Generating High-Resolution Hi-C Contact Maps of Bacteria

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Generation of high-resolution Hi-C contact maps in bacteria

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Summary

During the past decade, Chromosome Conformation Capture (3C/Hi-C)-based methods have been used to probe the 3D structure and organization of bacterial genomes, revealing fundamental aspects of chromosome dynamics. However, the current protocols are expensive, inefficient, and limited in their resolution. Here we present a simple, cost-effective Hi-C approach that is readily applicable to a range of Gram-positive and Gram-negative bacteria.

Key words

Chromosome conformation capture, Hi-C, 3C, Genome organization, Bacteria, Prokaryotes

1. Introduction

Hi-C relies on the proximity ligation of DNA fragments held together by cellular proteins and once processed, yields a snapshot of the 3D organization of the chromosome. The protocol comprises of six basic steps: 1) Cells are crosslinked with a chemical fixative to covalently link chromosomal regions that are in close proximity; 2) Following lysis, chromosomal DNA is fragmented by a frequently cutting restriction enzyme; 3) DNA overhangs are then filled in, with the incorporation of a biotinylated nucleotide; 4) The DNA is then subject to blunt-end ligation under dilute conditions to favor intra-molecular ligation; 5) Following reverse crosslinking, the DNA is purified and the biotinylated chimeric DNA molecules are enriched by streptavidin pull-down. 6) Finally, the Hi-C libraries are prepared for paired-end sequencing.

Despite several key improvements to eukaryotic Hi-C protocols during the past few years, including the development of commercial kits for non-specialists, the bacterial Hi-C protocol has remained largely unchanged. This means that prokaryotic Hi-C contact maps are still significantly limited in terms of the resolution they can offer. In this chapter, we have improved upon this by dissecting the different steps of published eukaryotic and prokaryotic Hi-C protocols, we identified several crucial areas where relatively simple modifications generated remarkably improved bacterial Hi-C contact maps and we used this information to generate an optimized Hi-C protocol (Figure 1). The protocol is suitable for a range of Gram-positive and Gram-negative bacteria and is readily applied to other organisms such as archaea. The resulting prokaryotic Hi-C experiments are much more cost and time-effective compared to their predecessors but most importantly facilitate contact matrices that can be resolved at higher resolutions, revealing new features of the bacterial chromosome architecture (Figure 1).

2. Materials

2.1 Equipment

1. Thermomixer
2. PCR machine
3. 65°C incubator
4. Variable temperature incubator (30 - 37°C)
5. Refrigerated centrifuge (for 50 ml falcon tubes)
6. Refrigerated centrifuge for 1.5 ml – 5ml microcentrifuge tubes.
7. Precellys Evolution tissue homogenizer (Ozyme)
8. Precellys Cryolys cooling attachment (optional)
9. Gel electrophoresis tank
10. Qubit Fluorometer
11. Magnet Rack for 1.5 ml microcentrifuge tubes
12. Tube rotator
13. Magnetic stirrer
14. S220 Focused-Ultrasonicator (Covaris)

2.2 Consumables for Hi-C library preparation

1. 50 ml disposable conical tubes
2. 0.22 µm filtration unit
3. 1.5 ml and 5 ml microcentrifuge tubes
4. Restriction enzyme and corresponding restriction enzyme buffer **(**see **Note 1)**
5. 30 U/µl T4 DNA ligase (Weiss units, Thermo Fisher Scientific)
6. 20 mg/ml Proteinase K in water (Eurobio)
7. 10 mg/ml DNase-free RNase A in water (Euromedex)
8. 37% Formaldehyde solution (w/v) (Sigma-Aldrich)

9. 2.5 M glycine (Filter sterilized)
10. 10% sodium dodecyl sulphate (w/v, SDS) solution (Thermo Fisher Scientific)
11. 10% Triton X-100 in water (Thermo Fisher Scientific)
12. 10x Ligation Buffer (-ATP): 500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT
13. 10 mg/ml bovine serum albumin (BSA, Sigma-Aldrich)
14. 100 mM ATP (pH 7.0, Sigma-Aldrich)
15. 500 mM EDTA (pH 8.0, Thermo Fisher Scientific)
16. 3 M sodium acetate (pH 5.2)
17. 10:9:1 phenol:chloroform:isoamylalcohol (pH 8.2, Interchim)
18. 100% ethanol (Thermo Fisher Scientific)
19. 70% ethanol (VWR international)
20. 1x TE buffer (pH 8.0)
21. cOmplete protease inhibitor cocktail (EDTA-free, Sigma-Aldrich)
22. 1x PBS
23. 2 ml Precellys tubes containing 0.5 mm glass beads (VK05, Ozyme)
24. Milli-Q water
25. Ultrapure Agarose (Thermo Fisher Scientific)
26. 10 mM dAGTP
27. Biotin-14-dCTP (Thermo Fisher Scientific)
28. DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs)

