfolobus. Our optimized Hi-C protocol described here permits delineation of how Sulfolobus species organize their chromosomes. For complete details on the use and execution of this protocol, please refer to Takemata et al. (2019).

BEFORE YOU BEGIN
Preparation of medium for Sulfolobus acidocaldarius

© Timing: 1 h

To grow S. acidocaldarius, prepare Brock’s medium (Brock et al., 1972) as follows.

1. To 900 mL of Milli-Q water, add
   a. 10 mL of 100 × Solution A (for recipe, see below)
   b. 5 mL of 200 × Solution B (for recipe, see below)
   c. 1 mL of 1,000 × Solution C (for recipe, see below)
   d. 2 g of sucrose
   e. 1 g of tryptone
2. Adjust pH to 3.2 with 50% (v/v) H2SO4.
3. Adjust the volume to 1 L with Milli-Q water.
4. Filter sterilize the medium. It can be stored for months at 4°C.

Preparation of medium for Sulfolobus islandicus

© Timing: 1 h

To grow S. islandicus, prepare TSVY medium (Xu et al., 2016) as follows.
5. To 800 mL of Milli-Q water, add
   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) H₂SO₄.
7. Adjust the volume to 1 L with Milli-Q water.
8. Filter sterilize the medium. It can be stored for months at 4°C.

Pre-culture of **Sulfolobus** cells

© Timing: 2–3 days

9. Inoculate cells of *S. acidocaldarius* or *S. islandicus* in an appropriate medium from your freeze stock. If you grow a 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. Culture can be transiently stored at 21°C for up to two weeks.

**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 | n/a |
| Sulfolobus islandicus REY15A | Lab stock | 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 |
| Protease K          | Gold Biotechnology | P-480-1 |
| 10 × NEBuffer 2     | New England Biolabs | B70025 |
| 10 × NEBuffer 2.1   | New England Biolabs | B72025 |
| Triton X-100        | Acros Organics | 327371000 |
| 100 U/μL HindIII    | New England Biolabs | R0104M |
| 20 U/μL HindIII     | New England Biolabs | R0104S |
| 10 U/μL Nhel       | New England Biolabs | R0131S |
| 100 mM dNTP Set    | Bioline  | BIO-39025 |
| 0.4 mM Biotin-14-dCTP | Thermo Fisher Scientific | 19518018 |
| 5 U/μL Klenow Large Fragment | New England Biolabs | M02105 |
| 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 |
| 20 mg/mL Glycogen   | USB     | 16445     |
| RNase A             | Sigma-Aldrich | R4875-500MG |
| 10× Cloned Pfu DNA Polymerase Buffer | Agilent | 200532 |
| 20 mg/mL BSA        | Thermo Fisher Scientific | B14 |
| 3 U/μL T4 DNA Polymerase | New England Biolabs | M02035 |
| Buffer EB           | QIAGEN  | 19086     |

(Continued on next page)
Continued

**MATERIALS AND EQUIPMENT**

### REAGENT or RESOURCE SOURCE IDENTIFIER

| Reagent/RESOURCE | SOURCE | IDENTIFIER |
|------------------|--------|------------|
| Qubit dsDNA HS Assay Kit | Thermo Fisher Scientific | Q32851 |
| AMPure XP beads | Beckman Coulter | A63880 |
| Dynabeads MyOne Streptavidin C1 | Thermo Fisher Scientific | 65001 |

### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| NEBNext Ultra DNA Library Prep Kit for Illumina | New England Biolabs | E7370S [https://international.neb.com/products/e7370-nebnextultra-dna-libraryprep-kit-forillumina#Product20Information](https://international.neb.com/products/e7370-nebnextultra-dna-libraryprep-kit-forillumina#Product20Information) |
| NEBNext Multiplex Oligos for Illumina | New England Biolabs | E7335S [https://international.neb.com/products/e7335-nebnextmultiplex-oligos-forillumina-indexprimers-set1#Product20Information](https://international.neb.com/products/e7335-nebnextmultiplex-oligos-forillumina-indexprimers-set1#Product20Information) |

