Chapter 13

Using Chromosome Conformation Capture Combined with Deep Sequencing (Hi-C) to Study Genome Organization in Bacteria

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Abstract

Genome organization is fundamental to all living organisms. Long DNA molecules are organized in hierarchical orders to be accommodated into eukaryotic nuclei or bacterial cells, which are thousands of folds shorter. Over the past two decades, chromosome conformation capture (3C) techniques substantially advanced our understanding of genome folding inside cells. 3C involves crosslinking and proximity ligation, and quantifies the physical contacts between two DNA regions within the genome. Coupled with high-throughput sequencing, 3C-seq and Hi-C techniques detect genome-wide DNA interactions, providing a comprehensive view of global genome organization. Here, we describe a detailed method to prepare Hi-C libraries using *Bacillus subtilis*, which includes procedures of crosslinking chromatin, digesting the crosslinked genome, labeling DNA ends with biotin, ligating DNA, and preparing the DNA library for sequencing using an Illumina platform.

Key words Hi-C, Chromosome conformation, Capture, Genome organization, Bacteria, *Bacillus subtilis*

1 Introduction

Genome organization plays critical roles in the regulation of gene expression and genome stability. Microscopy-based techniques have documented many architectural features of genomes, such as nucleus compartmentalization, chromatin fibers, and subnuclear positions of various chromosomal loci [1–3]. Complementary methods involving chromosome conformation capture (3C) assays have extend our understanding of genome organization [4]. Through chromatin crosslinking, genome digestion, re-ligation, and quantification of ligation frequency, 3C-based methods measure spatial proximity between DNA sequences and provide an understanding of the organization of a genome.

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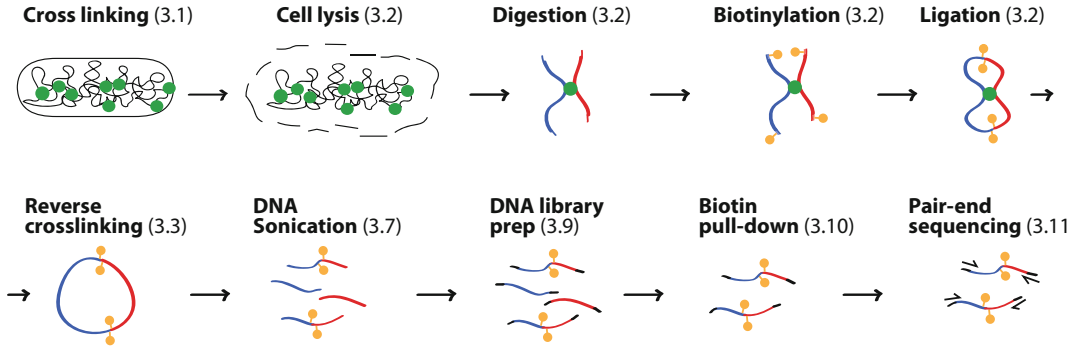

Fig. 1 Overview of the Hi-C experiment. Cells are crosslinked with formaldehyde to maintain protein–DNA and DNA–DNA interactions. After cell lysis, the chromatin is subjected to digestion with a restriction enzyme, resulting in 5′ overhangs which are subsequently filled in with biotinylated nucleotides. Blunt ends are ligated using T4 ligase. After the reversal of crosslinking and protein removal, DNA is purified and further fragmented by sonication. To prepare the DNA library for sequencing, necessary adaptors are ligated to the ends of sheared DNA. Biotinylated junctions are pulled down with streptavidin beads. Finally, the DNA products are amplified by PCR and analyzed by paired-end sequencing. Steps (**3.1**–**3.11**) in the Methods section are indicated above the schematics

While the original 3C method [5] only quantified the contact between two specific regions, when combined with deep sequencing, 3C-based methods can be used to measure the frequency of genome-wide DNA interactions at high resolution. These methods enable the reconstitution of three-dimensional architecture of an entire genome. While deep sequencing can be costly, one method called Hi-C [6] reduces the cost by enriching the ligation products before sequencing. Essentially, Hi-C involves the following steps: DNA fragments in proximity are covalently crosslinked, usually by formaldehyde, to preserve the DNA interactions; after cell lysis, the crosslinked chromatin is digested using restriction enzymes (either 4-bp or 6-bp cutters) to generate 5′ overhangs; protruding ends of the digestion products are filled with biotinylated nucleotides by the Klenow fragment; the blunt ends are ligated using T4 ligase; after the reversal of crosslinking, DNA is further fragmented by sonication; then the biotinylated fragments are enriched by streptavidin pull-down; and finally, the DNA is prepared for paired-end deep sequencing (Fig. 1).