2.3 Consumables for sequencing library preparation

1. 1.5 ml microcentrifuge tubes
2. Milli-Q water
3. Agencourt Ampure XP beads (Beckman)

4. 70% ethanol (VWR International)
5. 1x Tween Wash Buffer (TWB): 5 mM Tris-HCl pH7.5, 0.5 mM EDTA, 1M NaCl, 0.05% Tween
6. 2x Binding Buffer (BB): 10 mM Tris-HCl pH7.5, 1 mM EDTA, 2 M NaCl
7. 1x NEBuffer 2 (New England Biolabs)
8. Phusion 2x High Fidelity Master Mix (Thermo Fisher Scientific)
9. 1x T4 DNA ligase buffer (New England Biolabs)
10. T4 Polynucleotide Kinase (PNK, New England Biolabs)
11. T4 DNA polymerase (New England Biolabs)
12. Klenow Fragment (3'-5' exo-, New England Biolabs)
13. DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs)
14. 10mM dNTPs
15. 10mM dATP
16. Quick Ligation Kit (New England Biolabs)
17. Ultrapure Agarose (Thermo Fisher Scientific)
18. Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific)
19. Qubit dsDNA HS assay kit (Thermo Fisher Scientific)
20. microTUBE AFA Fiber Pre-Slit Snap-Cap (Covaris)

3. Methods

3.1 Generation of a bacterial Hi-C library

This protocol facilitates the generation of a Hi-C library and the subsequent sequencing library within two days. However, there are several steps where the library can be stored at -20°C or incubations extended and these are indicated throughout the protocol. We recommend that users initially prepare no more than 4 libraries at a

time, but the protocol can easily be scaled up once users become familiar with the process. Data analysis and methods for generating Hi-C contact maps have been explained previously[1,2].

3.1.1 Cell fixation

1. Grow bacterial culture in an appropriate medium until you have a total of ~2 x 10^8 cells growing in the exponential growth phase.
2. Chemically crosslink protein-DNA interactions by adding fresh formaldehyde (3% final concentration) for 30 min at room temperature with gentle agitation.
3. Quench formaldehyde by adding glycine (0.5 M final concentration), Incubate for 20 min at room temperature with gentle agitation.
4. Transfer culture to a 50 ml falcon tube and collect cells by centrifugation (4000.x g, 10 min, room temperature).
5. Carefully resuspend the cell pellet in 25 ml of 1x PBS and centrifuge again.
6. Resuspend the pellet in 1 ml of 1x PBS and transfer to a 1.5 ml microcentrifuge tube. Centrifuge again (4000 x g, 5 min, room temperature).
7. Carefully remove supernatant and store the cell pellet at -80°C. Pellets can be stored for approx. 12 months.

3.1.2 Hi-C library construction

1. Remove the cell pellet from the -80°C freezer and thaw on ice.
2. Resuspend the pellet in 1.2ml of 1x TE + cOmplete protease inhibitor cocktail and transfer to a 2 ml Precellys tube containing 0.5 mm glass beads.
3. Mechanically disrupt cells using the Precellys Evolution tissue homogenizer (V7500: 5 x 30s, 20s pause **(**see **Note 2)**.
4. Carefully transfer the lyase (~1 ml in volume) to a 5 ml microcentrifuge tube, avoid transferring any of the glass beads.