### Oligonucleotides

| Oligonucleotide | Source | Identifier |
|----------------|--------|------------|
| Sac_QC_F (5’-GGTGGGGCAAAAGTTATGTTACCTG-3’) | This study | n/a |
| Sac_QC_R (5’-AGAGGGGAGAGCAATAGGACCTG3’) | This study | n/a |
| Sis_QC_F (5’-ATGTATGCTCTGCTGCTTGTTAACACTATC-3’) | This study | n/a |
| Sis_QC_R (5’-CCTAAAGATGTTCCAGCTACGCCCAA-3’) | This study | n/a |

### Other

| COMPONENT | SOURCE | IDENTIFIER |
|-----------|--------|------------|
| 1.5-mL Safe-Lock Tubes | Eppendorf | 0030 120.086 |
| 1.5-mL DNA LoBind Tube | Eppendorf | 0030108051 |
| Bioruptor | Diagenode | UCD-300 |
| DynaMag™-2 Magnet | Thermo Fisher | 12321D |
| Agilent 4150 TapeStation System | Agilent Technologies | G2992AA |

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### 100 x Solution A

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Milli-Q water | n/a | X mL |
| (NH₄)₂SO₄ | 0.98 M | 130 g |
| MgSO₄·7H₂O | 0.1 M | 25 g |
| FeCl₃·6H₂O | 7 mM | 2 g |
| 50% (v/v) H₂SO₄ | 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 months.*

### 200 x Solution B

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Milli-Q water | n/a | X mL |
| MnCl₂·4H₂O | 2.8 mM | 566 mg |
| ZnSO₄·7H₂O | 270 μM | 78.3 mg |
| CuCl₂·2H₂O | 74.5 μM | 12.7 mg |
| VO(SO₄)₂·5H₂O | 36 μM | 9.32 mg |
| CoSO₄·7H₂O | 12.9 μM | 3.63 mg |
| Na₂MoO₄·2H₂O | 447 μM | 170 mg |
| Na₂WO₄·2H₂O | 29 μM | 7.05 mg |
| 50% (v/v) H₂SO₄ | 0.25% (v/v) | 5 mL |
| Total | n/a | 1 L |
Feature: 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   |
| CaCl₂·2H₂O             | 476 mM              | 14 g   |
| **Total**              | n/a                 | 200 mL |

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

### 10 × Base Salts

| Reagent                        | Final concentration | Amount |
|--------------------------------|---------------------|--------|
| Milli-Q water                  | n/a                 | X mL   |
| (NH₄)₂SO₄                      | 227 mM              | 30 g   |
| K₂SO₄                          | 28 mM               | 5 g    |
| KCl                             | 13 mM               | 1 g    |
| Glycine                        | 93 mM               | 7 g    |
| MnCl₂·4H₂O                     | 40 μM               | 8 mg   |
| Na₂B₄O₇·10H₂O                 | 55 μM               | 21 mg  |
| ZnSO₄·7H₂O                     | 3.8 μM              | 1.1 mg |
| CuSO₄·5H₂O                     | 1 μM                | 0.25 mg|
| Na₂MoO₄·2H₂O                  | 619 nM              | 0.15 mg|
| VOSO₄·5H₂O                    | 592 nM              | 0.15 mg|
| CoSO₄·7H₂O                    | 177 nM              | 0.05 mg|
| NiSO₄·6H₂O                    | 190 nM              | 0.05 mg|
| 1% (w/v) FeSO₄·7H₂O solution  | 0.002%              | 2 mL   |
| Ca(NO₃)₂·4H₂O                 | 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) H₂SO₄. Autoclave and store 10 × Base Salts at 21°C. It is stable for months.

### 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 x PBS**

1x 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**

2.5 or 5% Formaldehyde

5.4 or 10.8 mL 37% formaldehyde solution fill up to 80 mL with 1 x PBS.

