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Protocol

Generation of gene-level resolution chromosome contact maps in bacteria and archaea

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SUMMARY
Chromosome conformation capture (Hi-C) has become a routine method for probing the 3D organization of genomes. However, when applied to bacteria and archaea, current protocols are expensive and limited in their resolution. By dissecting the different steps of published eukaryotic and prokaryotic Hi-C protocols, we have developed a cost- and time-effective approach to generate high-resolution (down to 500 bp – 1 kb) contact matrices of both bacteria and archaea genomes.
For complete details on the use and execution of this protocol, please refer to Cockram et al. (2020).

BEFORE YOU BEGIN

© Timing: approximately 30 min

Considerations for designing the Hi-C experiment

1. Choosing the correct restriction enzyme:

The frequency of restriction sites correlates with the GC-content of a genome and thus the choice of restriction enzymes can introduce significant distortions to 3C/Hi-C contact maps (Cournac et al., 2012; Marbouty et al., 2017; Yaffe and Tanay, 2011). For this reason, eukaryotic Hi-C protocols now typically use the 4-cutter DpnII (\(^\text{GATC}\)), that offers a relatively uniform distribution of restriction sites along the genome (Rao et al., 2014). This is also what we use and recommend for prokaryotes (Marbouty and Koszul, 2017; Marbouty et al., 2015). However, DpnII is not an adequate solution for most bacterial species, whose genomes are subject to the Dam methylation of GATC sites. It is therefore important to consider other enzymes that will provide sufficient restriction positions within a particular genome, to reach at a proper resolution.

We recommend – as of today – using HpaII (\(^\text{CGG}\)) with NEBuffer 1 (10x NEBuffer 1: (200 mM Tris-HCl pH 7.5, 100 mM MgCl2, 10 mM DTT, 1 mg/mL BSA) for most applications. In case the investigated genome is particularly AT-rich, or if a specific AT-rich region is under investigation, then we suggest using MluCI (\(^\text{AATT}\)) with the same buffer.

The enzyme used will determine if either Biotin-14-dATP or Biotin-14-dCTP is used for the subsequent biotinylation step of the protocol.
Preparation of solutions and buffers

- **Timing:** approximately 1–2 h

2. Prepare cell culture medium and all solutions listed below in the “materials and equipment” section. Ensure there are enough for the number of samples being processed.

3. All experiments should be performed with fresh formaldehyde solution (<1 week). We recommend purchasing 25 mL bottles to limit waste.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| 2 ml VK05 microorganism Precelys tubes | Bertin Instruments | Cat# P000913-LYSK0-A |
| microTUBE AFA Fiber Pre-Slit Snap-Cap | Covaris | Cat# 520045 |
| 100 mM dNTPs (set of 4) | Dutscher | Cat# 755086 |
| Protease K (20 mg/mL) | Eurobio Scientific | Cat# GEXPRK01-B5 |
| Ethidium Bromide Solution | Eurobio Scientific | Cat# GEPBET02-AF |
| RNase A | Euromedex | Cat# R80473 |
| 36.5%–38% Formaldehyde solution | Sigma-Aldrich | Cat# F8775 |
| Glycine | Sigma-Aldrich | Cat# G8898 |
| 1 M MgCl2 | Sigma-Aldrich | Cat# M1028 |
| DL-Dithiothreitol solution | Sigma-Aldrich | Cat# 43816 |
| Bovine Serum Albumin | Sigma-Aldrich | Cat# A7906 |
| ATP | Sigma-Aldrich | Cat# 10519987001 |
| Sodium acetate | Sigma-Aldrich | Cat# S2889 |
| Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (pH 8.0) | Sigma-Aldrich | Cat# P2069 |
| cOmplete Mini EDTA-free protease inhibitor cocktail | Sigma-Aldrich | Cat# 11836170001 |
| T4 DNA ligase (30 U/ul) | Thermo Fisher Scientific | Cat# EL0013 |
| 20% SDS solution | Thermo Fisher Scientific | Cat# 10607633 |
| Triton-X-100 | Thermo Fisher Scientific | Cat# 10671652 |
| 1 M Tris-Hcl pH7.5 | Thermo Fisher Scientific | Cat# 10573145 |
| 0.5 M EDTA (pH 8.0) | Thermo Fisher Scientific | Cat# 10182903 |
| Absolute EtOH | Thermo Fisher Scientific | Cat# 10680993 |
| 10× PBS solution | Thermo Fisher Scientific | Cat# 10649743 |
| UltraPure Agarose | Thermo Fisher Scientific | Cat# 16500500 |
| 50× TAE solution | Thermo Fisher Scientific | Cat# 10490264 |
| Biotin-14-dCTP | Thermo Fisher Scientific | Cat# 19518018 |
| Biotin-14-dATP | Thermo Fisher Scientific | Cat# 19524016 |
| NaCl | Thermo Fisher Scientific | Cat# 10616082 |
| Tween 20 | Thermo Fisher Scientific | Cat# 10113103 |
| Dynabeads MyOne Streptavidin C1 | Thermo Fisher Scientific | Cat# 10202333 |
| GeneRuler 1 Kb Plus DNA ladder | Thermo Fisher Scientific | Cat# SM1331 |
| Orange DNA Loading Dye | Thermo Fisher Scientific | Cat# R0631 |

