iconHi-C

Nov 19, 2019

iconHi-C Protocol (ver. 1.0)

Mitsutaka Kadota[1], Osamu Nishimura[1], Hisashi Miura[2], Kaori Tanaka[1,3], Ichiro Hiratani[2], Shigehiro Kuraku[1]

[1]Laboratory for Phyloinformatics, RIKEN Center for Biosystems Dynamics Research (BDR), Kobe, 650-0047, Japan, [2]Laboratory for Developmental Epigenetics, RIKEN BDR, Kobe, 650-0047, Japan, [3]Division of Transcriptomics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, 812-0054, Japan (current address)

1 *Works for me* dx.doi.org/10.17504/protocols.io.4mjgu4n

Mitsutaka Kadota

ABSTRACT

Hi-C, a derivative of chromosome conformation capture (3C) targeting the whole genome, was originally developed as a means for characterizing chromatin conformation and is recently employed widely in scaffolding nucleotide sequences obtained by *de novo* genome sequencing. To gain insights into the best practice of Hi-C scaffolding, we performed a multifaceted methodological comparison and optimized various factors during sample preparation, sequencing, and computation. As a result, we have identified some key factors for efficient Hi-C data acquisition, including preparation of tissues, library preparation conditions, and quality controls of prepared samples. By incorporating those optimal conditions revealed by our experience, we release this customized protocol designated the 'inexpensive and controllable Hi-C (iconHi-C) protocol'.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Multifaceted Hi-C benchmarking: what makes a difference in chromosome-scale genome scaffolding? Mitsutaka Kadota, Osamu Nishimura, Hisashi Miura, Kaori Tanaka, Ichiro Hiratani, Shigehiro Kuraku bioRxiv 659623; doi: https://doi.org/10.1101/659623

GUIDELINES

This protocol consists of the following sections:

MATERIALS TEXT

Reagents and consumables

- 1.5 ml Protein LoBind tube (Eppendorf, cat. 0030108116)
  Note: For cell/tissue samples.
- 1.5 ml DNA LoBind tube (Eppendorf, cat. 0030108051)
  Note: For DNA samples.
- 2.0 ml Protein LoBind tube (Eppendorf, cat. 0030108132)
  Note: For cell/tissue samples.
- 50 ml tube (Thermo Fisher Scientific, cat. 14-432-22)
  Note: For cell/tissue samples.

- 0.2 ml PCR tube (INA OPTICA, cat. 3247-00)
- 384-well PCR plate (Applied Biosystems, cat. 4309849)
- Optical Adhesive Film (Applied Biosystems, cat. 4311971)
- Liquid nitrogen
- Mortar and pestle (AS ONE, cat. 2-9037-02)
- SK mill (Tokken, cat. SK-200)
- Stainless-steel tube (Tokken, cat. TK-AM5-SUS)
- Stainless-steel bullet (Tokken, cat. SK-100-DLC10)
- Tube holder (Tokken, cat. SK-100-TL)
- Douncer (Sigma-Aldrich, cat. D8938)
- PBS minus (Wako Pure Chemical, cat. 314-90185)
  Note: Make a 1X solution with H_2O.
- 16% formaldehyde (Pierce, cat. 28906)
- Glycine (Wako Pure Chemical, cat. 077-00735)
  Note: Make a 2.5 M solution with H_2O.
- 1 M Tris-HCl (pH 8.0) (Wako Pure Chemical, cat. 314-90065)
- 5 M NaCl (Nacalai Tesque, cat. 31334-51)
- 0.5 M EDTA (Invitrogen, cat. 15575-038)
- 10% SDS (Invitrogen, cat. 15553-035)
- Triton X-100 (Sigma-Aldrich, cat. T8787)
  Note: Make a 20% solution (w/v) with H_2O.
- IGEPAL CA-630 (NP40) (Sigma-Aldrich, cat. 18896)
  Note: Make a 10% solution (w/v) with H_2O.
- Tween 20 (Sigma-Aldrich, cat. P9416)
  Note: Make a 10% solution (w/v) with H_2O.
- Proteinase inhibitor cocktail (Sigma-Aldrich, cat. P8340)
- Proteinase K solution (Nacalai Tesque, cat. 15679-64)
- RNase A (Takara, cat. U0505S)
  Note: Make a 10 mg/ml solution with H_2O.
- Phenol/Chloroform/Isoamyl alcohol (25:24:1) (Wako Pure Chemical, cat. 311-90151)
- Glycogen solution (Thermo Fisher Scientific, cat. R0561)
- 2-Propanol (Nacalai Tesque, cat. 03065-35)
- Ethanol (Junsei, cat. 17065-1230)
  Note: Make 70% solution (for EtOH precipitaton) and 80% solution (for AMPure purification) with H_2O.
- TE (pH 8.0) (Wako Pure Chemical, cat. 314-90021)
- EB (Qiagen, cat. 19086)
- Qubit dsDNA High Sensitivity Kit (Thermo Fisher Scientific, cat. Q32851)
- Agilent Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, cat. 5067-4626)
- Agilent TapeStation Genomic DNA ScreenTape (Agilent Technologies, cat. 5067-5365)
- Agilent TapeStation Genomic DNA Reagents (Agilent Technologies, cat. 5067-5366)
- Agilent TapeStation High Sensitivity D1000 ScreenTape (Agilent Technologies, cat. 5067-5584)
- Agilent TapeStation High Sensitivity D1000 Reagents (Agilent Technologies, cat. 5067-5585)
- NEBuffer 2 (New England Biolabs, cat. B7002S)
- NEBuffer 2.1 (New England Biolabs, cat. B7202S)
- NEBuffer DpnII (New England Biolabs, cat. B0543)
- DpnII (New England Biolabs, cat. R0543M)
- HindIII (New England Biolabs, cat. R3104M)
- ClaI (Takara, cat. 1034A)
- NheI (New England Biolabs, cat. R0131S)
- BSA solution (New England Biolabs, cat. B9000S)
- dNTP set (Thermo Fisher Scientific, cat. LS10297018)
  Note: Make 1 mM and 10 mM solution with H_2O.
- Biotin-14-dATP (Thermo Fisher Scientific, cat. 19524016)
- Biotin-14-dCTP (Thermo Fisher Scientific, cat. 19518018)
- Klenow DNA polymerase (New England Biolabs, cat. M0210L)
- T4 DNA ligase (New England Biolabs, cat. M0202M)
- T4 DNA polymerase (New England Biolabs, cat. M0203S)

- Covaris microTUBE (Covaris, cat. 520045)
- Agencourt AMPure XP beads (Beckman coulter, cat. A63880)
- Magnet stand for the microtube (Thermo Fisher Scientific, cat. 12321D)
- Magnet stand for a PCR tube (Nippon Genetics, cat. FG-SSMAG2)
- Streptavidin beads (Thermo Fisher Scientific, cat. 11205D)
- KAPA LTP Library Preparation Kit (KAPA Biosystems, cat. KK8230)
- KAPA HiFi HotStart Ready Mix (KAPA Biosystems, cat. KK2600)
- KAPA HiFi HotStart Real-time PCR Master Mix (KAPA Biosystems, cat. KK2701)
- Illumina TruSeq compatible UDI adapter (PerkinElmer, cat. NOVA-514180)
- TPC mix (10 µM each)
  Note: Mix two oligos; 5'-AATGATACGGCGACCACCGAG-3' and 5'-CAAGCAGAAGACGGCATACGAG-3'.