*Note:* Prepare immediately before use.

**2.5 M glycine**

2.5 M Glycine

93.8 g Glycine, add 500 mL ddH₂O to 500 mL

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

**1 x PBS/1 mM EDTA**

1x PBS/1 mM EDTA

Combine 49.9 mL 1x 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 1 g 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.

**1 x TE buffer**

1 x TE buffer

Add 5 mL of 1 M 1 M Tris-HCl (pH 8) and 1 mL of 0.5 M EDTA (pH 8) to 494 mL Milli-Q water

*Note:* Autoclave and store the solution at 21°C. Solution is stable for several months.
Note: Store the solution at –20°C, it is stable for weeks of storage.

2 mM dNTP mix

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| 100 mM dATP| 2 mM                | 4 µL   |
| 100 mM dTTP| 2 mM                | 4 µL   |
| 100 mM dGTP| 2 mM                | 4 µL   |
| 100 mM dCTP| 2 mM                | 4 µL   |
| 1 x TE buffer | 0.92x              | 184 µL |
| Total      | n/a                 | 200 µL |

Note: Store the solution at –20°C, it is stable for weeks of storage.

Wash Buffer

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| 5 M NaCl        | 50 mM               | 0.5 mL |
| 1 M Tris-HCl (pH 8) | 10 mM             | 0.5 mL |
| 1 M MgCl₂      | 10 mM               | 0.5 mL |
| Milli-Q water  | n/a                 | 48.5 mL|
| Total           | n/a                 | 50 mL  |

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

B&W buffer

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| Milli-Q water   | n/a                 | 79.4 mL|
| 1 M Tris-HCl (pH 7.5) | 5 mM            | 0.5 mL |
| 0.5 M EDTA (pH 8) | 0.5 mM           | 0.1 mL |
| 5 M NaCl        | 1 M                 | 20 mL  |
| Total           | n/a                 | 100 mL |

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

0.1 x TE (pH 8)

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| 0.1 x 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.

△ CRITICAL: Phenol:chloroform:isoamyl alcohol is toxic.
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) for 16 h until the culture reaches a growth phase of your interest (mid-log phase, stationary phase, etc.). The 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, but these times could be variable if mutant strains are grown or different growth media employed.

△ 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 problems 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 Fixation Buffer (approximately 20°C). Use Fixation Buffer containing 2.5% formaldehyde for *S. acidocaldarius* (the final concentration is 2% after the mixture) and 5% for *S. islandicus* (the final concentration is 4% after the mixture).
   b. Incubate the mixture for 30 min at 25°C with agitation (110 rpm).
   c. Add 5.6 mL of 2.5 M glycine to quench the crosslink reaction. When fixation is carried out with 4% of formaldehyde, add 11.2 mL of 2.5 M glycine instead.
   d. Incubate the mixture for 10 min at 21°C without agitation.
4. Wash the fixed cells as follows.
   a. Dispense the mixture into two 50-mL tubes and centrifuge them at 3,120 × *g* for 30 min, 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.
DNA digestion followed by proximity ligation

**Timing:** 11 h or 1 day

This section describes how to digest crosslinked cellular DNA with HindIII. Generated 5’-overhangs are filled in using a biotin-conjugated nucleotide and then treated with ligase to join DNA fragments crosslinked to each other. Ligation junctions are purified with streptavidin at a later step.

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<sub>600</sub>.
8. Dilute the remainder of the cell suspension to an OD<sub>600</sub> of 4. 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 90 s.