**Critical commercial assays**

| Agencourt AMPure XP beads | Beckman Coulter | Cat# A63881 |
| Qubit dsDNA HS Assay Kit | Thermo Fisher Scientific | Cat# 10606433 |
| NextSeq 500/550 v2.5 High Output Kit (75 Cycles) | Illumina | Cat# 20024906 |
| TruSeq DNA CD Indexes (96 Indexes) | Illumina | Cat# 20015949 |
| Hpall | New England Biolabs | Cat# R0171M |
| MluCl | New England Biolabs | Cat# R0538L |

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### MATERIALS AND EQUIPMENT

#### Solutions

| Reagent                 | Final concentration | Amount       |
|-------------------------|---------------------|--------------|
| Glycine                 | 2.5 M               | 187.7 g      |
| ddH₂O                   | n/a                 | Up to 1 L    |

**Note:** Filter sterilize through a 0.22 μm filter, store (we use glycine solution stored for a maximum of 6 months; this indication and the others below are based on our experience, and doesn’t exclude longer conservation time: we typically consume all of these buffers before the indicated time) at 19°C–23°C.

| Reagent       | Final concentration | Amount       |
|---------------|---------------------|--------------|
| BSA           | 10 mg/mL            | 0.2 g        |
| ddH₂O         | n/a                 | Up to 20 mL  |

#### Hardware and software

- **Bowtie 2** ([Langmead and Salzberg, 2012](http://bowtie-bio.sourceforge.net/bowtie2/index.shtml))
- **SCN normalization procedure** ([Cournac et al., 2012](https://github.com/koszullab/hicstuff))
- **R** ([RStudio Team, 2020](https://rstudio.com))
- **Chromosight** ([Matthey-Doret et al., 2020](https://github.com/koszullab/chromosight))
- **HTseq - count** ([Anders et al., 2015](https://github.com/htseq/htseq))
- **DESeq2** ([Love et al., 2014](https://bioconductor.org/packages/release/bioc/html/DESeq2.html))
- **SAMtools** ([Li et al., 2009](http://www.htslib.org))
- **bamCoverage (deepTools)** ([Ramírez et al., 2016](https://github.com/deeptools/deepTools/blob/master/docs/content/tools/bamCoverage.rst))

#### Deposited data

- **Codes and functions for Hi-C analysis** ([Matthey-Doret et al., 2020](https://github.com/koszullab/hicstuff))
- **Raw data and processed data** ([Cockram et al., 2020](PRJNA587586))

#### Other

- **0.22 μm Filter, Nalgene filtration** (Thermo Scientific 596-3320)
- **Precellys Evolution tissue homogenizer** (Bertin Instruments P000062-PEVO0-A)
- **Covaris S220** (Covaris 500217)
- **NEXTFLEX DNA Barcodes Kits 48 (384 rxns)** (PerkinElmer NOVA 5141-13)

#### Experimental models: organisms/strains

- **E. coli K12 MG1655** ([Blattner et al., 1997](N/A))
- **H. volcanii H26 DpyrE2** ([Allers et al., 2004](N/A))

#### Notes

- Solutions
  - Note: Filter sterilize through a 0.22 μm filter, store (we use glycine solution stored for a maximum of 6 months; this indication and the others below are based on our experience, and doesn’t exclude longer conservation time: we typically consume all of these buffers before the indicated time) at 19°C–23°C.
**Note:** Filter sterilize through a 0.22 μm filter, aliquot into 1 mL tubes, store at −20°C (we use BSA solution for a maximum of 6 months).

| ATP solution | Reagent       | Final concentration | Amount |
|--------------|---------------|---------------------|--------|
| ATP          | 100 mM        | 1 g                 |        |
| NaOH (1 M)   | n/a           | ~ 1.6 mL            |        |
| ddH₂O        | n/a           | Up to 16.5 mL       |        |

**Note:** Volume of NaOH indicated is a guide, verify pH is 7.0 before adjusting to final volume with ddH₂O. Filter sterilize through a 0.22 μm filter, aliquot into 1 mL tubes, store at −20°C (we use ATP solution stored for a maximum of 6 months).

| 10× digestion buffer | Reagent                  | Final concentration | Amount |
|----------------------|--------------------------|---------------------|--------|
| Tris-HCl (1 M, pH7.5)| 200 mM                   | 10 mL               |        |
| MgCl₂ (1 M)          | 100 mM                   | 5 mL                |        |
| DTT (1 M)            | 10 mM                    | 0.5 mL              |        |
| BSA solution (10 mg/mL)| 1 mg/mL                | 5 mL                |        |
| ddH₂O                | n/a                      | Up to 50 mL         |        |

**Note:** Filter sterilize through a 0.22 μm filter, store at 19°C–23°C (we use TE stored up to 6 months).

| 1× TE solution | Reagent                  | Final concentration | Amount |
|----------------|--------------------------|---------------------|--------|
| Tris-HCl (1 M, pH7.5)| 10 mM                   | 5 mL                |        |
| EDTA (0.5 M)     | 1 mM                     | 1 mL                |        |
| ddH₂O            | n/a                      | Up to 500 mL        |        |