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

Section 1. (DAY 0) Preparation of cells/tissue

1 Follow sample preparation procedure that matches the sample type; dissociated cells (e.g., cultured cells and nucleated red blood cells), fibrous tissue (e.g. muscle, kidney, intestine, heart), or soft tissue (e.g., liver, brain, testis, and embryonic tissues).

step case

Dissociated cells

Sample preparation procedure for dissociated cells (e.g., cultured cells and nucleated red blood cells)

2 Dissociate cells, count cell number, and collect 1 ×10^7 cells in a microtube (1.5 or 2.0 ml).

3 Centrifuge the cells.

Centrifugation speed: **500 x g**

Centrifugation time: **00:05:00**

Centifugation temperature: **4 °C**

4 Remove the supernatant.

5 Put the tube on ice, and proceed immediately to fixation (Step 6).

Section 2. (DAY 0) Fixation of cells

6 Prepare the **fixing solution** and put on ice.

Reagent	Amount	Final Concentration
16% formaldehyde solution	1 ml	(1%)
PBS (-)	15 ml	
Total	**16 ml**	

Fixing solution

Make fresh every time.

7 Take the sample tube out from the ice bucket (from Step 5) and add **1 ml** - **1.5 ml** **ice-cold fixing solution** .

8 Vortex until the cells are fully resuspended in fixing solution.

9 Incubate the cell-suspension for **00:10:00** in a heat block or a water bath set at **25 °C** .

10 Quick spin the sample tube.

11 Add 1/20 volume of a **2.5 Molarity (M)** **glycine solution** to quench the formaldehyde.

12 Vortex mix.

13 Put the sample tube on ice for ~ **00:01:00** .

14 Centrifuge the sample tube.

Centrifugation speed: **1000 x g**

Centrifugation time: **00:05:00**

Centrifugation temperature: **4 °C**

15 Remove the supernatant.

16 Add **1 ml ice-cold PBS (-)** . (1/2)

17 Vortex mix. (1/2)

18 Centrifuge the sample tube. (1/2)

Centrifugation speed: **1000 x g**

Centrifugation time: **00:05:00**

Centrifugation temperature: **4 °C**

19 Remove the supernatant. (1/2)

20 Repeat the washing cycle (Steps 16-19) once more.

go to step #16

21 Resuspend the pellet in **1 ml** - **1.5 ml ice-cold PBS** and aliquot the cell-suspension into microtubes (1.5 ml) at 1-2 $\times 10^6$ cells/tube.

22 Centrifuge the sample tubes.

Centrifugation speed: **1000 x g**

Centrifugation time: **00:05:00**

Centrifugation temperature: **4 °C**

23 Remove the supernatant.

24 Store the sample tubes in an ultra-low temperature freezer (e.g., **-80 °C**) until use.

Section 3. (DAY 0) Pre-determination of the amount of tissue to use for Hi-C

25

The pre-determination section quantitates the amount of DNA contained in a cell/tissue pellet prepared in the previous section. Pre-determination should always be performed for tissue samples, but it is optional when the DNA content in a pellet is estimable,. e.g., for cultured cells and nucleated red blood cells.

Section 4. (DAY 1) Restriction enzyme digestion

26

This section includes the following steps.
1. Cell/tissue permeabilization - Steps 26 - 51
2. Restriction enzyme digestion - Steps 52 - 54

The number of cells to use for Hi-C is determined based on the amount of DNA.
Use 1×10^6 cells for an animal with the genome size of 3-3.5 Gb (e.g., human and mouse), or 2×10^6 cells for an animal with the genome size of 1-1.5 Gb (e.g., chicken and western clawed frog).
For tissue samples, use a pellet that contains 2-10 µg of DNA.

Follow cells/tissue resuspension steps as written, i.e., vortex mix or pipet mix.

Prepare the **permeabilization buffer 1 (PB1)**.

Reagent	Amount	Final Concentration
1 M Tris-HCl (pH 8.0)	400 µl	(10 mM)
5 M NaCl	80 µl	(10 mM)
10% (w/v) NP-40	800 µl	(0.2%)
H2O	38.72 ml	
Total	**40 ml**	

Permeabilization buffer 1 (PB1)

Filtrate and store at **4 °C** .

27 Take (**400 µl** ×n) + 5% extra volume of PB1 in a new tube, add 1/100 vol of a proteinase inhibitor cocktail (PI), and put on ice.

Add PI to PB1 just before use.

28 Take the frozen cells out from the freezer (from Step 24) and immediately add **400 µl PB1 (with PI)** .

29 Vortex mix.

30 Incubate the sample tube on ice for **00:20:00** with periodical mixing every **00:05:00** - **00:10:00** .

31 Centrifuge the sample tube.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

32 Remove **300 µl** of the supernatant using a P-1000 pipet.

33 Centrifuge the sample tube again.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

34 Remove the remaining supernatant using a P-100 or a P-200 pipet, and put the sample tube on ice.

35 .

Prepare the **permeabilization buffer 2 (PB2)** at **Room temperature** .

Reagent	Amount (x1 reaction)	Final Concentration
10X NEBuffer 2.1	25 µl	(1X)
10% (w/v) SDS	7.5 µl	(0.3%)
PI (100X)	2.5 µl	(1X)
H2O	215 µl	
Total	**250 µl**	

Permeabilization buffer 2 (PB2)

Make fresh every time.
Make 5% extra volume.