**Note:** A precipitate could form if the tube is left on ice too long.

15. Spin down condensate briefly and mix by pipetting.
16. Assemble the following reactions in 1.5-mL tubes.

### Sample U (undigested control)

| Reagent          | Final concentration | Amount  |
|------------------|---------------------|---------|
| Cell lysate      | n/a                 | 12.5 μL |
| 10 × NEBuffer 2  | n/a                 | 3.7 μL  |
| 10% Triton X-100| 2%                  | 10 μL   |
| Milli-Q water    | n/a                 | 23.8 μL |
| Total            | n/a                 | 50 μL   |

### Sample D (digestion reaction)

| Reagent                   | Final concentration | Amount  |
|---------------------------|---------------------|---------|
| Cell lysate               | n/a                 | 37.5 μL |
| 10 × NEBuffer 2           | n/a                 | 11.2 μL |
| 10% Triton X-100          | 2%                  | 30 μL   |
| Milli-Q MilliQ-water      | n/a                 | 56.3 μL |
| 100 U/μL HindIII          | 10 U/μL             | 15 μL   |
| Total                     | n/a                 | 150 μL  |
17. Incubate the samples for 4 h at 37°C with agitation (600 rpm).
18. Assemble two of the following reactions in 1.5-mL Eppendorf Safe-Lock Tubes to label restriction ends with biotin. The amount of Klenow Large Fragment is critical for optimal labeling efficiency. To avoid pipetting a small amount of the enzyme, we recommend making a pre-mixture of dNTPs, NEBuffer 2, and the enzyme for multiple samples.

| Sample L (sample for ligation) | Reagent                        | Final concentration | Amount |
|--------------------------------|--------------------------------|---------------------|--------|
| Sample D                       | n/a                            |                     | 50 μL  |
| 2 mM dATP                      | 59 μM                          | 2 μL                |
| 2 mM dTTP                      | 59 μM                          | 2 μL                |
| 2 mM dGTP                      | 59 μM                          | 2 μL                |
| 0.4 mM biotin-14-dCTP          | 59 μM                          | 10 μL               |
| 10 × NEBuffer 2                | n/a                            | 1.8 μL              |
| 5 U/μL Klenow Large Fragment   | 0.037 U/μL                     | 0.5 μL              |
| Total                          | n/a                            | 68.3 μL             |

19. Incubate Sample U, the remainder of Sample D, and the two Samples L for 30 min at 20°C with agitation (600 rpm).
20. Add reagents as follows to quench the digestion and fill-in reactions.

| To Sample U and Sample D       | Reagent                        | Final concentration | Amount |
|--------------------------------|--------------------------------|---------------------|--------|
| (Sample U or D)                | (n/a)                          | (50 μL)             |
| 1 × NEBuffer 2                 | n/a                            | 50 μL               |
| 10% SDS                        | 0.09%                          | 10 μL               |
| 0.5 M EDTA (pH 8)              | 22 mM                          | 5 μL                |
| Total                          | n/a                            | 115 μL              |

| To Sample L                    | Reagent                        | Final concentration | Amount |
|--------------------------------|--------------------------------|---------------------|--------|
| (Sample L)                     | (n/a)                          | (68.3 μL)           |
| 10% SDS                        | 1%                             | 7.7 μL              |
| 0.5 M EDTA (pH 8)              | 9.0 mM                         | 1.4 μL              |
| Total                          | n/a                            | 77.4 μL             |

21. Incubate the samples for 5 min at 21°C.
22. Add the following reagents to each tube of Sample L.

| Reagent                         | Final concentration | Amount  |
|---------------------------------|---------------------|---------|
| (Sample L)                      | (n/a)               | (77.4 μL)|
| Milli-Q water                   | n/a                 | 713 μL  |
| 10 × T4 DNA Ligase Reaction     | 1×                  | 100 μL  |
| Buffer                          |                     |         |
| 10% Triton X-100                | 1%                  | 100 μL  |
| 400 U/μL T4 DNA Ligase          | 4 U/μL              | 10 μL   |
| Total                           | n/a                 | 1,000 μL |
23. Incubate all samples for 4 h at 16 °C with agitation (600 rpm). Mix the Samples L by inverting the tubes every 30 min.

Optional: You can do ligation overnight (>12 h) instead of 4 h.

Cross-link reversal

© Timing: >12 h (overnight)

Reverse the crosslinks as follows for subsequent DNA purification.