5. Add 50 µl of 10% SDS solution (0.5% final concentration).
6. Incubate at room temperature for 10 min.
7. Add 3 ml dH_2O, 500 µl 10x Digestion Buffer, and 500 µl 10% Triton-X-100 and mix thoroughly.
8. Remove 400 µl of the sample and transfer it to a 1.5 microcentrifuge tube. This is the non-digested (ND) control.
9. Add 1000U of restriction enzyme to the remaining sample and incubate at 37°C for 3h with gentle agitation.
10. Following digestion, remove 400 µl of the sample and transfer it to a 1.5 microcentrifuge tube. This is the digested (D) control.
11. Centrifuge the remaining sample (16,000 x g, 20 min, room temperature) to pellet the insoluble fraction containing protein-DNA complexes of interest.
12. Remove the supernatant and carefully resuspend the pellet in 400 µl of dH_20.
13. Add the following to the tube: 50 µl 10x Ligation, 4.5 µl 10 mM dAGTTP, 37.5 µl Biotin-14-dCTP, 40 Units of DNA Polymerase I - Large Klenow Fragment.
14. Briefly mix the reaction and incubate at 37°C for 45 min with gentle agitation.
15. Set up the ligation reaction by adding: 120µl 10x Ligation Buffer, 12 µl 10 mg/ml BSA, 12 µl 100mM ATP, 540 µl dH2O, 480 U T4 DNA ligase.
16. Gently mix the reaction and incubate with gentle agitation for 3h at room temperature.

3.1.3 Reverse crosslinking and DNA purification

1. 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. Add 20 µl 500 mM EDTA, 20 µl 10 % SDS, and 10 µl 20mg/ml proteinase K to the ND and D controls.

2. Incubate all samples at 65°C overnight to reverse formaldehyde-mediated protein-DNA crosslinks.
3. Purify DNA by adding an equal volume of Phenol:Chloroform: Isoamyl alcohol to each sample, vortex for 30s, and then centrifuge (12,000 x g, 5 min, room temperature).
4. Carefully remove the upper aqueous phase and transfer to a new microcentrifuge tube.
5. Precipitate DNA by adding 2.5x volume of ice-cold 100% EtOH and 1/10 volume of 3 M NaOAc (pH 5.0).
6. Incubate at -80°C for 30 min and then pellet DNA by centrifugation (12,000 x g, 20 min, 4°C).
7. Carefully remove supernatant and wash pellets with 500 µl 70% EtOH. Centrifuge again (12,000 x g, 5 min, 4°C).
8. Remove EtOH and dry pellets on a 37°C heat block for 5-10 min.
9. Add 140 µl 1X TE buffer + 1mg/ml RNase to pellets and incubate for 30 min at 37°C with agitation.
10. Once the DNA has completely resuspended, run 10 µl of the Hi-C library and 20 µl of the ND and D controls on a 1 % agarose gel **(**see **Note 3)**.
11. Discard ND and D controls.

Safe stopping point: Hi-C library can be stored at -20°C.

3.2 Preparation of Hi-C sequencing libraries

3.2.1 DNA sonication and size selection

1. Transfer 130 µl of DNA to a sonication tube **(**see **Note 4)**.
2. Sonicate DNA to yield a fragment size of ~300 bp **(**see **Notes 3 and 5).**
3. Transfer sheared DNA to a 1.5 ml microcentrifuge tube.

4. Add an equal volume of AmPure XP beads. Mix sample 10x by gentle pipetting.
5. Incubate for 5 min at room temperature to allow DNA fragments to bind to the magnetic beads.
6. Transfer tube to a magnetic rack for 1 min or until the beads have separated to the wall of the tube.
7. Carefully remove the supernatant and wash 2x with 70% EtOH, keeping the tube on the magnet and ensuring that the beads are not disturbed.
8. Airdry pellet for 1 – 5 min to remove residual EtOH.
9. Remove beads from the magnet and resuspend in 320 µl of elution buffer. Mix sample 10x by gentle pipetting.
10. Incubate for 5 min at room temperature and then place back onto the magnetic rack for 1 min.
11. Carefully remove supernatant and transfer to a new 1.5 ml microcentrifuge tube.
12. Check sonication and size-selection by running 18 µl of the DNA on a 1% agarose gel **(**see **Note 4).**
13. The remaining 300 µl is used to prepare the sequencing library.

Safe stopping point: DNA can be stored at -20°C.

3.2.2 Biotin pull-down

1. Thoroughly mix Streptavidin C1 Dynabeads by pipetting.
2. Transfer 30 µl of beads to a 1.5ml microcentrifuge tube.
3. Place the tube on a magnet and wait 1 min to clear the supernatant.
4. Remove the supernatant and then resuspend the beads in 500 µl of 1x Tween Wash Buffer.