**Note:** Filter sterilize through a 0.22 μm filter, aliquot into 5 mL tubes, store at −20°C (we use digestion buffer stored up to 6 months).

| 10× ligation buffer | Reagent                  | Final concentration | Amount |
|---------------------|--------------------------|---------------------|--------|
| Tris-HCl (1 M, pH7.5)| 500 mM                   | 25 mL               |        |
| MgCl₂ (1 M)        | 100 mM                   | 5 mL                |        |
| DTT (1 M)          | 100 mM                   | 5 mL                |        |
| ddH₂O              | n/a                      | Up to 50 mL         |        |

**Note:** Filter sterilize through a 0.22 μm filter, aliquot into 5 mL tubes, store at −20°C (we use ligation buffer stored up to 6 months).
**1 x Tween Washing Buffer (TWB)**

| Reagent       | Final concentration | Amount     |
|---------------|---------------------|------------|
| Tris-HCl (1 M, pH 7.5) | 5 mM               | 0.5 mL     |
| EDTA (0.5 M)  | 0.5 mM              | 0.1 mL     |
| NaCl (5 M)    | 1 M                 | 20 mL      |
| Tween-20      | 0.05%               | 50 µL      |
| ddH₂O         | n/a                 | Up to 100 mL |

*Note:* Filter sterilize through a 0.22 µm filter, store at 19°C–23°C (we use TWB solution stored up to a year).

**2 x Binding Buffer (BB)**

| Reagent       | Final concentration | Amount     |
|---------------|---------------------|------------|
| Tris-HCl (1 M, pH 7.5) | 10 mM              | 1 mL       |
| EDTA (0.5 M)  | 1 mM                | 0.2 mL     |
| NaCl (5 M)    | 2 M                 | 40 mL      |
| ddH₂O         | n/a                 | Up to 100 mL |

*Note:* Filter sterilize through a 0.22 µm filter, store at 19°C–23°C (we use binding buffer stored up to 6 months).

**10 mM dNTP mix**

| Reagent       | Final concentration | Amount     |
|---------------|---------------------|------------|
| dATP (100 mM) | 10 mM               | 20 µL      |
| dTTP (100 mM) | 10 mM               | 20 µL      |
| dGTP (100 mM) | 10 mM               | 20 µL      |
| dCTP (100 mM) | 10 mM               | 20 µL      |
| ddH₂O         | n/a                 | Up to 200 µL |

*Note:* Store at ~20°C (we use dNTP solution stored up to a year).

**10 mM dAGTTP mix**

| Reagent       | Final concentration | Amount     |
|---------------|---------------------|------------|
| dATP (100 mM) | 10 mM               | 20 µL      |
| dGTP (100 mM) | 10 mM               | 20 µL      |
| dTTP (100 mM) | 10 mM               | 20 µL      |
| ddH₂O         | n/a                 | Up to 200 µL |

*Note:* Store at ~20°C (we use dAGTTP solution stored up to a year).

**10 mM dCGTTP mix**

| Reagent       | Final concentration | Amount     |
|---------------|---------------------|------------|
| dCTP (100 mM) | 10 mM               | 20 µL      |
| dGTP (100 mM) | 10 mM               | 20 µL      |
| dTTP (100 mM) | 10 mM               | 20 µL      |
| ddH₂O         | n/a                 | Up to 200 µL |
**Note:** Store at −20°C (we use dCGTTP solution stored up to a year).

| 10 mM dATP mix | Reagent          | Final concentration | Amount |
|----------------|------------------|---------------------|--------|
|                | dATP (100 mM)    | 10 mM               | 20 μL  |
|                | ddH2O            | n/a                 | Up to 200 μL |

**Note:** Store at −20°C (we use a dATP solution stored up to a year).

**STEP-BY-STEP METHOD DETAILS**

**Day 1: Cell fixation**

**Timing:** approximately 4–6 h

The subsequent steps describe a general overview of the growth and fixation of bacterial and archaeal cultures.

1. Grow bacterial or archaeal culture in an appropriate medium and at the required temperature until you have a total of ~1 × 10^8 cells growing in the exponential growth phase.

   **Note:** For *E. coli*, *Hbt. salinarum*, *H. volcanii*, and *T. kodakarensis* we use an OD_{600nm} of ~0.2 – 0.25, but this can be adjusted for other growth phases, whilst maintaining of ~1 × 10^8 cells. Note that trial and error will be needed for species requiring specific growth conditions. For instance, for hyperthermophile archaea crosslinking cannot be performed at high temperatures, and those cells will have to be cooled down immediately prior to fixation (see Cockram et al., 2020; Takemata et al., 2019). We recommend paying attention to this step, as it is a crucial one with respect to the quality of the resulting data.

2. Crosslink protein-DNA interactions by adding fresh formaldehyde (3% final concentration).

3. Incubate for 30 min at 19°C–23°C on an orbital shaker (50–100 rpm).

   **CRITICAL:** Always use fresh formaldehyde for crosslinking.

   **Optional:** Crosslinking can also be performed using a magnetic stirrer.

   **CRITICAL:** When crosslinking thermophilic organisms, such as the Euryarchaeota *T. kodakarensis*, we recommend mixing 20 mL of the cell culture with 80 mL of room temperature formaldehyde (19°C–23°C) diluted in 1 X PBS (4% final concentration, Cockram et al., 2020) see also (Takemata et al., 2019).

