The environmentally-regulated interplay between local three-dimensional chromatin architecture and gene expression

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Chapter 2:
Hi-C in bacteria and archaea

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Abstract

The three-dimensional structure of the chromosome is encoded within its sequence and regulates activities such as replication and transcription. Hi-C combines proximity ligation and high-throughput sequencing to resolve spatial chromosome organization in relation to the underlying sequence, hence, revealing the principles of structural regulation of genome transactions. This chapter describes the preparation of Hi-C sequencing libraries for bacteria and archaea.

Introduction

In prokaryotes, the genome sequesters into a phase-separated nucleoid in the cytoplasm. Within this compartment, chromosome(s) organise into domains — physically insulated regions of the chromosome in which the chromatin interacts more frequently with itself than with flanking chromatin. Domain organisation is hierarchical, with larger domains formed from the preferential interaction of two or more smaller domains (1–3).

Three-dimensional chromosome organisation is determined by the physical properties of the DNA polymer and by the architectural properties of nucleoid associated proteins (NAPs) bound to the macromolecule. These proteins maintain chromosome structure and remodel it in response to environmental stimuli, directly affecting genome processes. For example, in *Escherichia coli*, the formation of a DNA:H-NS:DNA bridge between the downstream regulatory element (DRE) and the end of the *proU* operon silences its expression. Disruption of this bridge upon a hyperosmotic shock activates the operon (Chapter 3, this thesis). In *Bacillus subtilis*, a pair of hairpins formed by the directional movement of SMC-ScpAB complexes along the chromatin bring distantly placed DnaA boxes in close proximity to OriC to initiate chromosome replication (1). Conversely, genome processes affect chromosome structure. In *Caulobacter crescentus*, long, highly-transcribed genes form domain boundaries by generating extended supercoil diffusion barriers that physically separate flanking DNA (4). Furthermore, gene transcription in the Ter>Ori direction slows the movement of SMC complexes towards the chromosome terminus (5, 6).

The intricate interplay between chromosome architecture and its processes is encoded within the genome sequence as, for instance, binding sites of varying affinity for the repertoire of NAPs available within a cell, transcription initiation and termination sites of varying strengths, replication origins and termini, and loading and offloading sites for motor proteins that track along the DNA (Reviewed in (7)). The inherent sequence dependence of chromosome architecture necessitates describing chromosome structure in terms of its underlying sequence. To that end, chromosome conformation capture (3C)-based techniques detect the physical proximity of chromosome segments in three-dimensional space (8). Hi-C uses proximity ligation and next generation sequencing to probe physical interactions amongst all segments of the chromosome in an unbiased manner (9). The technique, therefore, describes the three-dimensional structure of the chromosome in relation to genome sequence.

As an ensemble technique, Hi-C determines the probability that two regions of the chromosome lie in physical proximity (9). This highlights common architectural features of an organism's chromosome such as its domains and domain boundaries, compartmentalization into hetero- and eu-chromatin, and, in some prokaryotes, the presence of inter-arm interactions (1–4, 10). As a single-cell technique, Hi-C identifies the mutual inclusivity or exclusivity of these features (11–13).

This chapter builds on our earlier work (14) and describes protocols for the preparation of ensemble Hi-C libraries compatible for sequencing on an Illumina NGS platform from *Escherichia coli*, *Saccharolobus solfataricus*, and *Haloquadratum walsbyi* cultures.

Materials

Cell fixation

1. 37% w/v formaldehyde solution, stored at room temperature away from direct sunlight.
2. 1X TE Buffer: 10 mM Tris-HCl, 1 mM EDTA·Na_2, pH 8.0. Store the buffer at room temperature and cool it to 4 °C prior to use.
3. Liquid nitrogen.
4. Refrigerated benchtop centrifuge with rotors for 50 mL, 15 mL, and 1.5 mL tubes.
5. Roller bench.
6. -80°C freezer.
7. 50 mL and 15 mL conical tubes.
8. Serological pipettes.
9. Pipette pump.
10. Elongated Pasteur pipette (made of soda lime silica glass): Heat a 1 cm space of the Pasteur pipette above the tip and stretch the heated region to reduce the diameter of the pipette bore. Use a pair of tweezers to break the pipette at the elongated region.
11. 2.5 µL, 20 µL, 200 µL, and 1000 µL micropipettes.
12. Low-retention micropipette tips.
13. Low-retention 1.5 mL microfuge tubes.

Additional materials for *Escherichia coli* cell fixation:

14. Methanol, ACS reagent ≥99.8%. Store the reagent at room temperature and cool it to 4 °C prior to use.

15. 1X PBS solution: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4. Store 1X PBS at room temperature and cool it to 4 ºC prior to use.
16. 2.5 M Glycine solution filter-sterilized using a sterile, non-pyrogenic 0.2 µm filter *(see note 1)*.

Additional materials for *Saccharolobus solfataricus* cell fixation:

14. Thermomixer (e.g. Eppendorf Thermomixer® C) with a thermoblock for 15 mL conical tubes (e.g. Eppendorf SmartBlock™ 15 mL).
15. Filter-sterilised 2.5 M glycine *(see note 1)*.

Additional materials for *Haloquadratum walsbyi* cell fixation:

14. DSMZ medium 1091 for *H. walsbyi*.
15. 10X *H. walsbyi* dialysis buffer: 60 mM L-glutamic acid, 270 mM KCl, 115 mM sodium citrate dihydrate, 800 mM $MgSO_4 \cdot 7H_2O$, 2.0 mM $FeCl_2 \cdot 4H_2O$, 20.0 µM $MnCl_2 \cdot 2H_2O$, pH 6.3-6.4. Store the buffer at room temperature (20-25 °C), preferably in the dark.
16. Dialysis tubing with a 3.5 kDa molecular weight cut-off.
17. Dialysis tubing clamps and rubber bands.
18. 2 L beakers.
19. Cling film or aluminium foil.
20. (Heated) magnetic stirring plate and stir bar.
21. Optional: 20% ethanol.