5. Place the tube back on the magnet, remove the supernatant and resuspend the beads in 300 µl of 2x Binding Buffer.
6. Add 300 µl of Hi-C samples to the beads and incubate on a tube rotator for 15 min at room temperature.
7. Place the tube back on the magnet and wait 1 min to clear the supernatant.
8. Remove the supernatant and then resuspend the beads in 500 µl of 1x Tween Wash Buffer.
9. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
10. Place the tube back on the magnet and wait 1 min for the supernatant to clear.
11. Repeat steps 8-10.
12. Discard the supernatant, resuspend the beads in 100 µl of 1x T4 ligase buffer and transfer to a new 1.5 ml microcentrifuge tube.

3.2.3 End-repair

1. Prepare the end-repair mix by combining the following: 85 µl 1x T4 ligase buffer, 5 µl 10mM dNTPs, 50 U T4 PNK, 12 U T4 DNA polymerase, 5 U DNA Polymerase I, Large Klenow Fragment.
2. Place the Hi-C library on the magnet and wait 1 min to clear the supernatant.
3. Discard the supernatant and resuspend the beads in the end-repair mix.
4. Incubate for 30 min at room temperature, without agitation.
5. Place the tube on a magnet and wait 1 min to clear the supernatant.
6. Remove the supernatant and then resuspend the beads in 500 µl of 1x Tween Wash Buffer.
7. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
8. Place the tube back on the magnet and wait 1 min for the supernatant to clear.
9. Repeat steps 6-8.

10. Discard the supernatant, resuspend the beads in 100 µl of 1x NEB Buffer 2, and transfer to a new 1.5 ml microcentrifuge tube.

Safe stopping point: DNA can be stored at -20°C.

3.2.4 A-tailing

1. Prepare the A-tailing mix by combining the following: 90 µl 1xNEB 2 buffer, 5 µl 10mM dATP, 25 U Klenow Fragment (3'-5'exo-).
2. Place the Hi-C library on the magnet and wait 1 min to clear the supernatant.
3. Discard the supernatant and resuspend the beads in the A-tailing mix.
4. Incubate for 30 min at 37°C, without agitation.
5. Place the tube on a magnet and wait 1 min to clear the supernatant.
6. Remove the supernatant and then resuspend the beads in 500 µl of 1x Tween Wash Buffer.
7. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
8. Place the tube back on the magnet and wait 1 min for the supernatant to clear.
9. Repeat steps 6-8.
10. Discard the supernatant, resuspend the beads in 50µl of 1x Quick Ligase Buffer, and transfer to a new 1.5 ml microcentrifuge tube.

Adapter Ligation

1. Prepare the Ligation mix by combining the following: (48 µl 1x Quick Ligase Buffer, 2 µl Quick DNA Ligase. **(**see **Note 6).**
2. Place the Hi-C library on the magnet and wait 1 min to clear the supernatant.
3. Discard the supernatant and resuspend the beads in the Ligation mix.
4. Add 2 µl of sequencing adapter and mix by pipetting **(**see **Note 7)**.
5. Incubate at room temperature for 10 min.
6. Place the tube on a magnet and wait 1 min to clear the supernatant.

7. Remove the supernatant and then resuspend the beads in 500 µl of 1x Tween Wash Buffer.
8. Incubate in a thermomixer (2 min, 55°C, 1,000 rpm).
9. Place the tube back on the magnet and wait 1 min for the supernatant to clear.
10. Repeat steps 7-9.
11. Discard the supernatant, resuspend the beads in 50µl of 10mM Tris-HCl (pH 8) and transfer to a new 1.5 ml microcentrifuge tube.

Safe stopping point: beads containing the Hi-C library can be stored at -20°C.