4. Quench formaldehyde by adding glycine (0.5 M final concentration).

5. Incubate for 20 min at 19°C–23°C on an orbital shaker (50–100 rpm).

   **CRITICAL:** When working with halophilic organisms, such as the Euryarchaeota *H. volcanii*, which are very sensitive to salt concentrations, we recommend preparing 2.5 M glycine in 18% (w/v) saltwater solution (Cockram et al., 2020).

6. Transfer culture to a sterile centrifuge tube and collect cells by centrifugation (4000 × g, 10 min, at 19°C–23°C).

7. Decant supernatant.
**Critical:** Depending on the bacterial or archaeal species, pellets can be unstable and the supernatant may need to be removed by careful pipetting.

8. Carefully resuspend the cell pellet in 25 mL of 1× PBS by gentle pipetting and transfer to a 50 mL conical tube.
9. Collect cells by centrifugation (4000×g, 10 min at 19°C–23°C).
10. Resuspend the pellet in 1 mL of 1× PBS by gentle pipetting. Transfer to a 1.5 mL tube.
11. Centrifuge again (4000×g, 5 min at 19°C–23°C).
12. Carefully remove the supernatant using a pipette, so that the pellet is as dry as possible.
13. Store the cell pellet at −80°C.

**Pause point:** Pellets can be stored for at least 2 years at −80°C with no impact on the quality of the subsequent Hi-C library.

**Day 2: Hi-C library construction**

**Timing:** approximately 7 h

14. Remove the cell pellet from the −80°C freezer and place on ice until completely thawed.
15. Gently resuspend the pellet by pipetting in 1.2 mL of 1× TE containing complete protease inhibitor cocktail.
16. Transfer to a 2 mL VK05 Precellys tube.
17. Lyse cells using the Precellys Evolution tissue homogenizer (V7500: 5×30 s, 20 s pause).

**Note:** For Precellys machines without the Cryolys cooling attachment, the program is: V6700: 9×20 s, (30 s pause). Tubes should be removed every 3 cycles and placed on ice for 5 min to prevent sample degradation from overheating.

**Critical:** For Gram-negative species, such as *E. coli*, lysis can also be performed by incubating the cells with lysozyme (0.4 μg/mL final concentration) for 30 min at 37°C. This will produce similar results to mechanical disruption. However, formaldehyde-crosslinked Gram-positive bacteria, such as *B. subtilis*, can be difficult to lyse with lysozyme and the enzyme is inactive on archaea, since they do not contain peptidoglycan in their cell walls. In such cases, we recommend either disrupting cells with glass beads or a French Press (Marbouty et al., 2014).

18. Transfer the lysate (~1 mL in volume) to a 5 mL microcentrifuge tube. Take care not to transfer any of the glass beads.
19. Add 50 μL of 10% SDS solution (0.5% final concentration) and incubate for 10 min at 19°C–23°C.
20. Prepare the Digestion Mix:

| Reagent           | Volume |
|-------------------|--------|
| 10× Digestion Buffer | 500 μL |
| 10% Triton-X-100  | 500 μL |
| ddH₂O             | 3 mL   |

**Note:** If processing multiple samples, we recommend preparing a master mix.

21. Add the Digestion Mix to the tube containing the lysate and mix well by pipetting.
22. Remove 400 μL of the sample and transfer it to a 1.5 mL microcentrifuge tube.
Note: This is the non-digested (ND) control to check for sample degradation and should be stored on ice until required (troubleshooting 1 Figure 1).

23. Add 1000U of restriction enzyme to the remaining sample and incubate at 37°C for 3 h in a shaking incubator (~180 rpm).
24. Following digestion, remove 400 μL of the sample and transfer it to a 1.5 mL microcentrifuge tube.

Note: This is the digested (D) control to check for efficient cutting of the genomic DNA. Store on ice until required (troubleshooting 2 Figure 1).

25. Pellet the insoluble fraction by centrifugation (16,000 × g, 20 min at 19°C–23°C).

Note: This pellet contains protein-DNA complexes of interest

26. Decant the supernatant.
27. Carefully resuspend the pellet in 400 μL of dH2O by pipetting.

Note: Depending on the species, this pellet can be quite difficult to resuspend. Ensure it is fully resuspended before continuing.

28. Prepare the Biotinylation mix:

| Reagent                               | Volume   |
|---------------------------------------|----------|
| 10x Ligation Buffer                   | 50 μL    |
| 10 mM dAGTTP mix                      | 4.5 μL   |
| Biotin-14-dCTP                        | 37.5 μL  |
| DNA Polymerase I – Large (Klenow) Fragment (5U/μL) | 8 μL     |

Note: If processing multiple samples, we recommend preparing a master mix.

Note: If using MluCI for the digestion, exchange Biotin-14-dCTP for Biotin-14-dATP and replace the 10 mM dAGTTP mix with 10 mM dCGTTP mix.