Hi-C library preparation

1. 1X TE Buffer: 10 mM Tris-HCl, 1 mM EDTA·Na_2, pH 8.0.
2. **For *E. coli*:** 40,000 U/µL Ready-Lyse Lysozyme (Epicentre).
3. **For *E. coli*:** 1.0 M NaCl.
4. **For *S. solfataricus* and *H. walsbyi*:** Glass beads, 0.5 mm in diameter (e.g. Z250465-1PAK from Sigma Aldrich).
5. Nuclease-free water.
6. 10% w/v Sodium dodecyl sulfate (SDS) solution: Dissolve 1.0 g of SDS (ACS reagent, ≥99.0%) in 10 mL of nuclease-free water.
7. 10% v/v Triton X-100 solution: Dilute 1.0 mL of Triton X-100 (molecular biology grade) with 9.0 mL of nuclease-free water. Store the solution at room temperature (20-25 °C) in the dark.
8. Restriction enzyme: 10 U/µL PsuI (ThermoFisher Scientific) *(see note 2)*.

9. 10X restriction enzyme buffer, as supplied with the selected restriction enzyme *(see note 2)*: 10X Buffer B (ThermoFisher Scientific).
10. 100 mM deoxynucleotides (ultrapure dNTPs as separate solutions). Repeated freeze-thaw cycles may result in the degradation of dNTPs. To minimize degradation of the 100 mM stock, dilute 20.0 µL of 100 mM dNTP with 180.0 µL of nuclease-free water. Store the 10 mM dNTP solution at -20 °C as 10.0 – 50.0 µL aliquots.
11. Separate solutions of 1 mM dNTP: Dilute one 10 mM dNTP aliquot tenfold with nuclease-free water. Store the dNTP solution at -20 °C and thaw on ice or at 4 °C when necessary.
12. 0.4 mM Biotin-14-dATP (*see note 3*).
13. 20 mg/mL Bovine serum albumin (BSA).
14. 5 U/µL DNA Polymerase I, Large (Klenow) fragment.
15. Qubit® dsDNA HS Assay Kit and a Qubit® fluorometer from ThermoFisher Scientific.
16. 10X T4 DNA Ligase buffer: 0.5 M Tris-HCl, 0.1 M $MgCl_2$, 10 mM ATP, 0.1 M DTT, pH 7.5.
17. 2000 U/µL T4 DNA Ligase.
18. 0.5 M EDTA, pH 8.0.
19. 10 mg/mL RNase A.
20. 20 mg/mL Proteinase K.
21. 5.0 M NaCl.
22. 25:24:1 Phenol:chloroform:isoamyl alcohol solution *(see note 4)*.
23. Chloroform *(see note 4)*.
24. 1.0 M Sodium acetate (NaOAc), pH 8.0.
25. 5.0 mg/mL Glycogen.
26. 100% and 70% (v/v) Ethanol. Keep the solutions cold at -20 °C.
27. 10 mM Tris, pH 8.0.
28. 10X T4 DNA Polymerase buffer: 0.5 M NaCl, 0.1 M Tris-HCl, 0.1 M $MgCl_2$, 1 mg/mL BSA, pH 7.9 (available as NEBuffer 2.1).
29. 3 U/µL T4 DNA Polymerase.
30. Thermomixer (e.g. Eppendorf Thermomixer® C) with a thermoblock for 1.5 mL microfuge tubes (e.g. Eppendorf SmartBlock™ 1.5 mL).
31. Benchtop centrifuge.
32. Refrigerated benchtop centrifuge.
33. Vortex mixer.
34. Agarose gel electrophoresis set-up.
35. -20 °C freezer.

36. 2.5 µL, 20 µL, 200 µL, and 1000 µL micropipettes.
37. Low-retention micropipette tips.
38. Low-retention 1.5 mL microfuge tubes.
39. 15 mL conical tubes

NGS library preparation

1. Solid Phase Reverse Immobilization (SPRI) beads (e.g. KAPA HyperPure Beads from KAPA Biosystems). Store the beads as 1.0 mL aliquots at 4 °C. Ensure that the beads are equilibrated to 25 °C and vortexed before use.
2. Nuclease-free water.
3. 10 mM Tris, pH 8.0.
4. 10 mM Tris, pH 8.0 + 0.1% Tween 20.
5. Qubit® dsDNA HS Assay Kit and a Qubit® fluorometer from ThermoFisher Scientific.
6. Illumina® library preparation kit (e.g. KAPA HyperPlus kit from KAPA Biosystems)
7. Illumina®-compatible paired-end sequencing adapters at a concentration of 15 µM (e.g. KAPA Single-Indexed Adapter Sets A and B from KAPA Biosystems). The sequencing adapters should be barcoded to allow multiplexing during NGS.
8. Streptavidin-coupled magnetic beads optimized for the enrichment of biotin-labelled nucleic acids (e.g. Dynabeads® MyOne™ Streptavidin T1 from ThermoFisher Scientific). Store the beads at 4 °C. Equilibrate the beads to 25 °C and vortex well before use.
9. 2X Beads Wash Buffer (2X BWB): 10 mM Tris pH 7.5, 1 mM EDTA, 2 mM NaCl, 0.2% Tween 20.
10. 1X Beads Wash Buffer (1X BWB): 5 mM Tris pH 7.5, 0.5 mM EDTA, 1 mM NaCl, 0.1% Tween 20.
11. Magnetic racks (e.g. DynaMag™-PCR Magnet and DynaMag™-2 Magnet from ThermoFisher Scientific).
12. Thermomixer (e.g. Eppendorf Thermomixer® C) with a thermoblock for 1.5 mL microfuge tubes (e.g. Eppendorf SmartBlock™ 1.5 mL).
13. Thermal cycler with a heated lid.
14. Vortex mixer.
15. PCR tubes.
16. Tapestation (Agilent Technologies), or Bioanalyser (Agilent Technologies), or an agarose gel electrophoresis set-up.
17. 2.5 µL, 20 µL, 200 µL, and 1000 µL micropipettes.