35.1 Take **215 µl H2O** in a tube of appropriate size.

35.2 Add **25 µl 10X NEBuffer 2.1** .

35.3 Add **7.5 µl 10% (w/v) SDS** .

35.4 Add **2.5 µl PI (100X)** .

36 Add **250 µl PB2** to the sample tube and pipet mix.

37 Incubate/shake the sample tube in a thermal mixer for **00:10:00** at **37 °C** , **950 rpm** .

38 Add **28 µl 20% TritonX-100** to the sample tube.

39 Incubate/shake the sample tube in a thermal mixer for **00:10:00** at **37 °C** , **950 rpm** .

40 Prepare the **DNA-preparation buffer** .

Reagent	Amount	Final Concentration
5 M NaCl	250 µl	(250 mM)
0.5 M EDTA	50 µl	(5 mM)
10% (w/v) SDS	500 µl	(1%)
H2O	4.2 ml	
Total	**5 ml**	

DNA-preparation buffer

Store DNA-preparation buffer at **Room temperature** .

Warm the DNA-preparation buffer at **37 °C** to dissolve precipitates formed during storage.

41 Take **8 µl** (3%) aliquot (from Step 39) in a new 1.5 ml microtube as a pre-digest DNA control (ctr-1), add **42 µl DNA-preparation buffer** and store at **-20 °C** until the end of DAY2.

42 Centrifuge the sample tube.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

43 Remove **200 µl** of the supernatant using a P-200 pipet.

44 Centrifuge the sample tube again.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

45 Remove the remaining supernatant using a P-100 or a P-200 pipet, and put the sample tube on ice.

46 Prepare **washing buffer (WB)** with NEBuffer DpnII (for the DpnII digested sample).

Reagent	Amount (x1 wash cycle)	Final Concentration
10X NEBuffer DpnII	50 µl	(1X)
20 mg/ml BSA	2.5 µl	(0.1 mg/ml)
20% (w/v) TritonX-100	1.25 µl	(0.05%)
H2O	446.25 µl	
Total	**500 µl**	

Washing buffer (WB)

Do not use NEBuffer 3.1 for the samples for DpnII digestion.
Make WB for HindIII digestion with NEBuffer 2.1 (that contains BSA).
Make 5% extra volume.

46.1 Take **446.25 µl H2O** in a tube of appropriate size.

46.2 Add **50 µl 10X NEBuffer DpnII** .

46.3 Add **2.5 µl 20 mg/ml BSA** .

46.4 Add **1.25 µl 20% (w/v) TritonX-100** .

47 Add **500 µl WB** to the sample tube and vortex mix.

48 Centrifuge the sample tube.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

49 Remove **400 µl** of the supernatant using a P-1000 pipet.

50 Centrifuge the sample tube again.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

51 Remove the remaining supernatant using a P-100 pipet or a P-200 pipet, and put the sample tube on ice.

52 Prepare the **restriction-enzyme mix** (for DpnII digestion) on ice.

Reagent	Amount (x1 reaction)	Final Concentration
10X NEBuffer DpnII	20 µl	(1X)
20 mg/ml BSA	1 µl	(0.1 mg/ml)
DpnII (50 U/µl)	8 µl	(400 U)
20% Triton X-100	0.5 µl	(0.05%)
H2O	170.5 µl	
Total	**200 µl**	

Restriction-enzyme mix

Do not use NEBuffer 3.1 for DpnII because non-specific cleavage (star activity) may be induced.
Use NEBuffer 2.1 (that contains BSA) for HindIII digestion.
For restriction enzymes at different concentration, e.g., HindIII at 100U/µl, adjust the volume with H2O.
Make 5% extra volume.

52.1 Take **170.5 µl H2O** in a tube of appropriate size.

52.2 Add **20 µl 10X NEBuffer DpnII** .

52.3 Add **1 µl 20 mg/ml BSA** .

52.4 Add **8 µl DpnII (50 U/ul)** .

52.5 Add **0.5 µl 20% Triton X-100** .

53 Add **200 µl restriction-enzyme mix** to the sample tube and pipet mix.

54 Incubate the sample tube in a thermal mixer for ~ 16:00:00 at 37 °C , 1100 rpm .

55 Take 6 µl (3%) aliquot in a new 1.5 ml microtube as a digested-DNA control (ctr-2), add 44 µl **DNA-preparation buffer** and store at -20 °C until the end of DAY 2.

Section 5. (DAY 2) DNA fill-in and ligation

56

This section includes the following steps.
1. DNA fill-in reaction - Steps 56 - 76
2. Ligation reaction - Steps 77 - 79

Follow cells/tissue resuspension steps as written, i.e., vortex mix or pipet mix.

Centrifuge the sample tube.

Centrifugation speed: 2000 x g

Centrifugation time: 00:03:00 - 00:05:00

Centrifugation temperature: 4 °C

57 Remove 150 µl of the supernatant using a P-200 pipet.

58 Centrifuge the sample tube again.

Centrifugation speed: 2000 x g

Centrifugation time: 00:03:00 - 00:05:00

Centrifugation temperature: 4 °C

59 Remove the remaining supernatant using a P-200 pipet or a P-100 pipet, and put the sample tube on ice.

60 Prepare the **washing buffer (WB)** with NEBuffer 2.1.

Reagent	Amount (x1 wash cycle)	Concentration
10X NEBuffer 2.1	50 µl	(1X)
20% (w/v) TritonX-100	1.25 µl	(0.05%)
H2O	448.75 µl	
Total	**500 µl**	

Washing Buffer (WB)

Prepare buffer to perform total of four or two wash cycles (at Steps 61, 66 and 73), for the DpnII-digested sample or the HindIII-digested sample respectively.

60.1 Take **448.75 µl H2O** in a tube of appropriate size.

60.2 Add **50 µl 10X NEBuffer 2.1** .

60.3 Add **1.25 µl 20% (w/v) TritonX-100** .

61 Add **500 µl WB** to the sample tube and vortex mix.

62 Centrifuge the sample tube.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

63 Remove **400 µl** of the supernatant using a P-1000 pipet.

64 Centrifuge the sample tube again.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

65 Remove the remaining supernatant using a P-100 pipet or a P-200 pipet.

66 Repeat the washing cycle (Steps 61 - 65) twice more for the DpnII-digested sample (total of three wash cycles).

go to step #61

Additional washing cycle is not required for the HindIII-digested sample.