29. Add the Biotinylation mix to the resuspended pellet and mix by pipetting
30. Incubate at 37°C for 45 min in a shaking incubator (~180 rpm).
31. Prepare the ligation mix:

| Reagent                               | Volume   |
|---------------------------------------|----------|
| 10x Ligation Buffer                   | 120 μL   |
| 10 mg/mL BSA                          | 12 μL    |
| 100 mM ATP                            | 12 μL    |
| ddH2O                                 | 540 μL   |
| T4 DNA ligase (30U/μL)                | 16 μL    |

Note: If processing multiple samples, we recommend preparing a master mix.

32. Add ligation mix to the reaction and mix by pipetting.
33. Incubate with gentle agitation for 3 h at 19°C–23°C.
34. Following ligation, add 20 μL 500 mM EDTA, 80 μL 10% SDS, and 100 μL 20 mg/mL proteinase K to the Hi-C library.

35. Remove the ND and D controls from ice and add 20 μL 500 mM EDTA, 20 μL 10% SDS, and 10 μL 20 mg/mL proteinase K.

36. Incubate the Hi-C library, ND and D controls overnight (10–12 h) at 65°C to reverse formaldehyde-mediated protein-DNA crosslinks.

**Note:** we processed indiscriminately bacteria and archaea cells at this step, regardless of their normal growth condition.

### Day 3: DNA purification and sequencing library preparation

**Timing:** approximately 6 h

37. Purification of DNA by phenol extraction

**Timing:** approximately 1 h

a. Add an equal volume (~1.2 mL) of Phenol:Chloroform:Isoamyl alcohol to the Hi-C sample and vortex for 30 s.

CRITICAL: Always handle phenol under the hood, as it causes severe burns and eye damage and is toxic if inhaled or ingested.

b. Centrifuge (12,000 × g, 5 min at 19°C–23°C).

c. Carefully remove the upper aqueous phase (~1.2 mL) and transfer to a new 5 ml microcentrifuge tube.

d. Precipitate DNA by adding 2.5× volume (~3 mL) of ice-cold 100% EtOH and 1/10 volume (~120 μL) of 3 M NaOAc (pH 5.0).

e. Precipitate DNA of ND and D controls by adding 2.5× volume (~1 mL) of ice-cold 100% EtOH and 1/10 volume (~40 μL) of 3 M NaOAc (pH 5.0).

**Note:** to reduce the amount of phenol chloroform used, we proceed straight to ethanol precipitation for the ND and D controls.

f. Incubate all samples at −80°C for 30 min.

g. Pellet DNA by centrifugation (12,000 × g, 20 min, 4°C).

h. Carefully remove supernatant using a pipette

i. Wash pellets with 500 μL of ice-cold 70% EtOH.

j. Pellet DNA by centrifugation (12,000 × g, 5 min, 4°C).

k. Remove EtOH and dry pellets on a 37°C heat block for 5–10 min.

**Note:** Briefly centrifuge (~10 sec) after removing the ethanol to collect any residual EtOH on the side of the tube.

l. Add 140 μL 1× TE buffer containing 1 mg/mL RNase to pellets and incubate for 15–30 min at 37°C in a shaking incubator (~180 rpm).

m. Sample 10 μL for the ligation (L) control (troubleshooting 3 Figure 1).

n. Run 10 μL of the Hi-C library and 10 μL of the ND, D and L controls on a 1% agarose gel (Figure 1).

CRITICAL: These controls are important for determining that the Hi-C library construction was successful.

o. Discard ND and D controls.

|| Pause point: Hi-C library can be stored at −20°C.
38. Sonication and size-selection of DNA

**Timing:** approximately 45 min

a. Transfer 130 μL of DNA to a Covaris microtube.

*Note:* We recommend a maximum of 5 μg of DNA is used for sonication (as determined by Qubit analysis). Samples that have less DNA are OK, but if the Hi-C library exceeds 5 μg, then an aliquot should be taken and the remaining DNA stored at −20°C, as a backup. Once the correct quantity of DNA is obtained, the sample volume should be adjusted to 130 μL using 1 x TE buffer.

b. Sonicate DNA using a Covaris S220 machine and the following parameters:

| Parameter                  | Value  |
|----------------------------|--------|
| Peak Incidence Power (W)   | 140    |
| Duty Factor                | 10%    |
| Cycles per Burst           | 200    |
| Temperature                | 7°C    |
| Treatment Time             | 80 sec |

*Note:* These parameters will generate a fragment size of ~300 bp.

c. Transfer sheared DNA to a 1.5 mL microcentrifuge tube.

d. Add an equal volume of AMPure XP beads.

e. Mix sample 10 x by gentle pipetting.

f. Incubate for 5 min at 19°C–23°C to allow DNA fragments to bind to the magnetic beads.

g. Transfer tube to a magnetic rack to allow the beads to separate to the side of the tube (~ 1 min).

h. Carefully remove the supernatant and wash with 70% EtOH, keeping the tube on the magnet and ensuring that the beads are not disturbed.

i. Repeat step h.

j. Remove the EtOH, and airdry the pellet for 2 min to remove residual EtOH.