3.2.5 Library amplification by PCR

1. Prepare the PCR mix by combining the following: 40 µl of Phusion 2x High Fidelity Master Mix (New England Biolabs), 5 µl of 2 µM Primer Mix (NEXTflex, Bioo Scientific), 32 µl dH2O.
2. Add 3 µl of the Streptavidin-beads containing the HiC library.
3. Amplify the library for 12 cycles following the manufacturers' instructions.
4. Place the PCR reaction on a magnet and wait 1 min to clear the supernatant.
5. Transfer the supernatant to a new 1.5 ml microcentrifuge tube. Discard the Dynabeads.
6. Purify the PCR reaction by adding an equal volume of AmPure XP beads.
7. Mix sample 10x by gentle pipetting and incubate for 5 min at room temperature to allow DNA fragments to bind to the magnetic beads.
8. Transfer tube to a magnetic rack for 1 min or until the beads have separated to the wall of the tube.
9. Carefully remove the supernatant and wash 2x with 70% EtOH, keeping the tube on the magnet and ensuring that the beads are not disturbed.

10. Airdry pellet for 1- 5 min to remove residual EtOH but don't allow beads to over-dry.
11. Remove beads from the magnet and resuspend in 50 µl of 10 mM Tris-HCl pH 8.
12. Mix sample 10x by gentle pipetting.
11. Incubate for 5 min at room temperature and then place back onto the magnetic rack for 1 min.
12. Carefully remove the supernatant and transfer it to a new 1.5 ml microcentrifuge tube.
13. Check DNA on 1% agarose gel to determine the size of the final library and to check for the presence of primer-dimers **(**see **Note 3)**.
14. Determine DNA concentration using the Qubit dsDNA HS kit on a Qubit Fluorometer.
15. Store the final sequencing library at -20°C.
16. Prepare libraries for paired-end sequencing using the 75 cycle High-Output Kit v2.5. according to Illumina's instructions.

4. Notes

1. The choice of the restriction enzyme and buffer is an important parameter of a Hi-C experiment and can take some optimization. Some enzymes, such as DpnII (^GATC), are incompatible with many bacterial species because of Dam methylation. It is also important to determine the GC content and number of restriction sites within a particular genome, to check whether the required Hi-C resolution can be achieved. Currently, we recommend HpaII (C^CGG) with NEBuffer 1 (10X NEBuffer 1: (200mM Tris-HCl pH 7.5, 100mM MgCl2, 10mM

DTT, 1mg/ml BSA) for most applications but if a genome is particularly AT-rich, or if a specific AT-rich region is under investigation, we recommend using MluCI (^AATT) with the same buffer.

2. For Precellys machines without the Cryolys cooling attachment, the program is: V6700: 9 x 20s, (30s pause) and the tubes need to be removed every 3 cycles and placed on ice for 5 min to stop the sample overheating and any subsequent degradation. Lysis can also be performed with lysozyme and will produce comparable results for Gram-negative bacteria. However, formaldehyde-crosslinked Gram-positive bacteria, such as *B. subtilis*, can be difficult to lyse with lysozyme and in such cases, we recommend either disrupting cells with glass beads or a French Press [3,4]
3. See Figure 2
4. For efficient sonication, a maximum of 5 µg of DNA should be used (as determined by QuBit analysis), if the Hi-C library exceeds this, 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 is adjusted to 130 µl using 1x TE buffer.
5. The sonication parameters detailed in this protocol have been optimized for the amount of material used and the Covaris S220 ultrasonicator. Other sonicators will require additional optimization to ensure the average DNA fragment size is ~300bp.
6. Adapter ligation can also be performed using T4 DNA ligase (New England Biolabs) and performed either at room temperature for 2 hours or overnight at 16°C.

7. Adapters can be interchanged with any Illumina-compatible or custom sequences. We have previously used TruSeq DNA CD Indexes (Illumina) and Nextflex 48 barcodes (Perkin Elmer).