Hi-C studies have greatly advanced our understanding of genome organization across all domains of life. At the local level, genomes are folded into small domains known as topologically associating domains in eukaryotes [7] and chromosomal interaction domains in bacteria [8, 9]. At a higher level, eukaryotic chromosomes are organized in compartments while many bacteria have juxtaposed chromosome arms [6, 8, 10–13]. Furthermore, both eukaryotes and bacteria with multipartite genomes exhibited a plethora of inter-chromosomal interactions [14–17]. We believe that more secrets of genome biology await Hi-C to uncover. In

this chapter, we describe a detailed protocol to prepare a Hi-C library from *Bacillus subtilis*. The method can be adapted to other bacterial species.

2 Materials

2.1 Cell Culture Collection and Fixation

1. Defined rich casein hydrolysate (CH) medium [18].
2. 250 mL 3XD BAF Shake Flask.
3. Waterbath.
4. 50 mL Polypropylene conical centrifuge tube.
5. 15 mL Polypropylene conical centrifuge tube.
6. 37% formaldehyde (*see* **Note 1**).
7. Rocker.
8. 2.5 M glycine.
9. 1× Phosphate-buffered saline (PBS): dissolve 8 g of NaCl, 0.2 g of KCL, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 1 L of ddH_2O. Adjust pH to 7.4 with NaOH. Sterilize by autoclaving.
10. 1.5 mL Eppendorf DNA LoBind microcentrifuge safe-lock tube.

2.2 Cell Lysis, Genome Digestion, Biotin Labeling and Ligation

1. 1× TE buffer: 10 mM Tris, 1 mM EDTA in ddH_2O. Adjust pH to 8.0. Autoclaved.
2. Proteinase inhibitor cocktail.
3. Lysozyme, recommended Ready Lyse (VWR).
4. 10% sodium dodecyl sulfate (SDS).
5. Nuclease-free H_2O.
6. HindIII enzyme.
7. NEB buffer 2.1 (supplied with HindIII).
8. 10% Triton-X.
9. Nuclease-free PCR tubes.
10. 2 mM dGTP.
11. 2 mM dTTP.
12. 2 mM dCTP.
13. 1 mM Biotin-14-dATP (Axxora).
14. DNA Polymerase I, Large (Klenow) Fragment.
15. Thermocycler.
16. 2 mL Eppendorf DNA LoBind safe-lock tube.
17. T4 DNA ligase.

18. T4 DNA ligase reaction buffer.
19. 20 mg/mL Recombinant Albumin (rAlbumin, New England Biolabs).
20. 100 mM ATP.

2.3 Reverse Crosslinking

1. 0.5 M EDTA, pH 8.0.
2. Proteinase K.

2.4 DNA Extraction and RNA Digestion

1. Phenol/Chloroform/Isoamyl Alcohol (PCI, 25:24:1 mixture).
2. 1.7 mL microcentrifuge polypropylene tubes.
3. 3 M NaOAc: dissolve 246.1 g of $NaC_2H_3O_2$ in 800 mL of ddH_2O. Adjust pH to 5.2 with glacial acetic acid and increase volume to 1 L with ddH_2O. Sterilize by autoclaving.
4. 20 mg/mL glycogen.
5. Vortex mixer.
6. 100% ethanol.
7. Vacuum Aspirator.
8. 70% Ethanol.
9. QIAGEN elution buffer (EB).
10. RNaseA.

2.5 Removal of Biotin from Non-ligated Ends

1. 2 mM dATP.
2. T4 DNA polymerase.

2.6 DNA Sonication

1. 0.5 mL sonication tube (BrandTech).
2. Qsonica Q800R2 sonicator (Qsonica).
3. 2.2% agarose gel.