67 Put the sample tube on ice.

68 Prepare the **DNA fill-in mix** on ice.

Reagent	Amount (x1 reaction)	Final Concentration
10X NEBuffer 2	10 µl	(1X)
0.4 mM biotin-14-dATP	3.75 µl	(15 µM)
1 mM dCTP	1.5 µl	(15 µM)
1 mM dGTP	1.5 µl	(15 µM)
1 mM dTTP	1.5 µl	(15 µM)
Klenow DNA polymerase (5 U/µl)	6 µl	(30 U)
20% TritonX-100	0.25 µl	(0.05%)
H2O	**75.5 µl**	
Total	**100 µl**	

DNA fill-in mix for the DpnII-digested sample

Reagent	Amount	Final Concentration
10X NEBuffer 2	10 µl	(1X)
1 mM dATP	1.5 µl	(15 µM)
0.4 mM biotin-14-dCTP	3.75 µl	(15 µM)
1 mM dGTP	1.5 µl	(15 µM)
1 mM dTTP	1.5 µl	(15 µM)
Klenow DNA polymerase (5 U/µl)	3 µl	(15 U)
20% TritonX-100	0.25 µl	(0.05%)
H2O	78.5 µl	
Total	**100 µl**	

DNA fill-in mix for the HindIII-digested sample

Make a DNA fill-in mix that contains either biotin-14-dATP or biotin-14-dCTP, for the DpnII-digested sample or the HindIII-digested sample respectively.
Make 5% extra volume.

68.1 Take **75.5 µl H2O** in a tube of appropriate size.

Take **78.5 µl H2O** for the **DNA fill-in mix** for HindIII-digested sample.

68.2 Add **10 µl 10X NEBuffer 2** .

68.3 Add **3.75 µl 0.4 mM biotin-14-dATP** .

Add **1.25 µl 1 mM dATP** for the **DNA fill-in mix** for HindIII-digested sample.

68.4 Add **1.25 µl 1 mM dCTP** .

Add **3.75 µl 0.4 mM biotin-14-dCTP** for the **DNA fill-in mix** for HindIII-digested sample.

68.5 Add **1.25 µl 1 mM dGTP** .

68.6 Add **1.25 µl 1 mM dTTP** .

68.7 Add **6 µl Klenow DNA polymerase (5 U/µl)** .

Add **3 µl Klenow DNA polymerase (5 U/µl)** for the **DNA fill-in mix** for HindIII-digested sample.

68.8 Add **0.25 µl 20% TritonX-100** .

69 Add **100 µl DNA fill-in mix** to the sample tube and pipet mix.

70 Incubate the sample tube in a thermal mixer for **00:20:00** at **25 °C** , **1100 rpm** .

71 Centrifuge the sample tube.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

72 Remove the supernatant using a P-100 pipet or a P-200 pipet.

73 Add **500 µl WB** to the sample tube and vortex mix.

74 Centrifuge the sample tube.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

75 Remove **400 µl** of the supernatant using a P-1000 pipet.

76 Centrifuge the sample tube again.

Centrifugation speed: **2000 x g**

Centrifugation time: **00:03:00** - **00:05:00**

Centrifugation temperature: **4 °C**

77 Remove the remaining supernatant using a P-100 pipet or a P-200 pipet.

78 Put the sample tube on ice.

79 Prepare **ligation mix** on ice.

Reagent	Amount	Final Concentration
T4 DNA ligase buffer (10X)	10 µl	(1X)
T4 DNA ligase (2,000 U/ul)	2 µl	(4,000 CEU)
20% TritonX-100	0.25 µl	(0.05%)
H2O	87.75 µl	
Total	**100 µl**	

Ligation mix

Prepare T4 DNA ligase buffer in small aliquots and store them at **-20 °C** when the buffer is thawed for the first time.
Always use an aliquot that is not thawed and frozen repeatedly.
Use 2,000 CEU of T4 DNA ligase for the HindIII-digested sample.
Make 5% extra volume.

79.1 Take **87.75 µl H2O** in a tube of appropriate size.

79.2 Add **10 µl T4 DNA ligase buffer (10X)** .

79.3 Add **0.25 µl 20% TritonX-100** .

79.4 Add **2 µl T4 DNA ligase (2,000 U/ul)** .

80 Add **100 µl ligation mix** to the sample tube and pipet mix.

81 Incubate the sample tube in a thermal mixer for **04:00:00** - **06:00:00** at **16 °C** , **1100 rpm** .

Section 6.1. (DAY 2) Hi-C DNA purification

82 Prepare **DNA-extraction mix**.

Reagent	Amount (x1 reaction)	Final Concentration
1M Tris-HCl (pH 8.0)	2 µl	(10 mM)
0.5M EDTA	2 µl	(5 mM)
10% SDS	30 µl	(1.5%)
5M NaCl	15 µl	(375 mM)
20 mg/ml Proteinase K	10 µl	(1 mg/ml)
H2O	141 µl	
Total	**200 µl**	

DNA-extraction mix

Prepare DNA-extraction mix at **Room temperature** .
Make 5% extra volume.

82.1 Take **141 µl H2O** in a tube of appropriate size.

82.2 Add **2 µl 1M Tris-HCl (pH 8.0)** .

82.3 Add **2 µl 0.5M EDTA** .

82.4 Add **30 µl 10% SDS** .

82.5 Add **15 µl 5M NaCl** .

82.6 Add **10 µl 20 mg/ml Proteinase K** .

83 Add **200 µl DNA-extraction mix** to the sample tube (from Step 81) and pipet mix.

The total amount of the mixture will be **300 µl** .

84 Add **200 µl DNA-extraction mix** and **50 µl H2O** to the control tubes (ctr-1 from Step 41 and ctr-2 from Step 55) and pipet mix.

The total amount of the mixture will be **300 µl** .

85 Incubate in a thermal mixer for ~ **16:00:00** at **65 °C** , **350 rpm** .

Use a thermal mixer equipped with a heated lid to avoid condensation of water on the lid.
Alternatively, the incubation can be performed in a heated oven.

Section 6.2. (DAY 3) Hi-C DNA purification

86 Take the sample tube out from the thermal mixer (from Step 85) and let it cool down to **Room temperature** .

87 Add **5 µl RNase A (10 mg/ml)** to the sample tube and mix gently.

88 Incubate sample tube in a thermal mixer for **00:20:00** at **37 °C** , **800 rpm** .

89 Add **5 µl Proteinase K (20 mg/ml)** to the sample tube and mix gently.

90 Incubate the sample tube in a thermal mixer for **02:00:00** at **55 °C** , **800 rpm** .

91 Take the sample tube out from the thermal mixer, let it cool down to **Room temperature** , and proceed with DNA extraction.

92 Add **300 µl Phenol/Chloroform/Isoamyl alcohol solution** to the sample tube and mix gently.

93 Centrifuge the sample tube.

Centrifugation speed: **16000 x g**

Centrifugation time: **00:05:00**

Centrifugation temperature: **Room temperature**

94 Transfer ~ **250 µl** of the aqueous phase into a new 1.5 ml microtube.

95 Prepare **TE/NaCl solution**.

Reagent	Amount	Final concentration
1 M Tris-HCl (pH 8.0)	50 µl	(10 mM)
5 M NaCl	250 µl	(250 mM)
0.5 M EDTA (pH 8.0)	10 µl	(1 mM)
H2O	4.69 ml	
Total	5 ml	

TE/NaCl solution

Store TE/NaCl solution at **Room temperature** .