24. Add the following reagents to each Sample L.

| Reagent                      | Final concentration | Amount  |
|------------------------------|---------------------|---------|
| (Sample L)                   | (n/a)               | (1,000 μL) |
| 10% SDS                      | 0.09%               | 100 μL  |
| 0.5 M EDTA (pH 8)            | 22 mM               | 50 μL   |
| Total                        | n/a                 | 1,150 μL|

25. Add 5 μL of 20 mg/mL proteinase K to each of all samples.
26. 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 problems 3 for more detail).

DNA purification 1

© Timing: 8 h

This section describes how to purify DNA and confirm successful DNA digestion and ligation. Also described is how to estimate biotin labeling efficiency by PCR amplification of a ligation junction followed by restriction digestion.

27. Dispense the two Samples L into a total of four 1.5-mL tubes (~560 μL/tube).
28. 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.
29. 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.
30. (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.
31. Add 0.1 volume of 3 M sodium acetate (pH 5.2) to each tube.
32. 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. Addition of glycogen improves DNA yield and makes the DNA pellet more visible.
33. Add 1 volume of isopropanol and leave the samples for 1 h at −20 °C.
34. Spin down for 1 h at 21,000 × g (or the maximum speed), 4 °C.
35. Remove the supernatant and rinse the pellet in 500 μL of 70% ethanol.
36. Spin down for 5 min at 21,000 × g (or the maximum speed), 4 °C.
37. Remove the supernatant and spin down briefly to remove residual ethanol.
38. Leave the tubes open for 10 min at 21 °C to air-dry the pellets.
39. For Samples U and D, dissolve the pellet in 20 μL of 1× NEBuffer 2 containing 0.1 mg/mL RNase A. For Sample L, dissolve the three pellets together in 40 μL of 1× NEBuffer 2 containing 0.1 mg/mL RNase A.

40. Incubate the samples for 30 min at 37°C.

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

41. Run 5 μL of each sample on a 0.7% agarose gel for quality control. Sample U (undigested control) should run as a tight band above 10 kb in size (Figure 1A). You will also see larger DNA stuck in the well. Sample D (digested, but not ligated) should run as a smear that starts from slightly above 10 kb to ~0.5 kb. Sample L (digested and then ligated) should generate a band above 10 kb as observed for Sample U. It is acceptable to see a small amount of smear in Sample L. 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.

42. Estimate labeling efficiency by restriction digestion of a PCR-amplified ligation junction. Ligation of two biotin-labeled ends, generated by complete fill-in of HindIII sites, forms a Nhel

![Restriction digestion of a PCR-amplified ligation junction](image)

Figure 1. Quality check of Hi-C DNA by gel electrophoresis
(A) Hi-C 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).
(B) Size distribution of a typical Hi-C DNA library from *S. acidocaldarius*. The 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.
site. On the other hand, ligation of unlabeled ends regenerates a HindIII site. Thus, labeling efficiency can be estimated as how much of the PCR product is cleavable by Nhel. If the DNA labeling was successful, 30%–60% of the product will be cleaved by Nhel alone relative to that cleaved by the double digestion.

a. Perform PCR in a total volume of 60 μL using Hi-C DNA (Sample L) as a template, a PCR enzyme of your choice, and the appropriate primer set for the species you are working on (Sac_QC_F and Sac_QC_R for S. acidocaldarius or Sis_QC_F and Sis_QC_R for S. islandicus). We routinely amplify 0.6 μL of the template DNA using 0.2 mM each of dNTPs, 0.3 μM each of the primers, and 2.5 units of Pfu DNA polymerase. The PCR cycling conditions are as follows.

| PCR cycling conditions | Temperature | Time   | Cycles |
|------------------------|-------------|--------|--------|
| Initial Denaturation   | 98°C        | 2 min  | 1      |
| Denaturation           | 98°C        | 15 s   | 35–40 cycles |
| Annealing              | 60°C        | 15 s   |        |
| Extension              | 72°C        | 1 min  |        |
| Hold                   | 15°C        | forever|        |

b. Assemble four digestion reactions as follows.