2.7 Cleanup of Fragmented DNA

1. AMPure XP beads (Beckman).
2. 1.5 mL low adhesion microcentrifuge tubes.
3. Magnetic stand.
4. 80% Ethanol.
5. 0.1× TE.

2.8 DNA Library Preparation for Illumina

1. NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs).
2. Tris/NaCl (10 mM Tris-HCl pH 8.0, 10 mM NaCl).
3. NEBNext Multiplex Oligos for Illumina (New England Biolabs).
4. USER enzyme (New England Biolabs).

5. 2× NTB buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 2 M NaCl in ddH_2O.
6. 1× NTB buffer.
7. Dynabeads MyOne Streptavidin C1 beads (Fisher Scientific).
8. Qubit dsDNA HS assay kit (Fisher Scientific).
9. Qubit Assay Tubes (Fisher Scientific).
10. Qubit Fluorometers (Fisher Scientific).

3 Methods

3.1 Cell Culture Collection and Fixation

1. Inoculate cells in 5 mL of CH medium at 22 °C overnight with aeration.
2. The next morning, in a 250 mL flask, set up a subculture of 30 mL by diluting the overnight culture in fresh CH medium to an optical density at 600 nM (OD_{600}) of 0.02.
3. Put the flask in a 37 °C shaking waterbath. Grow cells to mid-exponential-growth phase, to an OD_{600} of ~0.3 (*see* **Note 2**).
4. Transfer 20 mL of the cells to a 50 mL conical centrifuge tube containing 1.8 mL of 37% formaldehyde (3% final concentration). Incubate at room temperature (RT) for 30 min with gentle mixing on a rocker (*see* **Note 3**).
5. Add 1 mL of 2.5 M glycine (125 mM final concentration). Incubate at RT for 5 min with gentle mixing on a rocker.
6. Pellet cells by centrifugation at 12,000 × g for 10 min at 4 °C. Remove the supernatant.
7. Wash cells by resuspending the pellet using 10 mL of ice-cold 1× PBS. Transfer into a 15 mL conical tube. Centrifuge cells again at 12,000 × g for 10 min at 4 °C. Remove the supernatant.
8. Resuspend the pellet in 500 μL of ice-cold 1× PBS.
9. Make a 1:40 dilution (25 μL resuspension in 975 μL of PBS) and measure its OD_{600}. Save aliquots of 1 OD unit of cells (~5×10^8 cells) in 1.5 mL tubes.
10. Freeze cells using liquid nitrogen and store at −80 °C.

3.2 Cell Lysis, Genome Digestion, Biotin Labeling and Ligation

1. Thaw the frozen cells on ice.
2. Centrifuge cells at 21,000 × g for 3 min at 4 °C. Carefully remove the supernatant (*see* **Note 4**).
3. Resuspend the cell pellet in 50 μL of lysis buffer (50 μL of TE + 0.5 μL of proteinase inhibitor cocktail +3 μL of lysozyme) and incubate at RT for 60 min. Pipette the lysate to mix.

4. Add 5 μL of 10% SDS (1% final concentration) and incubate at RT for 30 min (*see* **Note 5**).
5. Transfer 12 μL of the cell lysate into digestion buffer (84 μL of nuclease-free H_2O, 12 μL of NEB buffer 2.1, and 12 μL of 10% triton-X). Mix the reaction well and incubate at RT for 10 min.
6. Add 6 μL of HindIII into the mixture, and incubate at 37 °C for 2 h.
7. Once digestion is finished, cool the reaction down on ice for 5 min (*see* **Note 6**).
8. To label the overhangs of the digested fragments with biotin, in a PCR tube, set up a 120 μL of reaction by mixing the following components: 1.8 μL of 2 mM dGTP, 1.8 μL of 2 mM dTTP, 1.8 μL of 2 mM dCTP, 3.6 μL of 1 mM Biotin-14-dATP, 2.4 μL of DNA Polymerase I, Large (Klenow) Fragment, 11 μL of cold nuclease-free H_2O, and 100 μL of cooled digestion product. Pipette to mix thoroughly.
9. Incubate the reaction at 25 °C for 75 min in a thermocycler.
10. Stop the reaction by adding 6 μL of 10% SDS (0.5% final concentration). Pipette to mix thoroughly. Incubate the reaction at RT for 10 min.
11. Set up a ligation reaction in a 2 mL safe-lock tube on ice. Prepare ligation mixture by mixing the following components: 623 μL of cold nuclease-free H_2O, 80 μL of 10% Triton, 93 μL of ligase buffer, 2.3 μL of 20 mg/mL rAlbumin, and 1.9 μL of 100 mM ATP.
12. Add all SDS-treated digestion products from **Step 10** into the mixture and incubate on ice for 10 min.
13. Add 6 μL of T4 DNA ligase into the mixture and invert to mix.
14. Incubate the tube at 16 °C overnight (*see* **Note 7**).