96 Add **300 µl TE/NaCl solution** to the sample tube containing Phenol and mix gently.

97 Centrifuge the sample tube.

Centrifugation speed: **16000 x g**

Centrifugation time: **00:05:00**

Centrifugation temperature: **Room temperature**

98 Transfer ~ **300 µl** of the aqueous phase into the microtube containing the first aqueous phase.

The total volume of the collected aqueous phase will be ~ **550 µl** .

99 Add **1 µl glycogen solution (20 mg/ml)** to the collected aqueous phase and mix gently.

100 Add **600 µl 2-propanol** to the collected aqueous phase and mix gently until the solution becomes homogeneous.

101 Centrifuge the sample tube.

Centrifugation speed: **20000 x g**
Centrifugation time: **00:30:00**
Centrifugation temperature: **4 °C**

102 Decant the supernatant, add **1 ml 70% EtOH** , and mix gently to rinse the DNA pellet.

103 Centrifuge the sample tube.

Centrifugation speed: **20000 x g**
Centrifugation time: **00:10:00**
Centrifugation temperature: **4 °C**

104 Decant the supernatant.

105 Centrifuge the sample tube again.

Centrifugation speed: **20000 x g**
Centrifugation time: **00:05:00**
Centrifugation temperature: **4 °C**

106 Remove the supernatant completely with a P-100 or a P-200 pipet, and keep the lid open for ~ **00:01:00** to allow the ethanol to evaporate.

Do not over dry the pellet.

107 Add **30 µl** - **50 µl EB** and **10 µl EB** to the Hi-C DNA and the control DNAs (ctr-1 and ctr-2) respectively.

Mix gently to avoid shearing of the DNA.
Store DNA samples at **4 °C** or **-20 °C** .

108 Quantitate DNA using **1 µl** of the DNA sample with the Qubit dsDNA High Sensitivity Kit.

Section 7. (DAY 3) Hi-C DNA QC (QC1)

109 Take **1 µl** - **2 µl** of the DNA sample (ctr-1, ctr-2, and Hi-C DNA) from the previous section (Step 107) in a new PCR tube and add **EB** to adjust the concentration to 2-20 ng/µl.

The concentration of the DNA samples in a trio (ctr-1, ctr-2, and Hi-C DNA) should be within two-fold of difference, to accurately observe the size shift by Agilent Bioanalyzer or Agilent TapeStation.

110 Analyze **1 µl** of the DpnII-digested DNA sample (ctr-2 and Hi-C DNA) using the Agilent Bioanalyzer with the DNA High Sensitivity chip, and **1 µl** of the DpnII-digested DNA sample (ctr-1) using the Agilent TapeStation with the genomic tape.

Only ctr-2 and Hi-C DNA of the DpnII digested samples are analyzed using the Agilent Bioanalyzer with the DNA High Sensitivity chip, because ctr-1 (pre-digested DNA), >50 kb in size, exceeds the limit of the DNA size that can be analyzed. Alternatively, the DpnII-digested DNA samples can be analyzed using the Agilent TapeStation with the genomic tape.

For the HindIII-digested DNA samples, analyze **1 µl** of ctr-1, ctr-2, and Hi-C DNA using Agilent TapeStation with the genomic tape.

Only qualified Hi-C DNA showing the expected pattern of size shift, i.e., reduction in ctr-2 and recovery in Hi-C DNA, with no or minimum degree of DNA degradation in ctr-1, ctr-2 and Hi-C DNA, is used for the preparation of the Hi-C library.

111 Store Hi-C DNA at **-20 °C** or proceed to the next section (Step 112).

Section 8. (DAY 3) Removal of biotin from un-ligated DNA ends

112 Take **250 ng** - **2 µg** of Hi-C DNA from the previous step (Step 111) in a 0.2 ml PCR tube and adjust the total volume to **30 µl** with H_2O.

113 Prepare **T4 DNA polymerase mix** on ice.

Reagent	Amount	Final Concentration
10X NEBuffer 2.1	5 µl	(1X)
10 mM GTP	0.5 µl	(100 µM)
T4 DNA pol (3 U/µl)	1.67 µl	(5 U)
H2O	12.83 µl	
Total	**20 µl**	

T4-DNA-polymerase mix for the DpnII-digested sample

Reagent	Amount	Final Concentration
10X NEBuffer 2.1	5 µl	(1X)
10 mM dATP	0.5 µl	(100 µM)
10 mM dGTP	0.5 µl	(100 µM)
T4 DNA pol (3 U/µl)	0.84 µl	(2.5 U)
H2O	13.16 µl	
Total	**20 µl**	

T4-DNA-polymerase mix for the HindIII-digested sample

Make 5% extra volume.

113.1 Take **12.83 µl H2O** in a tube of appropriate size.

Take **13.16 µl H2O** for the **T4-DNA-polymerase mix** for the HindIII-digested sample.

113.2 Add **5 µl 10X NEBuffer 2.1** .

113.3 Add **0.5 µl 10 mM dGTP** .

Add **0.5 µl 10 mM dATP** and **0.5 µl 10 mM dGTP** for the **T4-DNA-polymerase mix** for the HindIII-digested sample.

113.4 Add **1.67 µl T4 DNA pol (3 U/µl)** .

Add **0.84 µl T4 DNA pol (3U/µl)** for the **T4-DNA-polymerase mix** for the HindIII-digested sample.

114 Add **20 µl T4-DNA-polymerase mix** to the Hi-C DNA and mix gently.

115 Incubate the sample in a PCR machine for **00:30:00** at **37 °C** , and **00:15:00** at **75 °C** .

Section 9. (DAY 3) Fragmentation and size selection of the Hi-C DNA

116 Turn on the Covaris (S220 or E220) and the chiller.

117 Transfer the entire reaction from the previous section (Step 115) into a Covaris microTUBE and add **80 µl TE buffer** .

The total volume will be **130 µl** .

118 Perform sonication

Duty factor: 5%
Peak incident power: 175
Cycles per burst: 200
Time: **00:01:00** ×2
Bath temperature: **7 °C**

119 Transfer **120 µl** of the fragmented DNA into a 1.5 ml microtube.

120 Add **72 µl** (×0.6 amount) of AMPure XP beads to the fragmented DNA, vortex and incubate **00:05:00** at **Room temperature** .

DNA larger than 600 bp are removed at Steps 120-122.