18. Low-retention micropipette tips.
19. Low-retention 1.5 mL microfuge tubes.

Methods

The protocols described here are optimized for HiC in *Escherichia coli*, *Saccharolobus solfataricus*, and *Haloquadratum walsbyi*. To process other prokaryotes using these protocols, optimize the cell lysis and solubilization steps by adjusting the treatment duration – and, if relevant, the concentrations – of Ready-Lyse Lysozyme, SDS, and physical shearing with glass beads.

Cell fixation

Escherichia coli

1. Transfer an aliquot of an *Escherichia coli* culture equivalent to ~$5.0x10^9$ cells into a chilled 50 mL conical tube.
2. Harvest the cells by centrifugation at 3000 xg for 5 minutes at 4 °C.
3. Decant the supernatant and carefully remove the rest with a micropipette. Resuspend the cell pellet in 4.0 mL of 1X PBS.
4. Add 16.0 mL of methanol to the cell suspension *(see note 5)*. Mix well by inversion and incubate the cells at 4 °C for 10 minutes on a roller bench at 30 rpm.
5. Collect the cells at 3000 xg for 5 minutes at 4 °C.
6. Discard the supernatant and resuspend the cells in 20.0 mL of 1X PBS to wash.
7. Centrifuge the cell suspension at 3000 xg for 5 minutes at 4 °C.
8. During the centrifugation, prepare 40 mL of 3% formaldehyde in 1X PBS by adding 3.2 mL of 37% formaldehyde solution to 36.8 mL of cold 1X PBS in a 50 mL conical tube. Mix well by inversion and place the solution on ice or at 4 °C.
9. Discard the supernatant of the centrifugation performed at step 7 of this section. Resuspend the cell pellet in 40.0 mL of 3% formaldehyde. Incubate the cells at 4 °C for 1 hour on a roller bench at 30 rpm.
10. Add 6.4 mL of 2.5 M glycine to the fixation reaction (to a final concentration of 0.375 M) to quench excess formaldehyde. Incubate the cells for 15 minutes at 4 °C on a roller bench at 30 rpm.
11. Collect the fixed cells by centrifugation at 3000 xg for 10 minutes at 4 °C.
12. Decant the supernatant and carefully remove the rest with a micropipette. Resuspend the pellet in 5.0 mL of 1X TE and divide the suspension into

five aliquots of 1.0 mL in sterile 1.5 mL microfuge tubes. Each aliquot contains approximately 10^9 cells.

13. Pellet the fixed cells by centrifugation at 10000 xg for 2 minutes at 4 °C.
14. Remove the supernatant with an elongated Pasteur pipette.
15. Proceed immediately with Hi-C library preparation or flash freeze the cells in liquid nitrogen. Frozen pellets can be stored at -80 °C.

Saccharolobus solfataricus

1. Heat up a 15 mL conical tube to 80 ºC in a Thermomixer.
2. Transfer an aliquot of a *Saccharolobus solfataricus* culture equivalent to ~5.0x10^9 cells to the conical tube at 80 ºC.
3. Add 37% formaldehyde solution to the culture to a final concentration of 1%. This corresponds to 28.8 µL of 37% formaldehyde per mL of culture. Mix the reaction by inversion and return the conical tube to the Thermomixer.
4. Change the set temperature of the Thermomixer to 75 ºC and allow the fixation reaction to cool to 75 ºC. This takes approximately 6 minutes with the Eppendorf Thermomixer® C.
5. Add 2.5 M glycine to the fixation reaction to a final concentration of 0.125 M. This corresponds to 54.1 µL of 2.5 M glycine per mL of culture. Mix well by inversion and incubate the reaction at 4 ºC on a roller bench at 30 rpm for 15 minutes.
6. Collect the cells by centrifugation at 3000 xg for 5 minutes at 4 ºC.
7. Discard the supernatant and resuspend the cell pellet in 10.0 mL of 1X TE to wash.
8. Centrifuge the cells at 3000 xg for 5 minutes at 4 °C.
9. Pour out the supernatant and carefully remove the rest with a micropipette. Resuspend the cell pellet in 5.0 mL of 1X TE and separate the suspension of fixed cells into five 1.0 mL aliquots in low-retention 1.5 mL microfuge tubes.
10. Pellet the fixed cells by centrifugation at 10000 xg for 2 minutes at 4 °C.
11. Remove the supernatant with an elongated Pasteur pipette.
12. Use the fixed cells immediately for Hi-C library preparation, or, flash freeze the pellets in liquid nitrogen and store the cells at -80 °C.