3.3 Reverse Crosslinking

1. After the overnight ligation, add 20 μL of 0.5 M EDTA to inactivate the enzymes (~10 mM final concentration) and invert the tube to mix.
2. Add 10 μL proteinase K and invert the tube to mix.
3. Add 20 μL of 10% SDS and invert the tube to mix.
4. Incubate the sample at 65 °C overnight (*see* **Note 8**).

3.4 DNA Extraction and RNA Digestion

1. After overnight incubation, add 1 mL of PCI to the 2 mL tube. Vortex vigorously for 30 s. Then, centrifuge at 21,000 × g for 5 min at RT.
2. Carefully transfer the aqueous phase to a new 2 mL tube. Add 1 mL of PCI and repeat vortex and centrifugation as in **Step 1**.

3. Transfer the aqueous phase to a 1.7 mL microcentrifuge tube (*see* **Note 9**).
4. Add 2 μL of glycogen and vortex vigorously for 10 s. Then, add 200 μL of 3 M NaOAc and vortex vigorously for 10 s.
5. Split the mixture in half by transferring ~550 μL of the mixture to 1.7 mL microcentrifuge tubes.
6. Add 1.1 mL of ice-cold 100% EtOH and vortex vigorously for 10 s.
7. Incubate at −80 °C for 1 h.
8. Pellet DNA by centrifugation at 21,000 × g for 30 min at 4 °C. Carefully remove the supernatant by aspiration.
9. Add 1 mL of freshly prepared 70% ethanol (EtOH) to each tube. Vortex vigorously for 10 s.
10. Centrifuge at 21,000 × g for 30 min at 4 °C. Carefully remove the supernatant by aspiration.
11. Air-dry pellets for 3 min.
12. Add 20 μL of EB buffer.
13. Pipette to resuspend. Then, combine the two tubes of the same sample into one tube.
14. Add 2 μL of RNase A and then incubate at 65 °C for 30 min.
15. Cool down the sample on ice for 5 min before setting up the following reaction.

3.5 Removal of Biotins from Non-ligated Ends

1. Set up the reaction in a PCR tube on ice by adding 10.3 μL of nuclease-free H_2O, 6.4 μL of NEBuffer 2.1, 1.6 μL of 2 mM dATP, 1.6 μL T4 DNA polymerase, and all purified DNA.
2. Mix by pipetting followed by a pulse spin to collect all liquid from the side of the tube. Incubate at 20 °C for 4 h in a thermocycler.

3.6 DNA Extraction

1. Transfer the reaction to a 1.7 mL microcentrifuge tube.
2. Add 200 μL of PCI and vortex vigorously for 10 s to inactivate enzymes.
3. Add 150 μL of nuclease-free H_2O to bring up the volume. Vortex vigorously for 30 s.
4. Centrifuge at 21,000 × g for 5 min.
5. Carefully transfer aqueous phase to a new 1.7 mL microcentrifuge tube (*see* **Note 9**).
6. Add 1 μL of glycogen and vortex vigorously for 10 s. Then, add 40 μL of 3 M NaOAc and vortex vigorously for 10 s.
7. Add 1 mL of ice-cold 100% EtOH and vortex vigorously for 10 s.

8. Incubate at −80 °C for 45 min.
9. Pellet the DNA by centrifugation at 21,000 × g for 30 min at 4 °C. Carefully remove the supernatant by aspiration.
10. Add 1 mL of freshly prepared 70% EtOH to the tube. Vortex vigorously for 10 s.
11. Centrifuge at 21,000 × g for 10 min at 4 °C. Carefully remove the supernatant by aspiration.
12. Air-dry the pellet for 3 min. Avoid over-drying.
13. To resuspend DNA, add 105 μL of nuclease-free H_2O. Pipette to mix.