121 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

122 Transfer the supernatant into a new 1.5 ml microtube.

The supernatant contains DNA smaller than 600 bp.

123 Add **108 µl** (×0.9 amount) of AMPure XP beads to the supernatant, vortex mix, and wait **00:05:00** at **Room temperature** .

DNA larger than 150 bp are collected.

124 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

125 Remove the supernatant using a P-200 pipet while the sample tube is still on the magnet.

126 Add **200 µl** **80% EtOH** to the beads while the sample tube is still on the magnet and wait **00:00:30** . (1/2)

127 Remove EtOH with P-200 pipet while the sample tube is still on the magnet. (1/2)

128 Repeat the washing cycle (Steps 126-127) once more.
go to step #126

129 Quick spin the sample tube, put on magnet and remove the residual EtOH completely using a P-10 or a P-20 pipet.

130 Air dry the beads for ~ **00:01:00** at **Room temperature** with the lid kept open.

131 Add **60 µl** **EB** to the beads, vortex mix and incubate **00:02:00** at **Room temperature** .

132 Quick spin the sample tube, put on magnet and collect the eluate in a new PCR tube.

Section 10. (DAY 3) Enrichment of biotin-containing DNA

133 Prepare the **2X binding-and-washing buffer (BWB)**.

Reagent	Amount	Final Concentration
1 M Tris-HCl (pH 7.5)	400 µl	(10 mM)
0.5 M EDTA (pH 8.0)	80 µl	(1 mM)
5 M NaCl	16 ml	(2 M)
10% Tween20	80 µl	(0.02%)
H2O	23.44 ml	
Total	**40 ml**	

2X binding-and-washing buffer (BWB)

Store at **Room temperature** .

133.1 Take **23.44 ml H2O** in a tube of appropriate size.

133.2 Add **400 µl 1 M Tris-HCl (pH 7.5)** .

133.3 Add **80 µl 0.5 M EDTA (pH 8.0)** .

133.4 Add **16 ml 5 M NaCl** .

133.5 Add **80 µl 10% Tween20** .

134 Prepare 1X BWB by diluting the 2X BWB with H_2O.

Store at **Room temperature** .

135 Mix the bottle of the streptavidin beads and transfer (**25 µl** × n) + 5% extra volume of beads into a new 1.5 ml microtube.

136 Put the tube on the magnetic and wait until the supernatant becomes clear.

137 Remove the supernatant, take off the tube from the magnet, add 1 ml **1X BWB** , and vortex mix.

138 Quick spin the tube, put on magnet and wait until the supernatant becomes clear.

139 Remove the supernatant, take off the tube from the magnet, add (60 µl × n) + 5% extra volume of **2X BWB**, and pipet mix.

140 Add 60 µl **beads** (in 2X BWB) to the size-selected Hi-C DNA prepared in previous section (from Step 132) and vortex mix.

The total volume will be 120 µl .

141 Incubate the sample tube in a thermal mixer for 00:15:00 at 20 °C with periodical mixing for 00:00:10 at 2000 rpm every 00:03:00 .

142 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

143 Remove the supernatant while the tube is still on the magnet.

144 Take off the sample tube from the magnet.

145 Add 100 µl **1X BWB** to the sample tube and vortex mix. (1/4)

146 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear. (1/4)

147 Remove the supernatant while the tube is still on the magnet.(1/4)

148 Take off the sample tube from the magnet.(1/4)

149 Repeat the washing cycle (Steps 145-148) three more times.
go to step #145

150 Add 100 µl **EB** and vortex mix.

151 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

152 Remove the supernatant while the tube is still on the magnet.

153 Add **100 µl EB** with the tube kept on the magnet.

154 Remove the supernatant, take off the sample tube from the magnet, quick spin, and put the sample tube back on the magnet.

155 Remove the residual EB with a P-10 or a P-20 pipet.

156 Resuspend the beads in **50 µl EB** and store the sample tube at **4 °C** until the next day (DAY 4).

Section 11. (DAY 4) Hi-C library preparation

157

This section consists of the following steps.
1. End repair - Steps 157 - 175
2. A-tailing - Steps 176 - 193
3. Adapter ligation - Steps 194 - 212
4. Pre-PCR (releasing DNA off the streptavidin beads) - Steps 213 - 226
5. PCR cycle pre-determination - Steps 227 - 231
6. Amplification of the library for Hi-C library QC (QC2) - Steps 232 - 234
7. Library amplification - Steps 235 - 249

Library preparation is performed using the KAPA LTP DNA library kit but in a 1/5 reaction volume of the original protocol and with an additional step for PCR cycle pre-determination.

Prepare the **end-repair mix** on ice.

Reagent	Amount
10X KAPA End Repair Buffer	1.4 µl
KAPA End Repair Enzyme Mix	1.0 µl
H2O	11.6 µl
Total volume	**14 µl**

End-repair mix

Make 5% extra volume.

157.1 Take **11.6 µl H2O** in a tube of appropriate size.

157.2 Add **1.4 µl 10X KAPA End Repair Buffer** .

157.3 Add **1 µl KAPA End Repair Enzyme Mix** .

158 Quick spin the samle tube prepared in the previous section (from Step 156), put on magnet and wait until the supernatant becomes clear.

159 Remove the supernatant, add **14 µl end-repair mix** to the sample tube, pipet mix, and proceed immediately to the next step.

160 Incubate for **00:30:00** at **20 °C** (followed by a hold at **4 °C**) in a PCR machine.

161 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

162 Remove the supernatant while the tube is still on the magnet.

163 Take off the sample tube from the magnet.

164 Add **100 µl 1X BWB** to the sample tube and vortex mix. (1/4)

165 Remove the supernatant while the tube is still on the magnet.(1/4)

166 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear. (1/4)

167 Take off the sample tube from the magnet.(1/4)

168 Repeat the washing cycle (Steps 164-167) three more times.
go to step #164

169 Add **100 µl EB** and vortex mix.

170 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

171 Remove the supernatant while the tube is still on the magnet.

172 Add **100 µl EB** with the tube kept on the magnet.

173 Remove the supernatant, take off the sample tube from the magnet, quick spin, and put the sample tube back on the magnet.

174 Remove the residual EB with a P-10 or a P-20 pipet.

175 Close the lid and put on ice.

176 Prepare the **A-tailing mix** on ice.

Reagent	Amount
10X KAPA A-Tailing Buffer	1.0 µl
KAPA A-Tailing Enzyme	0.6 µl
H2O	8.4 µl
Total volume	**10 µl**

A-tailing mix

Make 5% extra volume.