Haloquadratum walsbyi

1. Transfer 25 mL of a *Haloquadratum walsbyi* culture into a 50 mL conical tube.

2. Collect the cells by centrifugation at 2300 xg for 20 minutes at 25 ºC.
3. Decant the supernatant and gently resuspend the cell pellet in 1.0 mL of DSMZ medium 1091 for *H. walsbyi*. Vigorous pipetting or vortexing will rupture the cells.
4. Transfer the cell suspension into a 1.5 mL low retention microfuge tube containing 156.3 µL of 37% formaldehyde (final concentration: 5% formaldehyde). Mix by pipetting and incubate the fixation reaction in a Thermomixer at 25 ºC for 3 hours.
5. Dilute 300 mL of 10X *H. walsbyi* buffer in 2700 mL of MiliQ water (final concentration: 1X *H. walsbyi* buffer).
6. Pour out 1.5 L of 1X *H. walsbyi* dialysis buffer into each of two 2 L beakers. Cover one beaker with cling film or aluminium foil to be used at step 10 of this section.
7. Cut a ~5 cm strip of a dialysis tubing with a 3.5 kDa molecular weight cut-off and soak it in 1X *H. walsbyi* dialysis buffer for 20 seconds. Fold 1 cm of one end of the tubing onto itself and clamp the fold. Using a micropipette, fill the tubing with 1X dialysis buffer from the open end and verify that the clamped end does not leak. Leakages can be addressed by tightening the clamps using rubber bands.
8. Empty the dialysis tubing and pipette in the suspension of fixed *H. walsbyi* cells. Fold the open end of the tubing onto itself and clamp the fold. Use rubber bands if necessary. Place the tubing in the dialysis buffer and cover the beaker with cling film or aluminium foil.
9. Dialyse the fixed cells for 1 hour at 25 ºC with stirring. Check the set-up every 15 minutes for leakages. A well-clamped set-up will swell during dialysis.
10. Quickly transfer the dialysis tube to the second beaker of 1X *H. walsbyi* dialysis buffer set aside earlier (step 6 of this section). Cover the set-up with cling film or aluminium foil and let the dialysis continue overnight at 25 ºC with stirring.
11. Transfer the contents of the tubing into a 1.5 mL low-retention microfuge tube.
12. Pellet the fixed cells by centrifugation at 10000 xg for 2 minutes at 4 ºC.
13. Remove the supernatant with an elongated Pasteur pipette and proceed immediately with Hi-C library preparation.
14. Optional: Wash the dialysis tubing thoroughly with MiliQ water and store it in 20% ethanol. The tubing can be reused for the dialysis of fixed *H.*

walsbyi cells as described from steps 7 to 11 of this section following thorough washing with MiliQ water and 1X *H. walsbyi* buffer.

Hi-C library preparation

Lysis and solubilization: *Escherichia coli*

1. Dilute 0.5 µL of 40,000 U/µL Ready-Lyse Lysozyme (Epicentre) with 4.0 µL of 1X TE and 0.5 µL of 1.0 M NaCl. Place the dilution on ice.
2. Resuspend a pellet of fixed *Escherichia coli* cells in 50.0 µL of 1X TE and add 0.5 µL of the diluted lysozyme (2000 U) to the cell suspension. Mix by pipetting and incubate the tube at 37 ºC in a Thermomixer at 1000 rpm for 15 minutes.
3. Add 2.5 µL of 10% SDS to the lysis reaction and mix by pipetting. Incubate the tube for 15 minutes at 37 °C in a Thermomixer at 1000 rpm.
4. Add 45.0 µL of 1X TE to 5.0 µL of the lysed and solubilised cells. Store the preparation at -20 ºC as 'Chromatin'. This control is used to qualitatively examine the efficiency of cell lysis and solubilisation – (steps 2 and 3 of this section; Figure 2.1). Continue with processing Chromatin at step 20 of Hi-C library preparation.
5. Make up the volume of the lysed and solubilised cells to 53.0 µL with 5.0 µL of 1X TE and continue with Hi-C library preparation (step 6 of Hi-C library preparation).

Lysis and solubilization: *Saccharolobus solfataricus*, and *Haloquadratum walsbyi*

1. Resuspend a pellet of fixed cells in 60.0 µL of 1X TE. Add 25-30 glass beads to the cell suspension and vortex vigorously for 5-7 minutes to shear the cells. Ensure that the beads flow with the cell suspension on the sides of the tube during vortexing.
2. Centrifuge the tube for 5 seconds to spin down the disrupted cells and sediment the glass beads.
3. Add 2.8 µL of 10% SDS to the lysate and mix by briefly vortexing the tube. Incubate the tube for 15 minutes at 37 ºC in a Thermomixer at 1000 rpm.
4. Mix 5.0 µL of the lysed and solubilised cells with 45.0 µL of 1X TE in a 1.5 mL low-retention microfuge tube. Store the preparation at -20 ºC as the Chromatin control. Continue with processing this sample at step 20 of Hi-C library preparation.

5. Transfer 53.0 µL of the lysed and solubilised cells into a 1.5 mL low-retention microfuge tube to continue with Hi-C library preparation (step 6 of Hi-C library preparation).

Restriction digestion

6. Add 136.0 µL of nuclease-free water, 25.0 µL of 10% Triton X-100, and 25.0 µL of 10X Buffer B (ThermoFisher Scientific) to 53.0 µL of lysed and solubilised cells (step 5 of Hi-C library preparation).
7. Mix by inversion and incubate the sample at 37 °C for 10 minutes in a Thermomixer at 1000 rpm to sequester SDS in the cell lysate with Triton X-100.
8. Add 10.0 µL of 10 U/µL PsuI (ThermoFisher Scientific) *(see note 2)* to the digestion mix and incubate the reaction at 37 °C for 3 hours at 450 rpm.