3.7 DNA Sonication

1. Transfer all DNA into a 0.5 mL sonication tube. Cool down the sample on ice for 5 min.
2. Using Qsonica Q800R2 sonicator, sonicate for 6 min without pause at 20% amplitude (*see* **Note 10**).
3. Vortex and quickly spin the tube, and then repeat sonication one more time using the same conditions.
4. Check the sample by DNA electrophoresis using 2.2% agarose gel. A DNA smear below 1 kb is desired. Sonicate more if needed.

3.8 Cleanup PF Fragmented DNA

1. Warm AMPure XP beads up to RT for at least 30 min.
2. Transfer the fragmented DNA into a 1.5 mL low adhesion microcentrifuge tube.
3. Vortex the AMPure XP beads to resuspend. Add 160 μL of AMPure XP beads to the DNA, mix well, and incubate for 5 min at RT (*see* **Note 11**).
4. Quickly spin the tube and place it on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 min), carefully remove and discard the supernatant (*see* **Note 12**).
5. Add 200 μL of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at RT for 30 s, and then carefully remove and discard the supernatant.
6. Repeat **Step 5** for a total of 2 washes.
7. Air-dry beads for 5 min while the tube is on the magnetic stand with the lid open (*see* **Note 13**).
8. Elute the DNA target from the beads into 53 μL of 0.1× TE. Mix well on a vortex mixer or by pipetting up and down. Let sit at RT for 5 min, and then vortex and quickly spin the tube.
9. Place the tube back on a magnetic stand. After the solution is clear (about 5 min), transfer 50 μL to a PCR tube and leave the tube on ice (*see* **Note 14**).

3.9 DNA Library Preparation for Illumina Sequencing

3.9.1 NEBNext End Prep

1. Add 3 μL of NEBNext Ultra II End Prep Enzyme Mix and 7 μL of 10× NEBNext Ultra II End Repair Reaction Buffer into the above PCR tube containing 50 μL of purified fragmented DNA.
2. Mix by pipetting followed by a pulse spin to collect all liquid from the side of the tube.
3. Place the PCR tube in a thermocycler. Run the following program:
   - 30 min at 20 °C.
   - 30 min at 65 °C.
   - Hold at 4 °C.

3.9.2 Adaptor Ligation

1. Dilute 15 μL of adaptors using 60 μL of a Tris/NaCl solution to achieve 75 μL of a 1:5 dilution of adaptors (*see* **Note 15**).
2. Add 2.5 μL of diluted adaptors directly to the End Prep reaction mixture from Subheading 3.9.1, **Step 3** and pipette to mix thoroughly (*see* **Note 16**).
3. Add 1 μL of NEBNext Ultra II Ligation Enhancer and 30 μL of NEBNext Ultra II Ligation Master Mix into the reaction mixture. Mix by pipetting followed by a pulse spin to collect all liquid from the side of the tube.
4. Incubate at 20 °C for 15 min in a thermocycler.
5. Add 3 μL of USER enzyme to the ligation mixture.
6. Pipette to mix thoroughly and incubate at 37 °C for 30 min in a thermocycler. Put the tube on ice when done.
7. Cleanup the DNA using 87 μL well-resuspended AMPure XP beads for each reaction, following the same procedure as described in Subheading 3.8. Elute the DNA from the beads into 28 μL of 0.1× TE. Use 25 μL of the DNA for the next step.

3.10 Biotin Pull-Down

1. Vortex streptavidin beads in the vial for 30 s to mix. Transfer 5 μL of beads into a 1.5 mL low adhesion microcentrifuge tube (*see* **Note 17**).
2. Add 400 μL of 1× NTB buffer to the beads and pipette to mix. Transfer the resuspension to a new 1.5 mL low adhesion microcentrifuge tube.
3. Place the tube on an appropriate magnetic stand for 3 min to isolate the beads. Carefully remove and discard the supernatant.
4. Repeat **Steps 2** and **3** two more times for a total of 3 washes.
5. Resuspend the beads in 25 μL of 2× NTB buffer per 5 μL of beads used in **Step 1** and transfer to a 1.5 mL low adhesion

microcentrifuge tube (*see* **Note 18**). Place the tube on ice while preparing DNA.