176.1 Take **8.4 µl H2O** in a tube of appropriate size.

176.2 Add **1 µl 10X KAPA A-Tailing Buffer** .

176.3 Add **0.6 µl KAPA A-Tailing Enzyme** .

177 Add **10 µl A-tailing mix** to the sample tube (from Step 175), pipet mix, and proceed immediately to the next step.

178 Incubate **00:30:00** at **30 °C** (followed by a hold at **4 °C**) in a PCR machine.

179 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

180 Remove the supernatant while the tube is still on the magnet.

181 Take off the sample tube from the magnet.

182 Add **100 µl** **1X BWB** to the sample tube and vortex mix. (1/4)

183 Remove the supernatant while the tube is still on the magnet.(1/4)

184 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear. (1/4)

185 Take off the sample tube from the magnet.(1/4)

186 Repeat the washing cycle (Steps 182-185) three more times.
go to step #182

187 Add **100 µl** **EB** and vortex mix.

188 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

189 Remove the supernatant while the tube is still on the magnet.

190 Add **100 µl** **EB** with the tube kept on the magnet.

191 Remove the supernatant, take off the sample tube from the magnet, quick spin, and put the sample tube back on the magnet.

192 Remove the residual EB with a P-10 or a P-20 pipet.

193 Close the lid and put on ice.

194 Prepare the **ligation-buffer mix** (without the ligase enzyme) on ice.

Reagent	Amount
5X KAPA Ligation Buffer	2.0 µl
H2O	6.0 µl
Total volume	**8 µl**

Ligation-buffer mix

Make 5% extra volume.

194.1 Take **6 µl H2O** in a tube of appropriate size.

194.2 Add **2 µl 5X KAPA Ligation Buffer** .

195 Add **8 µl ligation-buffer mix** and **1 µl 1 µM Illumina TruSeq compatible adapter** to the sample tube (from Step 191) pipet mix, and put on ice.

In the case of multiplexed sequencing, adaptors should be balanced for their nucleotide composition at each sequence position of the index sequence.
When sequencing a single library, it is better that the library is made using a mixed index adapter (e.g., **0.5 µl 1 µM Illumina TruSeq compatible adapter 1** + **0.5 µl 1 µM Illumina TruSeq compatible adapter 2**) to balance the nucleotide composition of the index sequence.
We recommend using the UDI (unique dual-indexed) adapter to identify and remove index-hopped reads in the Illumina sequencing platform.

196 Add **1 µl KAPA T4 DNA ligase** to the sample tube, pipet mix, and proceed immediately to the next step.

197 Incubate for **00:15:00** at **20 °C** (followed by a hold at **4 °C**) in a PCR machine.

198 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

199 Remove the supernatant while the tube is still on the magnet.

200 Take off the sample tube from the magnet.

201 Add **100 µl 1X BWB** to the sample tube and vortex mix. (1/4)

202 Remove the supernatant while the tube is still on the magnet.(1/4)

203 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear. (1/4)

204 Take off the sample tube from the magnet.(1/4)

205 Repeat the washing cycle (Steps 201-204) three more times.
go to step #201

206 Add **100 µl EB** and vortex mix.

207 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

208 Remove the supernatant while the tube is still on the magnet.

209 Add **100 µl EB** with the tube kept on the magnet.

210 Remove the supernatant, take off the sample tube from the magnet, quick spin, and put the sample tube back on the magnet.

211 Remove the residual EB with a P-10 or a P-20 pipet.

212 Close the lid and put on ice.

213 Prepare the **pre-PCR mix** on ice.

Reagent	Amount
2X KAPA HiFi Ready Mix	10 µl
10 µM TPC mix	0.9 µl
H2O	9.1 µl
Total volume	**20 µl**

Pre-PCR mix

Make 5% extra volume.

213.1 Take **9.1 µl H2O** in a tube of appropriate size.

213.2 Add **10 µl 2X KAPA HiFi Ready Mix** .

213.3 Add **0.9 µl 10 µM TPC mix** .

214 Add **20 µl pre-PCR mix** to the sample tube (from Step 212), pipet mix, and proceed immediately to the next step.

215 Perform 4 cycles of PCR amplification at 98°C 45 sec, 4 cycles of (98°C 15 sec, 60°C 30 sec, 72°C 30 sec), 72°C 1 min, and a hold at 4°C.

216 Put the sample tube on the magnetic and wait until the supernatant becomes clear.

217 Transfer the supernatant to a new PCR tube, add **20 µl** (×1 volume) of AMPure XP beads, vortex mix, and wait **00:05:00** at **Room temperature** .

DNA larger than 200 bp will bind to the AMPure XP beads.

218 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

219 Remove the supernatant using a P-200 pipet while the sample tube is still on the magnet.

220 Add **200 µl 80% EtOH** to the beads while the sample tube is still on the magnet and wait **00:00:30** . (1/2)

221 Remove EtOH with a P-200 pipet while the sample tube is still on the magnet. (1/2)

222 Repeat the washing cycle (Steps 220-221) once more.
go to step #220

223 Quick spin the sample tube, put on magnet and remove the residual EtOH completely using a P-10 or a P-20 pipet.

224 Air dry the beads for ~ 00:01:00 at Room temperature with the lid kept open.

225 Add 11 µl **EB** to the beads, vortex mix, and incubate 00:02:00 at Room temperature .

226 Quick spin the sample tube, put on magnet and collect the eluate in a new PCR tube.

227 Prepare the **real-time PCR mix** on ice

Reagent	Amount (x1 reaction)
2X KAPA HiFi HS real-time Mix	5 µl
10 µM TPC mix	0.35 µl
H2O	3.15 µl
Total volume	**8.5 µl**

Real-time PCR mix

Make one extra reaction for the negative control that does not contain any DNA template.
Make 5% extra volume.

227.1 Take 3.15 µl **H2O** in a tube of appropriate size.

227.2 Add 5 µl **2X KAPA HiFi HS real-time Mix** .

227.3 Add 0.35 µl **10 µM TPC mix** .

228 Dispense 8.5 µl **real-time-PCR mix** in a well of a PCR plate (384 well or 96 well) and add 1.5 µl of the pre-PCR product (from Step 224).

229 Dispense **10 µl** of each Fluorescence Standards (FS1~4) in the same plate in separate wells.

230 Run real-time PCR in a ROX minus condition at, 98°C 45 sec, 20 cycles of (98°C 15 sec, 60°C 30 sec, 72°C 30 sec), 72°C 1 min.

231 Determine the threshold-PCR-cycle (Ct) that reaches Fluorescence Standard 1 (FS1) but does not exceed Fluorescence Standard 2 (FS2).