Fill-in with biotin-labelled nucleotides

9. To 250 µL of the digestion reaction, add 3.0 µL of 1.0 mM dTTP, 3.0 µL of 1.0 mM dGTP, 3.0 µL of 1.0 mM dCTP, 7.5 µL of 0.4 mM biotin-14-dATP, 1.5 µL of 20 mg/mL BSA, 26.0 µL of nuclease-free water, and 6.0 µL of 5 U/µL DNA Polymerase I, Large (Klenow) fragment (*see note 3*). Mix well by pipetting.
10. Incubate the reaction for 45 minutes at 25 °C in a Thermomixer at 450 rpm.
11. Add 15.8 µL of 10% SDS to the fill-in reaction to a final concentration of 0.5%. Incubate the sample for 20 minutes at 25 °C in a Thermomixer at 1000 rpm to terminate the reaction. Avoid inactivation at higher temperatures to minimize premature reverse cross-linking of the chromatin fragments.

Fractionation:

12. Centrifuge the digested, biotin-labelled chromatin at 25000 xg for 1 hour at 4 °C.
13. Carefully remove the supernatant with an elongated Pasteur pipette and resuspend the gel-like pellet in 200.0 µL of nuclease-free water by vigorous pipetting and vortexing.
14. Use 1.0 µL of the sample to determine DNA concentration using the Qubit® dsDNA HS Assay Kit. Proceed with Hi-C library preparation if at least 1300 ng of digested chromatin is available.

15. Keep a 200-500 ng aliquot aside as the R+ control to visualize the efficiency of restriction digestion (Figure 2.1). Make up the volume of the control to 50.0 µL using 1X TE and store it at -20 °C. Continue with processing the R+ control at step 20 of Hi-C library preparation.

Ligation:

16. Adjust the volume of an aliquot of digested chromatin containing between 1.0-3.0 µg of DNA to 895 µL with nuclease-free water.
17. Add 100.0 µL of 10X T4 DNA Ligase Buffer, 5.0 µL of 20 mg/mL BSA, and 2.0 µL of 2000 U/µL T4 DNA Ligase to the sample and mix well by inversion. Multiple reactions can be prepared to use up all the fractionated chromatin.
18. Incubate the reaction(s) in a Thermomixer for 16 hours at 16 °C at 0 rpm, and for 1 hour at 25 °C at 450 rpm.
19. Terminate the ligation reaction(s) with 20.5 µL of 0.5 M EDTA.

Reverse cross-linking and DNA purification

20. Add 16.6 µL of 10 mg/mL RNase A to the ligation reaction(s). For Chromatin and R+ (steps 4 and 15 of Hi-C library preparation), use 0.8 µL of 10 mg/mL RNase A. Mix by pipetting and incubate the samples for 30 minutes at 37 °C in a Thermomixer at 450 rpm.
21. Add 12.5 µL of 20 mg/mL Proteinase K and 120.8 µL of 5.0 M NaCl to the ligation reaction(s). Use 0.64 µL of 20 mg/mL Proteinase K and 6.2 µL of 5.0 M NaCl for Chromatin and R+ (steps 4 and 15 of Hi-C library preparation). Incubate the samples for 16 hours at 65 °C in a Thermomixer at 450 rpm.
22. Transfer the ligation sample into a 15 mL conical tube. Pool corresponding samples if applicable (step 17 of Hi-C library preparation).
23. Add 1.0 volume of 25:24:1 phenol:chloroform:isoamyl alcohol to ligation sample and controls *(see note 4)*. Vortex vigorously for 10 seconds.
24. Centrifuge the samples for 10 minutes at 13000 xg at 4 °C.
25. Transfer the top aqueous layer into sterile 1.5 mL or 15 mL tubes, as necessary *(see note 4)*.
26. Repeat steps 23 to 25 of Hi-C library preparation.
27. Add 1.0 volume of chloroform to the ligation sample and controls and vortex vigorously for 10 seconds *(see note 4)*.
28. Centrifuge the tubes for 10 minutes at 13000 xg at 4 °C.

29. Transfer the top aqueous layer into sterile 1.5 mL or 15 mL tubes, as necessary *(see note 4)*.
30. Add 0.1 volumes of 1.0 M sodium acetate, pH 8.0, 0.025 volumes of 5.0 mg/mL glycogen, and 2.5 volumes of cold 100% ethanol to the ligation sample and controls. Vortex well and incubate the solutions overnight at -20 °C.
31. Centrifuge the solutions for 20 minutes at 25000 xg at 4 ºC.
32. Remove the supernatant with an elongated Pasteur pipette. A white pellet should be visible at the base of the tubes.
33. Add 500 µL of cold 70% ethanol to the tubes to wash the pellets.
34. Centrifuge the tubes at 25000 xg for 5 minutes at 4 °C.
35. Remove the supernatant with an elongated Pasteur pipette.
36. Repeat steps 33 to 35 of Hi-C library preparation.
37. Dry the pellets in a 50 °C incubator.
38. Dissolve each pellet in 31.0 µL of nuclease-free water in a Thermomixer at 60 ºC for 20 minutes at 1000 rpm.
39. Use 1.0 µL of the controls and the purified ligation sample, hereafter referred to as the Hi-C library, to determine DNA concentration using the Qubit® dsDNA HS Assay Kit.
40. Visualize between 100 and 500 ng of each sample on a 1.5% Agarose gel to qualitatively assess Hi-C library preparation. Typically, the Chromatin migrates as a heavy >10 kb band on a 1.5% agarose gel, R+ runs as a smear of digestion products, and a well-ligated Hi-C library shows a shift of the R+ profile to higher molecular weights (Figure 2.1). Ensure that at least 1.0 µg of the Hi-C library is available for the next steps of the protocol.