### Digestion by HindIII

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| PCR reaction     | n/a                 | 10 μL  |
| Milli-Q water    | n/a                 | 7 μL   |
| 10 × NEBuffer 2.1| 1×                  | 2 μL   |
| 20 U/μL HindIII  | 1 U/μL              | 1 μL   |
| **Total**        | n/a                 | 20 μL  |

### Digestion by Nhel

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| PCR reaction     | n/a                 | 10 μL  |
| Milli-Q water    | n/a                 | 7 μL   |
| 10 × NEBuffer 2.1| 1×                  | 2 μL   |
| 10 U/μL Nhel     | 0.5 U/μL            | 1 μL   |
| **Total**        | n/a                 | 20 μL  |

### Double digestion

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| PCR reaction     | n/a                 | 10 μL  |
| Milli-Q water    | n/a                 | 6 μL   |
| 10 × NEBuffer 2.1| 1×                  | 2 μL   |
| 20 U/μL HindIII  | 1 U/μL              | 1 μL   |
| 10 U/μL Nhel     | 0.5 U/μL            | 1 μL   |
| **Total**        | n/a                 | 20 μL  |
c. Incubate the four reactions for 1 h at 37°C.

d. Run 10 μL on a 2% agarose gel. Labeling efficiency can be estimated as the fraction of the PCR product digested by NheI relative to that digested by NheI and HindIII. Note that even the double digestion will not cleave all the PCR product, likely due to inefficient digestion, mutations at the ligation junction, and so on (Figure 2). If the DNA was labeled efficiently, 30%–60% of the product will be cleaved by NheI alone compared with that cleaved by the double digestion.

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

**Removal of biotin from unligated ends**

© Timing: 4.5 h

This section describes how to remove biotin from free DNA ends. This procedure is required to enrich biotin-conjugated ligation junctions in a subsequent purification step using streptavidin.

43. Assemble a reaction as follows. The amount of T4 DNA Polymerase is critical for optimal results. To avoid pipetting a small amount of the enzyme, we recommend making a pre-mixture of NEBuffer 2, BSA, dCTP, and the enzyme for multiple samples.

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Sample L | n/a | 30 μL |
| Milli-Q water | n/a | 36 μL |
| 10 × NEBuffer 2 (New England Biolabs) | n/a | 4.5 μL |
| 20 mg/mL BSA (Thermo Fisher Scientific) | 0.1 mg/mL | 0.375 μL |
| 2 mM dCTP | 0.1 mM | 3.75 μL |
| 3 U/μL T4 DNA Polymerase (New England Biolabs) | 0.015 U/μL | 0.375 μL |
| Total | n/a | 75 μL |

44. Incubate the reaction for 4 h at 20°C with agitation (600 rpm). Immediately proceed to the next phenol extraction step to inactivate the enzyme.

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**Figure 2. Estimation of biotin labeling efficiency**

Hi-C DNA from *S. acidocaldarius* was used as a template for PCR reaction followed by restriction digestion. The DNA was run on a 2% agarose gel. Note that the digestion generates two DNA fragments but they are too close in size to separate. DNA marker: 100 bp DNA Ladder (New England Biolabs N3231S).
DNA purification 2 and DNA shearing

Timing: 4 h

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

45. Adjust the volume to 100 μL by adding Buffer EB.
46. Add 1 volume of phenol:chloroform:isoamyl and mix by vortexing.
47. Spin down for 10 min at 10,000 × g, 21°C.
48. Transfer 80 μL of the aqueous phase to a new 1.5-mL tube.
49. 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, 21°C. Combine 80 μL of the aqueous phase with the extract previously taken for a total of 160 μL.
50. Add 0.1 volume of 3 M sodium acetate (pH 5.2).
51. Add 2 volume of 100% ethanol. Leave the sample for 1 h at −20°C.
52. Spin down for 30 min at 21,000 × g, 4°C.
53. Remove the supernatant and rinse the pellet in 500 μL of 70% ethanol.
54. Spin down for 5 min at 21,000 × g, 4°C.
55. Remove the supernatant and spin down briefly to remove residual ethanol.
56. Leave the tubes open for 10 min at 21°C to air-dry the pellets.
57. Dissolve the pellet in 90 μL of Buffer EB.