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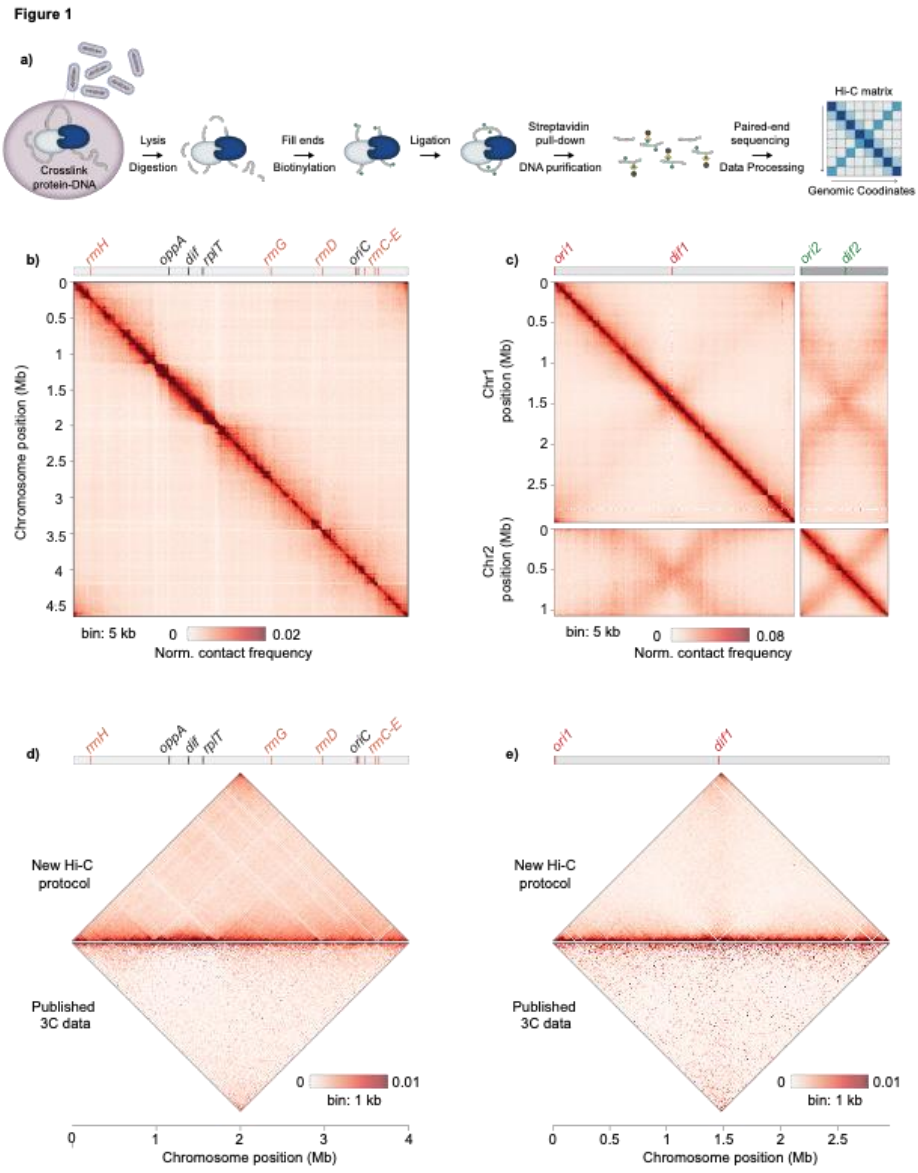

Fig. 1 (a) Overview of the Hi-C protocol. Normalized Hi-C contact maps of asynchronously growing populations of WT bacterial cells. The X and Y axes represent the coordinates of the chromosome and the colorscale reflects the frequency of contacts between two regions of the genome (arbitrary units), from white (rare contacts) to dark red (frequent contacts). Features of interest are indicated along the top axis. (b) Hi-C contact matrix of WT *E. coli* (5 kb bins). (c) Hi-C contact matrix of WT *V. cholera* (5 kb bins). (d) Side-by-side comparison of the *E.coli* contact maps generated using either the new Hi-C or previously published 3C approaches [1] (1 kb bins). (e) Side-by-side comparison of chromosome 1 from *V. cholera* contact maps generated using either the new Hi-C or previously published 3C approaches [5] (1 kb bins).

Figure 2

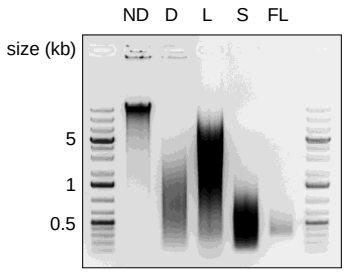

Fig. 2. Typical results obtained for the different steps of the bacterial Hi-C protocol. DNA extracted from fixed cells (ND) is digested with a 4-base cutting restriction enzyme for 3 hours at 37°C (D). The DNA ends are then filled in with a biotinylated nucleotide and blunt-end ligation performed for 3 hours at room temperature (L). Following reverse crosslinking, the DNA is purified, sonicated (S), and then prepared for sequencing using a modified 'on-bead' approach. The size and quality of the final library (FL) are then checked on gel before pair-end sequencing.