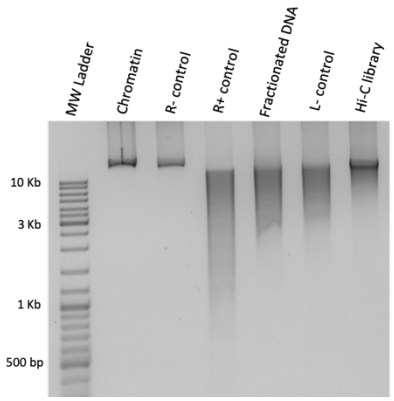

Figure 2.1: Typical results obtained for Hi-C library preparation. Chromatin: Chromatin extracted from fixed cells during lysis and solubilization runs as a high molecular weight band >10 kb. **R- control:** Chromatin that is incubated in the restriction digestion mix for three hours in the absence of

a restriction enzyme should appear as a high molecular weight band comparable to the Chromatin. A smear in the R- control indicates that the chromatin undergoes degradation during restriction digestion. **R+ control:** Chromatin digested with a 6-cutter should run as a smear with a bulk of the fragments having a size > 2 kb. **Fractionated DNA:** Fractionation pellets DNA that is cross-linked to protein (16). 'Free floating' DNA fragments that may contribute to random ligation and self-circularized products are eliminated. This step may be associated with a loss of shorter fragments. **L- control:** Fractionated chromatin in the ligation mix in the absence of T4 DNA Ligase should run on the agarose gel with a profile comparable to fractionated DNA. Degradation will be observed as a shift of the profile towards shorter DNA lengths. **Hi-C library:** A well-ligated Hi-C library shows a shift of the DNA profile towards high molecular weights.

Removal of biotin-labelled nucleotides from unligated restriction fragment ends

41. To 30.0 µL of the Hi-C library, add 8.75 µL of nuclease-free water, 5.0 µL of 10X NEBuffer 2.1, 5.0 µL of 1 mM dGTP, 0.25 µL of 20 mg/mL BSA, and 1.0 µL of 3 U/µL T4 DNA Polymerase. Mix by pipetting and incubate the reaction at 16 °C for 3 hours in a Thermomixer at 450 rpm (*see note 6*).

Hi-C library purification

42. Add 1.0 volume of 25:24:1 phenol:chloroform:isoamyl alcohol to Hi-C library and vigorously vortex the tube for 10 seconds *(see note 4)*.
43. Centrifuge the tube at 13000 xg for 10 minutes at 4 °C.
44. Transfer the top aqueous layer into a low-retention 1.5 mL microfuge tube *(see note 4)*.
45. Add 0.1 volumes of 3.0 M NaOAc pH 5.6, 0.025 volumes of 5.0 mg/mL glycogen, and 2.5 volumes of cold 100% ethanol. Mix well by vortexing and incubate the solution at -20 °C for at least 1 hour.
46. Centrifuge the solution for 20 minutes at 25000 xg at 4 °C.
47. Remove the supernatant with an elongated Pasteur pipette. A white pellet should be visible at the base of the tube.
48. Add 500 µL of cold 70% ethanol to the microfuge tube to wash the pellet.
49. Centrifuge the tube at 25000 xg for 5 minutes at 4 °C.
50. Remove the supernatant with an elongated Pasteur pipette.
51. Dry the pellet in a 50 °C incubator.
52. Dissolve the pellet in 21.0 µL of nuclease-free water in a Thermomixer at 60 °C for 20 minutes at 1000 rpm.
53. Use 1.0 µL of the library to determine DNA concentration using the Qubit® dsDNA HS Assay Kit. Ensure that at least 750 ng of the Hi-C library is available for NGS Library preparation.

NGS library preparation

1. Remove glycogen and excess salt from the Hi-C library by adding 60.0 µL of KAPA HyperPure beads with 20.0 µL of the Hi-C library in a low-retention 1.5 mL microfuge tube. Vortex to mix. Incubate the sample at 25 ºC in a Thermomixer for 5 minutes.
2. Wash the beads according to the manufacturer's instructions and elute the Hi-C library in 36.0 µL of 10 mM Tris, pH 8.0 (*see note 7*).
3. Use 1.0 µL of the eluted Hi-C library to determine DNA concentration using the Qubit® dsDNA HS Assay Kit. Proceed to the next step if more than 500 ng of the Hi-C library is available *(see note 8)*.
4. Add 5.0 µL of 10X KAPA Frag buffer and 10.0 µL of the KAPA Frag enzyme to the Hi-C library on ice. Vortex gently to mix and incubate the tube in a Thermomixer set at 37 ºC for 45 minutes.
5. Transfer the fragmentation reaction to ice and set the temperature of the Thermomixer to 65 ºC.
6. Mix 7.0 µL of the End Repair and A-Tailing Buffer and 3.0 µL of the End Repair and A-Tailing Enzyme Mix on ice (supplied with the KAPA HyperPlus kit). Transfer the mix (10.0 µL) to the fragmentation reaction and vortex gently.
7. Place the reaction in the Thermomixer at 65 ºC for 30 minutes.
8. Transfer the reaction to ice and set the temperature of the Thermomixer to 20 ºC.
9. Add the following to 60.0 µL of end repaired and A-tailed DNA: 5.0 µL of 15 µM Illumina® paired-end sequencing adapter (barcoded), 5.0 µL of nuclease-free water, 30.0 µL of ligation buffer (KAPA HyperPlus), and 10.0 µL of DNA Ligase (KAPA HyperPlus). To sequence several Hi-C libraries in a single Illumina® lane, ligate each library to a different sequencing adapter.
10. Vortex gently and incubate the reaction at 20 °C for 2 hours. Continue processing this sample at step 14 of NGS library preparation.
11. Transfer 20.0 µL of Dynabeads® MyOne™ Streptavidin T1 into a low-retention microfuge tube. Collect the beads with a magnetic rack and carefully pipette off the supernatant.
12. Re-suspend the beads in 50.0 µL of 1X BWB by vortexing. Place the beads back on the magnetic rack until the solution clears and pipette off the supernatant. Perform this step twice.
13. Re-suspend the beads in 50.0 µL of 2X BWB by vortexing.