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

58. Transfer the solution to a 0.6-mL tube and chill it on ice for 10 min.
59. Shear the DNA into fragments of 200–300 bp on average using Bioruptor and the following parameters. Power: Low, ON time: 30 s, OFF time: 30 s, Cycles: 60.

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

Library construction

Timing: 6 h

This section describes how to prepare a Hi-C 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) but also includes a step for enrichment of biotin-conjugated ligation junctions.

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

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

| Reagent                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| Sheared DNA                                  | n/a                 | 55.5 μL |
| 10 × End Repair Reaction Buffer (provided in the library prep kit) | 1×                  | 6.5 μL  |
| End Prep Enzyme Mix (provided in the library prep kit) | 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.

61. Perform Adapter Ligation as follows.

   a. Mix the following reagents in a 1.5-mL DNA LoBind Tube.

   | Reagent                                           | Final concentration | Amount |
   |---------------------------------------------------|----------------------|--------|
   | End Prep reaction                                 | n/a                  | 65 µL  |
   | 15 µM NEBNext Adaptor for Illumina (provided in the multiplex oligo kit) | 0.45 µM              | 2.5 µL |
   | Ligation Enhancer (provided in the library prep kit) | n/a                  | 1 µL   |
   | Blunt/TA Ligase Master Mix (provided in the library prep kit)      | 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. We do not heat or cool the lid.
   c. Add 3 µL of USER Enzyme (provided in the multiplex oligo kit).
   d. Incubate the sample for 15 min at 37°C. We do not heat or cool the lid.

62. Perform Size Selection of Adapter Ligated DNA as follows to purify ~320-bp (insert + adapter) DNA.

   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 55 µL of resuspended AMPure XP Beads.
   d. Incubate the mixture for 5 min at 21°C.
   e. Place the tube on 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 25 µL of resuspended AMPure XP Beads.
   g. Incubate the mixture for 5 min at 21°C.
   h. Place the tube on 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 s 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 DynaMag™-2 Magnet for 5 min. Over-drying of the beads could lead to low DNA yield.
   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 new 1.5-mL DNA LoBind Tube.

63. Purify ligation junctions labeled with biotin as follows.

   a. Transfer 10 µL of Dynabeads MyOne Streptavidin C1 to a 1.5-mL tube.
   b. Add 1 mL of B&W Buffer to the tube containing the beads.
c. Place the tube on DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
d. Resuspend the beads in 10 μL of B&W Buffer. Place the tube on DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
e. Repeat sub-step d twice.
f. Resuspend the beads in 135 μL of B&W Buffer. Add the suspension to the tube containing the size-selected DNA.
g. Rotate the DNA-bead mixture for 30 min at 21°C, 13 rpm.
h. Spin down and place the tube on DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
i. Resuspend the beads in 100 μL of B&W Buffer by tapping. Spin down and place the tube on DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
j. Repeat sub-step i twice.
k. Resuspend the beads in 100 μL of Wash Buffer by tapping. Spin down and place the tube on DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
l. Resuspend the beads in 15 μL of 0.1 × TE (pH 8).

64. Perform PCR Enrichment of Adapter Ligated DNA as follows.
   a. Mix the following reagents.

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

   b. Do PCR as follows.

   | PCR cycling conditions                        | Temperature | Time | Cycles |
   |-----------------------------------------------|-------------|------|--------|
   |Initial Denaturation                           | 98°C        | 30 s | 1      |
   |Denaturation                                   | 98°C        | 10 s | 14 cycles |
   |Annealing/ Extension                           | 65°C        | 75 s |        |
   |Final extension                                | 65°C        | 5 min| 1      |
   |Hold                                           | 15°C        | forever |

65. Perform Cleanup of PCR Amplification 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 DynaMag™-2 Magnet to separate the beads. Remove the supernatant.
   f. For 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 DynaMag™-2 Magnet during the wash.
   g. Repeat sub-step f.
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h. To air-dry the beads, leave the tube with the lid opened on DynaMag™-2 Magnet for 5 min. Over-drying of the beads could lead to low DNA yield.