△ **CRITICAL:** do not allow the pellet to over-dry as this will reduce the elution efficiency.

k. Remove beads from the magnet and resuspend in 320 μL of 10 mM Tris-HCl (pH 7.5).

l. Mix sample 10 x by gentle pipetting.

m. Incubate for 5 min at 19°C–23°C and then place back onto the magnetic rack for 1 min.
n. Carefully remove supernatant and transfer to a new 1.5 mL microcentrifuge tube.
o. Check sonication and size-selection by running 18 μL of the DNA on a 1% agarose gel (sonication control (S), troubleshooting 4 Figure 1).
p. The remaining 300 μL is used to prepare the sequencing library.

Pause point: DNA can be stored at −20°C.

39. Biotin pull-down

இ Timing: approximately 30 min
a. Thoroughly mix the Streptavidin C1 Dynabeads before use.
b. Transfer 30 μL of beads to a 1.5 ml microcentrifuge tube.
c. Place the tube on a magnet and wait ~1 min to clear the supernatant.
d. Remove the supernatant and then resuspend the beads in 500 μL of 1× TWB.
e. Place the tube back on the magnet and wait ~1 min to clear the supernatant.
f. Remove the supernatant and resuspend the beads in 300 μL of 2× BB.
g. Add 300 μL of the Hi-C sample to the beads.
h. Incubate on a tube rotator (~10 rpm) for 15 min at 19°C–23°C.
i. Place the tube back on the magnet and wait ~1 min to clear the supernatant.
j. Remove the supernatant and then resuspend the beads in 500 μL of 1× TWB.
k. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
l. Place the tube back on the magnet and wait ~1 min for the supernatant to clear.
m. Repeat steps j–l.
n. Discard the supernatant, resuspend the beads in 100 μL of 1× T4 ligase buffer and transfer to a new 1.5 mL microcentrifuge tube.

40. End repair

இ Timing: approximately 45 min
a. Prepare the end-repair mix by combining the following:

| Reagent                                                   | Volume       |
|-----------------------------------------------------------|--------------|
| 1× T4 ligase buffer                                       | 85 μL        |
| 10 mM dNTPs                                               | 5 μL         |
| T4 Polynucleotide Kinase (10U/μL)                         | 5 μL         |
| T4 DNA polymerase (3U/μL)                                 | 4 μL         |
| DNA Polymerase I, Large (Klenow) Fragment (5U/μL)         | 1 μL         |

Note: If processing multiple samples, we recommend preparing a master mix.
b. Place the Hi-C library on the magnet and wait ~1 min to clear the supernatant.
c. Discard the supernatant and resuspend the beads in the end-repair mix.
d. Incubate for 30 min at 19°C–23°C, without agitation.
e. Place the tube on the magnet and wait ~1 min to clear the supernatant.
f. Remove the supernatant and then resuspend the beads in 500 μL of 1× TWB.
g. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
h. Place the tube back on the magnet and wait 1 min for the supernatant to clear.
i. Repeat steps f–h.
j. Discard the supernatant, resuspend the beads in 100 μL of 1× NEB Buffer 2, and transfer to a new 1.5 mL microcentrifuge tube.

Pause point: DNA can be stored at −20°C.

41. A-tailing
© Timing: approximately 45 min

a. Prepare the A-tailing mix by combining the following:

| Reagent                        | Volume |
|--------------------------------|--------|
| 1X NEBuffer 2                  | 90 µL  |
| 10 mM dATP solution            | 5 µL   |
| Klenow Fragment (3’-5’ exo-, 5U/µL) | 5 µL   |

**Note:** If processing multiple samples, we recommend preparing a master mix.

b. Place the Hi-C library on the magnet and wait 1 min to clear the supernatant.
c. Discard the supernatant and resuspend the beads in the A-tailing mix.
d. Incubate for 30 min at 37°C, without agitation.
e. Place the tube on a magnet and wait ~1 min to clear the supernatant.
f. Remove the supernatant and then resuspend the beads in 500 µL of 1X TWB.
g. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
h. Place the tube back on the magnet and wait ~1 min for the supernatant to clear.
i. Repeat steps f–h.
j. Discard the supernatant, resuspend the beads in 50 µL of 1X Quick Ligase Buffer, and transfer to a new 1.5 mL microcentrifuge tube.

Optional: adapter ligation can also be performed using T4 DNA ligase for 2 h at 19°C–23°C or overnight (10 – 12 h) at 16°C.

If doing this, resuspend in 50 µL 1X T4 DNA ligase buffer instead and follow the manufacturer’s instructions (https://www.thermofisher.com/order/catalog/product/EL0013?fr&en#/EL0013?fr&en) for setting up the reaction.

42. Adapter ligation

© Timing: approximately 30 min

a. Prepare the Ligation mix by combining the following:

| Reagent                  | Volume |
|--------------------------|--------|
| 1X Quick Ligase Buffer   | 48 µL  |
| Quick Ligase             | 2 µL   |

**Note:** If processing multiple samples, we recommend preparing a master mix.

b. Place the Hi-C library on the magnet and wait ~1 min to clear the supernatant.
c. Discard the supernatant and resuspend the beads in the Ligation mix.
d. Add 2 µL of sequencing adapter and mix by pipetting.
e. Centrifuge a few seconds to collect sample at bottom of tube.
f. Incubate at 19°C–23°C for 10 min.
g. Place the tube on a magnet and wait ~1 min to clear the supernatant.
h. Remove the supernatant and then resuspend the beads in 500 µL of 1X TWB.
i. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
j. Place the tube back on the magnet and wait 1 min for the supernatant to clear.
k. Repeat steps h–j.
l. Discard the supernatant, resuspend the beads in 50 µL of 10 mM Tris-HCl (pH 8) and transfer to a new 1.5 mL microcentrifuge tube.