14. Clean-up the adapter-ligated Hi-C library (steps 9 and 10 of NGS library preparation) with 220.0 µL of KAPA HyperPure beads. Wash the beads according to the manufacturer's instructions and elute the library in 50.0 µL of nuclease-free water.
15. Mix the eluted library with 50.0 µL of Dynabeads® MyOne™ Streptavidin T1 in 2X BWB (step 13 of NGS library preparation). Incubate the sample for 45 minutes at 37 °C in a ThermoMixer at 500 rpm.
16. Collect the beads (now coupled to the adapter-ligated Hi-C library) using a magnetic rack and pipette off the supernatant.
17. Re-suspend the beads in 100.0 µL of 10 mM Tris pH 8.0 + 0.1% Tween 20 by vortexing. Collect the beads with the magnetic rack and remove the supernatant. Perform this step three times.
18. Re-suspend the beads in 20.0 µL of 10 mM Tris pH 8.0 + 0.1% Tween 20 and transfer the suspension into a PCR tube.
19. To the adapter-ligated Hi-C library on beads, add: 25.0 µL of 2X KAPA HiFi HotStart ReadyMix, and 5.0 µL of 10X Library Amplification Primer Mix as supplied with the KAPA HyperPlus kit. Run the following program in a thermocycler to amplify the library:

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98 °C	45 seconds	1
Denaturation	98 °C	15 seconds	6–8 (*see note 9*)
Annealing	60 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	1 minute	1
Hold	4 °C	∞	1

20. Collect the beads with a magnetic rack and transfer the supernatant (hereafter referred to as the NGS library) into a 1.5 mL low-retention microfuge tube. Place the tube on ice until processing at step 23 of NGS library preparation.
21. Re-suspend the beads in 100.0 µL of 10 mM Tris pH 8.0 + 0.1% Tween 20 by vortexing. Place the tube on a magnetic rack and pipette off the supernatant. Perform this step three times.
22. Re-suspend the beads in 20.0 µL of 10 mM Tris pH 8.0 + 0.1% Tween 20 and store the sample at 4 °C. This adapter-ligated Hi-C library on beads can be re-used on a later date to prepare the NGS library as outlined in step 19 of NGS library preparation.

23. Clean-up the NGS library (step 20 of NGS library preparation) with 100.0 µL of KAPA HyperPure beads and elute it in 20.0 µL of nuclease-free water (*see note 10*).
24. Add 10.0 µL of KAPA HyperPure beads to the eluted library. Vortex to mix and incubate the suspension at 25 °C in a Thermomixer for 5 minutes. *(see notes 7 and 10)*.
25. Place the tube on a magnetic rack and transfer the supernatant to a fresh low-retention microfuge tube. Discard the beads *(see note 10)*.
26. Purify the supernatant with 60.0 µL of KAPA HyperPure beads and elute the size-selected NGS library in 11.0 µL of nuclease-free water.
27. Use 1.0 µL of the NGS library to determine DNA concentration using the Qubit® dsDNA HS Assay Kit. The concentration of the library should be between 5 - 20 ng/µL.
28. Assess the quality of the NGS library by determining the size distribution of the DNA fragments using a Tapestation or Bioanalyzer (Agilent Technologies), or by agarose gel electrophoresis (*see note 11*) (Figure 2.2). 1.0-2.0 µL of the NGS library is sufficient for this purpose.
29. Libraries that are marked with distinct Illumina® paired-end sequencing adapters can be pooled for sequencing. The ratio at which the libraries are pooled determines the fraction of total reads available for each library.

Data analysis and generation of contact maps.

Reads from Illumina® paired-end sequencing of a Hi-C library are mapped to restriction fragments of the reference genome. Paired reads that correspond to self-circularized DNA molecules, unligated restriction fragments *(see note 6)*, and duplicates from PCR amplification of the adapter-ligated library on beads (step 19, NGS library preparation) are filtered out, and each valid junction is assigned to a pair of 'bins' by the midpoint coordinate of its corresponding restriction fragments. In this context, a 'bin' refers to a segment of a computationally partitioned genomic sequence that spans between one to several Kbs, for instance, the 4.64 Mb chromosome of *E. coli* can be partitioned into 464 bins spanning 10 kb. The interaction data is contained in symmetric square matrix where every value represents the interaction frequency between a pair of bins. The final, normalised contact map is obtained after balancing the matrix by equalising the sum of all its rows and columns. Detailed methods for processing Hi-C data are available in (15).

The resolution of a Hi-C contact map is determined by its bin size. Smaller bins increase the resolution of the map at the cost of a decreased signal-to-noise ratio. Thus, the selected bin size is a trade-off between the two factors and is heavily influenced by read depth. In our experience, 15 million valid junction reads are sufficient to generate a normalized contact map of the *E. coli* chromosome with a resolution of 5 kb.

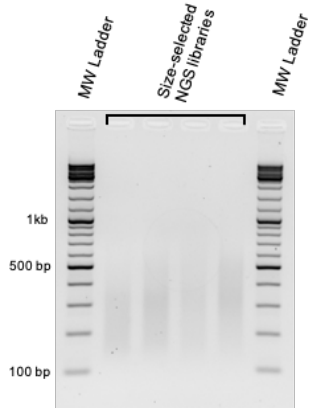

Figure 2.2: Typical run of a size-selected NGS library on a 2% agarose gel. A size-selected NGS library runs as a smear of DNA fragments between 150 – 600 bp.