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 DynaMag™-2 Magnet to separate the beads. Transfer 22 μL of the supernatant to a new 1.5-mL DNA LoBind Tube.

66. Use Agilent 4150 TapeStation System and a D1000 ScreenTape to check the size and concentration of library DNA.

67. Perform paired-end sequencing using an Illumina sequencing platform. We routinely use NextSeq for sequencing of Hi-C libraries. We typically aim to obtain more than 40 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 with a peak at 300–350 bp (Figure 1B). Below are statistics of a typical library from *S. acidocaldarius*. Reads were analyzed using HiC-Pro (Servant et al., 2015) (Table 1). On average, 30%–40% of the total reads are categorized as valid ligation products, which can be used to generate a contact map. It should be noted, however, that this percentage is variable depending on strains and growth conditions (Takemata et al., 2019).

| Table 1. Read statistics of a typical Hi-C library |
|-----------------------------------------------|
| Total read pairs                              | 109,205,167 |
| Reported pairs after mapping                  | 87,968,755  (80.6%) |
| Invalid pairs                                 |             |
| Dangling-end pairs                            | 13,996,730  (12.8%) |
| Religation pairs                              | 2,460,847   (2.3%) |
| Self-circle                                    | 8,062,948   (7.4%) |
| Singletons                                     | 0           (0.0%) |
| Dumped pairs                                   | 21,216      (0.0%) |
| Valid pairs                                    |             |
| Before removal of PCR duplicates              | 63,427,014  (58.1%) |
| After removal of PCR duplicates               | 50,453,724  (46.2%) |

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

**LIMITATIONS**

Since this protocol uses the 6-bp cutter HindIII to fragment the genome, the data resolution is ~10 kb at best. Use of a 4-bp cutter commonly used in eukaryotic Hi-C studies (Mbol or DpnII, for example) could improve the resolution, but we found that Mbol and DpnII digest the Sulfolobus genome with very low efficiency (data not shown). This poor digestion may cause a bias or artifact in the contact map.

Recently, we were able to generate contact maps of Sulfolobus chromosomes with higher resolution (2 kb at maximum) using the 4-bp blunt cutter AluI at the expense of omitting the biotin enrichment step Takemata and Bell, 2021a. For more detail, see that work or our recent STAR Protocols paper Takemata and Bell, 2021b.

**TROUBLESHOOTING**

**Problem 1**

Digestion/ligation efficiency of DNA is variable between replicates (step 41).

**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 41).

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 HindIII is added.

Problem 3
A large amount of smear is observed for Hi-C DNA (step 41).

Potential solution

- If you also see a smear for the undigested control (Sample U), DNA may be degraded during the fixation because of a 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
Labeling efficiency of Hi-C DNA is low (step 42).

Potential solution

Try using fresh Klenow Large Fragment or optimize reaction conditions for the labeling step. Since the percentage of PCR duplicates in the library is usually small in our protocol (see Table 1), it will also be okay to add a few additional PCR cycles in the library construction step to compensate for the low labeling efficiency.

Problem 5
The percentage of valid read pairs is small in the library (step 67).

Potential solution

First, check the read statistics generated by your mapping program. If there is a large fraction of dangling-end pairs (reads from unligated DNA), the biotin removal by T4 DNA polymerase would have been inefficient. In this case, you may need to repurchase the enzyme. If there is a large fraction of PCR duplicates, the number of PCR cycles would have been too large for your experiments, and you may need to reduce the number of PCR cycles.

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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