6. Transfer 25 μL of DNA from Subheading 3.9.2, **Step 7** into the above tube containing streptavidin beads, mix well.
7. Incubate the tube at RT for 30 min with gentle agitation on a vortexer.
8. Place the tube on a magnetic stand for 3 min to isolate the beads. Remove and discard the supernatant.
9. Add 400 μL of 1× NTB buffer to resuspend the beads, transfer the resuspension to a new 1.5 mL low adhesion microcentrifuge tube, and agitate for 3 min.
10. Repeat **Steps 8** and **9** for a total of 2 washes.
11. Add 400 μL of nuclease-free H_2O to resuspend the beads and transfer to a new 1.5 mL low adhesion microcentrifuge tube.
12. Place the tube on a magnetic stand for 3 min to isolate the beads. Remove and discard the supernatant.
13. Add 100 μL of nuclease-free H_2O to resuspend the beads and transfer to a new 1.5 mL low adhesion microcentrifuge tube.
14. Place the tube on a magnetic stand for 3 min to isolate the beads. Remove and discard the supernatant.
15. Add 21 μL of nuclease-free H_2O to resuspend the beads. Transfer the beads to a PCR tube and place on ice.

3.11 PCR Amplification

1. Mix the following components in a PCR tube: 25 μL of NEBNext Ultra II 2× Q5 Master Mix, 4 μL of Index Primers Mix, and 21 μL of streptavidin beads.
2. Pipette to mix thoroughly.
3. Place the tube in a thermocycler and set up conditions as in Table 1.

Table 1
PCR cycling conditions

Step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	N/A
Denaturation	98 °C	10 s	
Annealing & Extension	65 °C	1 min and 15 s	14 cycles
Final extension	65 °C	5 min	N/A
Hold	4 °C	∞	N/A

4. Quick spin the PCR tube to pellet beads. Desired DNA are in the supernatant.
5. Check the PCR product by DNA electrophoresis using a 2.2% gel. Expect to see a smear below 500 bp.
6. Transfer 45 μL of the PCR reaction to a 1.5 mL low adhesion microcentrifuge tube. Avoid taking the streptavidin beads.
7. Clean up the DNA using 45 μL of well-resuspended AMPure XP beads for each reaction as described previously in Subheading 3.8. Elute the DNA from the beads into 33 μL of 0.1× TE. Then, transfer 30 μL to a clean 1.5 mL tube.
8. Determine DNA concentration. Qubit dsDNA HS assay kit is recommended for accurate measurement.
9. Submit the DNA samples to a sequencing facility for paired-end sequencing.

4 Notes

1. Use formaldehyde within 6 months after opening to minimize oxidation.
2. Collect the cells in your desired growth phase. For most studies, exponential growth might be desired.
3. If cells are grown at a lower temperature such as 30 °C, increase crosslinking time to 45 min.
4. It is important to remove all of the supernatant. Repeat centrifugation if necessary.
5. Because of the presence of SDS, bubbles easily form during pipetting. Try to minimize it by pipetting slowly.
6. It is important to cool the reaction down before setting up the biotin labeling reaction.
7. During the incubation at 16 °C, invert the tube multiple times. We recommend a ligation time over 15 h.
8. Incubate the sample at 65 °C for more than 17 h.
9. Use cut tips to avoid pulling the organic phase.
10. Perform the sonication at 4 °C.
11. Beads can be mixed either by pipetting up and down 10 times or by vortexing for a few seconds.
12. Be careful not to disturb the beads which contain the desired DNA.
13. Over-drying the beads may result in low recovery of DNA.
14. Cool down the DNA on ice before adding the enzyme mix and the buffer in the following step.

15. As DNA input is low, the manufacturer recommends that the NEBNext adaptor for Illumina be diluted 1:5 before using.
16. Mix adaptors with the End Prep reaction mixture before adding ligation enhancer and ligation mix. This action will avoid self-ligation of adaptors.
17. If you have more than one reaction, process all the beads in the same tube.
18. If you have more than one reaction, resuspend beads with 2× NTB buffer accordingly and then distribute 25 μL of resuspension to individual 1.5 mL low adhesion microcentrifuge tubes.

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