Notes

1. 2.5 M Glycine should be stored at 20-25 °C. Glycine crystallises out of solution at lower temperatures.
2. Restriction enzymes define the resolution of the Hi-C library. The enzyme is selected based on the criterion that it produces a non-degenerate 5' overhang upon cleavage of its restriction site. This is necessary as 3' overhangs cannot be filled in, and degenerate sticky ends may interfere with biotin tagging. It is necessary that the enzyme functions optimally at 37 °C (or at an even lower temperature; for instance, the optimal temperature for CviAII activity is 25 °C) as restriction digestion at higher temperatures leads to premature reverse cross-linking of DNA-protein complexes. To ensure a good digestion efficiency during library preparation, it is important that the activity of the selected enzyme is not blocked by DNA methylation. The size distribution of the predicted restriction fragments generated by the enzyme should also be evaluated to ensure that the enzyme generates limited, if any, restriction fragments with sizes greater than the chosen Hi-C map resolution; long restriction fragments introduce biases in the contact matrix.

3. During the preparation of Hi-C libraries, restriction fragment overhangs are filled in with biotin-labelled nucleotides to tag ligation junctions generated during the subsequent proximity ligation step. Ensure that the fill-in of the 5' overhang generated by restriction digestion allows the incorporation of the selected biotin-labelled nucleotide. For instance, do not use biotin-14-dATP to fill-in overhangs generated by HpaII (C^CGG).
4. 25:24:1 phenol:chloroform:isoamyl alcohol and chloroform are neurotoxic organic solvents. Perform all steps requiring these solvents under a fume hood with necessary personal protective equipment.
5. Ensure that the *E. coli* cells are completely resuspended in 1X PBS prior to the addition of methanol. In an 80% methanol solution, even vigorous pipetting and vortexing only break a bacterial pellet into large clumps.
6. Some chromatin fragments may have been filled in with biotin-labelled nucleotides but may not have undergone ligation to give a chimeric product. Unligated 'dangling ends' that are bound by streptavidin-coated magnetic beads and thereafter sequenced reduce the number of useful reads per sequencing run. Biotin removal from such ends is performed with T4 DNA Polymerase under conditions that favor the 3' exonuclease activity of the enzyme in removing nucleotides past blunted ends. This condition is provided by maintaining the concentration of selected free nucleotides in the reaction mix below 100 µM. For instance, for the removal of biotin-14-dATP from the dangling ends of PsuI- or BglII-digested Hi-C libraries (RGA*TC), 100 µM of dGTP is added to the reaction mix, whereas dATP, dCTP, and dTTP are not (step 41 of Hi-C library preparation). This way, nucleotides up to but excluding G will be removed as a result of the activation of the 5' polymerase function of the enzyme at that site. The 3' exonuclease activity of T4 DNA Polymerase is also favored by increasing enzyme concentration, extending reaction times, and incubation at higher temperatures, for instance, 16 °C – as opposed to 12 °C – to favor the breathing of DNA ends.
7. Solid Phase Reverse Immobilization (SPRI) beads reversibly bind DNA fragments in solution in the presence of polyethylene glycol (PEG). The immobilization is size selective and depends on the concentration of PEG in the DNA-bead solution. Since PEG is present in the SPRI beads storage buffer (as supplied by the manufacturer), the size selective immobilization of DNA onto the SPRI beads is determined by the volumetric ratio rather than the concentration ratio of beads to DNA. Short DNA fragments can only be immobilized at high PEG concentrations. Therefore, to remove glycogen and excess salts from the HiC library for NGS library preparation, a 3.0x volumetric

ratio of beads to DNA solution is used (step 1 of NGS library preparation). Larger DNA fragments bind to the beads at lower PEG concentrations. Thus, a 0.5x volumetric ratio of beads to DNA solution is used to separate long DNA fragments from the sequencing library during NGS library preparation (step 24 of NGS library preparation).

8. It is important to ensure that >750 ng of a purified Hi-C library is available to begin NGS library preparation. Depending on the restriction enzyme used for Hi-C, and the efficiencies of restriction digestion, biotin-labelled nucleotide fill-in, ligation, removal of biotin labels from unligated restriction fragment ends and coupling to magnetic beads, a significant fraction of the starting material is lost during the enrichment of biotin-labelled ligation junctions with streptavidin-coated magnetic beads (steps 15 and 16 of NGS library preparation). Hence, the effective quantity of the library is much lower. Small quantities of starting material increase the number of PCR cycles that are necessary to sufficiently amplify the library for Illumina® sequencing, hence, increasing the fraction of duplicate reads in the NGS dataset. While duplicates are filtered out during processing, the fraction of 'useful' Hi-C reads is minimized.
9. These are the recommended number of cycles for 750 – 500 ng of input DNA as measured in step 3 of NGS library preparation.
10. 2.0x SPRI-based clean-up of the adapter-ligated Hi-C library amplification reaction purifies fragments up to several kbs in length. Fragments longer than 600 bp interfere with cluster formation during NGS and reduce the quality of the sequencing run. These fragments are removed from the library with a 0.5x SPRI clean-up. This purification is performed in two steps as opposed to a single 0.5x SPRI clean-up to eliminate the interference of isostabilisers present in the 2X KAPA HiFi HotStart Ready Mix on the immobilization of DNA on beads.
11. On a 2% agarose gel, a purified, size-selected NGS library runs as a smear between 150 – 600 bp (Figure 2.2). A 'laddered' pattern in the smear or a strong DNA signal at ~120 bp indicate the presence of PCR duplicates and adapter dimers, respectively, both of which consume a significant proportion of the read depth and reduce the quality of the sequencing run.

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