**Pause point:** beads containing the Hi-C library can be stored at –20°C for at least 2 years

43. PCR amplification
© Timing: approximately 1 h

a. Prepare the PCR mix by combining the following:

| Reagent                                      | Amount |
|----------------------------------------------|--------|
| Phusion 2× High Fidelity Master Mix          | 40 µL  |
| 2 µM Primer Mix (NEXTflex, PerkinElmer)      | 5 µL   |
| Streptavidin beads containing Hi-C library   | 3 µL   |
| ddH2O                                        | 32 µL  |

Note: If processing multiple samples, we recommend preparing a master mix.

b. Amplify the library using the following conditions:

| Steps                      | Temperature | Time | Cycles |
|----------------------------|-------------|------|--------|
| Initial denaturation       | 98°C        | 30 sec | 1      |
| Denaturation               | 98°C        | 10 sec | 12     |
| Annealing                  | 61°C        | 15 sec |        |
| Extension                  | 72°C        | 15 sec |        |
| Final extension            | 72°C        | 10 min | 1      |
| Hold                       | 4°C         | forever|        |

c. Place the PCR reaction on a magnet and wait ~1 min to clear the supernatant.
d. Transfer the supernatant to a new 1.5 mL microcentrifuge tube. Discard the Dynabeads.
e. Purify the PCR reaction by adding an equal volume (~80 µL) of AMPure XP beads.

Note: if multiple PCR reactions were performed (i.e., for low concentration samples), the reactions can be combined at this step and the volume of AMPure XP beads should be adjusted accordingly.
f. Mix sample 10× by gentle pipetting
g. Incubate for 5 min at 19°C–23°C to allow DNA fragments to bind to the magnetic beads.
h. Transfer tube to a magnetic rack to allow the beads to separate to the side of the tube (~ 1 min).
i. Carefully remove the supernatant and wash with 70% EtOH, keeping the tube on the magnet and ensuring that the beads are not disturbed.
j. Repeat step i.
k. Remove the EtOH, and airdry the pellet for 2 min to remove residual EtOH.

CRITICAL: do not allow the pellet to over-dry as this will reduce the elution efficiency.
l. Remove beads from the magnet and resuspend in 50 µL of 10 mM Tris-HCl pH 8.
m. Mix sample 10× by gentle pipetting.
n. Incubate for 5 min at 19°C–23°C and then place back onto the magnetic rack for ~1 min.
o. Carefully remove the supernatant and transfer it to a new 1.5 mL microcentrifuge tube.
p. Remove a 5 µL aliquot and transfer to a new tube. This is the final library (FL) control.
q. Run the FL sample on a 1% agarose gel to determine the fragment size of the sequencing library and to check for the presence of primer-dimers (troubleshooting 5, Figure 1).
r. Determine DNA concentration using the Qubit dsDNA HS kit on a Qubit Fluorometer.
s. Store the final sequencing library at ~20°C.

Note: Samples can be stored at ~20°C for more than 2 years with no impact on the quality of the Hi-C data.
t. Prepare libraries for paired-end sequencing using the 75 cycle High-Output Kit v2.5. according to Illumina’s instructions (https://support.illumina.com/content/dam/illumina-support/
Day 4: Data processing

**Timing:** approximately 1–2 days

44. All data were processed into contact maps accordingly to standard Hi-C analysis that include iterative alignment, filtering, and normalization (Cournac et al., 2012; Imakaev et al., 2012; Le et al., 2013; Liou et al., 2018; Servant et al., 2015). Details relating to the datasets shown in Figure 2 are described in (Cockram et al., 2020).

**EXPECTED OUTCOMES**

The typical results obtained for the different steps of the bacterial/archaeal Hi-C protocol are shown in Figure 1. Following cell lysis and the preparation of the digestion reaction, we remove an aliquot and designate it as the non-digested (ND) control. This control monitors the amount of DNA degradation during these first steps. Next, we use the digested (D) control to check for efficient cutting of the genomic DNA by the restriction enzyme, this is shown by a DNA smear that is absent in the ND control. Following end-filling with a biotinylated nucleotide and blunt-end ligation, the efficiency of the ligation can be determined by checking that the DNA smear (L) has shifted to a higher molecular weight. Following reverse crosslinking, DNA purification and sonication, the average size of the DNA fragments is checked (S) before continuing with the preparation of the sequencing library. The size and quality of the final library (FL) are then checked on gel before pair-end sequencing. DNA concentration is determined by Qubit analysis. When starting with ~\(3 \times 10^8\) cells, we typically obtain a concentration of 5–15 ng/ul for the final Hi-C library. Figure 2 shows the typical Hi-C maps obtained from wild-type, exponentially growing cultures of the bacterium *E. coli* (Figure 2A) and the Euryarchaeota *H. volcanii* (Figure 2B) (Cockram et al., 2020; see also Le et al., 2013 for an earlier example of bacteria contact map). This protocol is capable of generating 0.5 – 1 kb Hi-C matrices for species of both domains of life, offering gene-level resolution contact maps (Figures 2C and 2D).

**LIMITATIONS**

The Hi-C method relies on a population of cells and as a result the contact maps reflect an average of the population which may mask the presence of cell-cell heterogeneity in different conditions and mutant backgrounds. In addition, archaeal species are generally cultured in extreme conditions, such as high-salt or high-temperature. These species-specific growth conditions may require specific optimization. Finally, the Euryarchaeota and some bacterial species, such as *D. radiodurans*, are polyploid, so inter-chromosomal contacts between replicated chromosomes cannot be determined using the Hi-C technique.

**TROUBLESHOOTING**

**Problem 1**
Degradation of DNA in the non-digested (ND) control (step 22).

**Potential solution**
If some DNA degradation is observed, then the final Hi-C library will likely be fine, but if there is significant degradation, we recommend starting the protocol again with new buffers.

**Problem 2**
Incomplete digestion of genomic DNA (step 23).
Potential solution

In our experience, some restriction enzymes work better than others and there isn’t always a clear reason for this. We have tested HpaII and MluCI in a range of bacteria and archaea and both seem to work very well. However, if your organism requires a specific enzyme, we suggest performing steps 1–35 with a test sample first to ensure efficient digestion. A second reason for incomplete digestion could be that there is an issue with the digestion buffer, so we would suggest re-making this and trying the digestion again. Finally, it may be that you have too much DNA, so perhaps try diluting the lysate before digestion.

Problem 3

No shift in higher molecular weight following ligation (Figure 1; control L; steps 31–34).

Potential solution

This step may fail for one of two reasons. Either the end-fill step didn’t work, or the blunt-end ligation was unsuccessful. Unfortunately, the only solution is to repeat the Hi-C library preparation from the beginning. We would recommend re-making all solutions for both the end-fill and ligation steps.

Figure 2. Representative examples of the quality of Hi-C matrices obtained using the optimized protocol for bacteria and archaea. b

Normalized Hi-C contact maps of asynchronously growing populations of WT E. coli and H. volcanii cells. The X and Y axes represent the coordinates of the chromosome and the color scale reflects the frequency of contacts between two regions of the genome (arbitrary units), from white (rare contacts) to dark red (frequent contacts). Features of interest are indicated along the top axis.

(A) Hi-C contact matrix of WT E. coli binned at 5 kb.

(B) Hi-C contact matrix of WT H. volcanii binned at 5 kb.

(C) Magnification of a 50 kb region surrounding the E. coli fliF-R operon (coordinates: 1988 – 2038 kb, bin = 0.5 kb).

(D) Magnification of a 100 kb region of the main H. volcanii chromosome (coordinates 1474 kb – 1574 kb, bin = 1 kb).
the digestion (D) smear looks correct, then the buffers for this step are fine and do not need to be
remade.

**Problem 4**
Poor sonication efficiency (step 38).

**Potential solution**
If the average fragment size is too high following sonication, we suggest re-checking the DNA con-
centration by Qubit analysis. The Covaris parameters detailed in this protocol are suitable for a
maximum of 5 μg of DNA. If this is exceeded, the efficiency of sonication will be reduced and the
fragment size will be too large. If you’re using a different sonication method, we suggest checking
the manufacturer’s instructions and performing a time course experiment to determine the optimal
conditions.

**Problem 5**
Low DNA yield in the final Hi-C library (step 43, r)

**Potential solution**
This is why we strongly encourage all users to take aliquots at the steps suggested and run the con-
trols on an agarose gel (Figure 1). This will help them to identify which step went wrong. For example,
if the gel shows a high concentration of DNA following sonication and size-selection, but the final
library has a poor yield, then it suggests a problem occurred during the preparation of the
sequencing library. We would first suggest repeating the final PCR amplification step, as it’s the
easiest and least-expensive problem to fix. If this doesn’t solve the issue, then it suggests the
DNA was lost in one of the previous steps, such as binding of the Hi-C library to the streptavidin
beads and means the sequencing library needs to be prepared again. Alternatively, if there was
already a low amount of material following sonication and size-selection, then it may mean that
you need to scale up and perform two PCR reactions instead of one, we do not recommend
increasing the number of PCR cycles. In our experience, as long as the final Hi-C library has a
DNA concentration >1 ng/μl by Qubit analysis, then it should be ok to sequence.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be ful-
filled by the lead contact, Romain Koszul (romain.koszul@pasteur.fr).

**Materials availability**
This study did not generate new unique reagents

**Data and code availability**
The datasets generated during using protocol are detailed in (Cockram et al., 2020) and are avail-
able at PRJNA587586. Open-access versions of the programs and pipelines used for downstream
data analysis (E. coli analysis pipeline and Chromosight; Matthey-Doret et al., 2020) are available
online via the lab Github account (https://github.com/koszullab/hicstuff).

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**AUTHOR CONTRIBUTIONS**
C.C. and A.T. adapted the Hi-C protocol. C.C. and R.K. wrote the manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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