DNA for the library QC (QC2) is prepared by amplifying a small aliquot (1 µl) of the pre-PCR product with (Ct +3) cycles of PCR.
Hi-C library for sequencing is prepared by amplifying the remaining 8.5 µl of the pre-PCR product with the pre-determined Ct cycle.

232 Prepare the **PCR-mix for Hi-C library QC (QC2)** on ice.

Reagent	Amount
2X KAPA HiFi HotStart Ready Mix	5 µl
10 µM TPC	0.45 µl
H2O	3.55 µl
Total volume	**9 µl**

PCR-mix for Hi-C library QC (QC2)

Make 5% extra volume.

232.1 Take **3.55 µl H2O** in a tube of appropriate size.

232.2 Add **5 µl 2X KAPA HiFi HotStart Ready Mix** .

232.3 Add **0.45 µl 10 µM TPC** .

233 Dispense **9 µl** of the **PCR-mix for Hi-C library QC (QC2)** in a PCR tube, and add **1 µl** of the pre-PCR product (from Step 224).

234 Perform PCR amplification at, 98°C 45 sec, (Ct +3) cycles of (98°C 15 sec, 60°C 30 sec, 72°C 30 sec), 72°C 1 min, and a hold at 4°C.

Proceed to Step 250 in the next section after the PCR.

235 Prepare the **PCR-mix for Hi-C library amplification**.

Reagent	Amount
2X KAPA HiFi HotStart Ready Mix	10 µl
10 µM TPC	0.9 µl
H2O	0.6 µl
Total volume	**11.5 µl**

PCR-mix for Hi-C library amplification

Make 5% extra volume.

235.1 Take **0.6 µl** **H2O** in a tube of appropriate size.

235.2 Add **10 µl** **2X KAPA HiFi HotStart Ready Mix** .

235.3 Add **0.9 µl** **10 µM TPC** .

236 Add **11.5 µl** of the **PCR-mix for Hi-C library amplification** to the tube containing the pre-PCR product (from Step 226).

237 Perform PCR amplification at, 98°C 45 sec, (Ct) cycles of (98°C 15 sec, 60°C 30 sec, 72°C 30 sec), 72°C 1 min, and a hold at 4°C.

238 Add **20 µl** (×1 volume) of AMPure XP beads, vortex mix, and wait **00:05:00** at **Room temperature** .

239 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

240 Remove the supernatant using a P-200 pipet while the sample tube is still on the magnet.

241 Add 200 µl **80% EtOH** to the beads while the sample tube is still on the magnet and wait 00:00:30 . (1/2)

242 Remove EtOH with a P-200 pipet while the sample tube is still on the magnet. (1/2)

243 Repeat the washing cycle (Steps 241-242) once more.
go to step #241

244 Quick spin the sample tube, put on magnet and remove the residual EtOH completely using a P-10 or a P-20 pipet.

245 Air dry the beads for ~ 00:01:00 at Room temperature with the lid kept open.

246 Add 30 µl **EB** to the beads, vortex mix, and incubate 00:02:00 at Room temperature .

247 Quick spin the sample tube, put on magnet and collect the eluate in a new microtube (1.5 ml).

248 Quantitate the DNA using 1 µl of the DNA sample with the Qubit dsDNA High Sensitivity Kit.

249 Analyze the size-distribution using 2 µl of the library DNA using the Agilent TapeStation with the High Sensitivity D1000 tape.

Section 12. (DAY 4) Quality control of the Hi-C library (QC2)

250 Add 10 µl (×1 volume) of AMPure XP beads to the PCR product from the previous section (Step 234), vortex mix, and wait 00:05:00 at Room temperature .

DNA larger than 200 bp will bind to the AMPure XP beads.

251 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

252 Remove the supernatant using a P-200 pipet while the sample tube is still on the magnet.

253 Add **200 µl** **80% EtOH** to the beads while the sample tube is still on the magnet and wait **00:00:30** . (1/2)

254 Remove EtOH with a P-200 pipet while the sample tube is still on the magnet. (1/2)

255 Repeat the washing cycle (Steps 253-254) once more.
go to step #253

256 Quick spin the sample tube, put on magnet and remove the residual EtOH completely using a P-10 or a P-20 pipet.

257 Air dry the beads for ~ **00:01:00** at **Room temperature** with the lid kept open.

258 Add **5 µl** **EB** to the beads, vortex mix, and incubate **00:02:00** at **Room temperature** .

259 Quick spin the sample tube, put on magnet and collect the eluate in a new PCR tube.

260 Quantitate the DNA using **1 µl** of the DNA sample with the Qubit dsDNA High Sensitivity Kit.

261
Prepare the **restriction-mix for QC2** with or without the restriction enzyme (RE)

Reagent	Amount
10X buffer	1 µl
RE (10 U/µl) or H2O	0.3 µl
20 mg/ml BSA	0.05 µl
H2O	µl
Total volume	**µl**

Restiction-mix for QC2

Adjust the volume of H_2O based on the concentration of the amplified library DNA.
The restriction enzyme for the DpnII-digested library is ClaI.
The restriction enzyme for the HindIII-digested library is NheI.
Make 5% extra volume.

262 Dispense the **restriction-mix for QC2** (with or without the RE) to a PCR tube, add **10 ng** - **20 ng** of the amplified library DNA (from Step 259), adjust the total volume of the reaction to **10 µl** , and incubate **00:30:00** at **37 °C** .

263 Add **18 µl** (×1.8 volume) of AMPure XP beads, vortex mix, and wait **00:05:00** at **Room temperature** .

DNA larger than 100 bp will bind to the AMPure XP beads.

264 Quick spin the sample tube, put on magnet and wait until the supernatant becomes clear.

265 Remove the supernatant using a P-200 pipet while the sample tube is still on the magnet.

266 Add **200 µl** **80% EtOH** to the beads while the sample tube is still on the magnet and wait **00:00:30** . (1/2)

267 Remove EtOH with a P-200 pipet while the sample tube is still on the magnet. (1/2)

268 Repeat the washing cycle (Steps 266-267) once more.
go to step #266

269 Quick spin the sample tube, put on magnet and remove the residual EtOH completely using a P-10 or a P-20 pipet.

270 Air dry the beads for ~ **00:01:00** at **Room temperature** with the lid kept open.

271 Add **10 µl** **EB** to the beads, vortex mix, and incubate **00:02:00** at **Room temperature** .

272 Quick spin the sample tube, put on magnet and collect the eluate in a new PCR tube.

273 Analyze the size difference between the RE(-) and the RE(+) samples using **2 µl** of DNA with the Agilent TapeStation using the High Sensitivity D1000 tape.

Place RE(-) and RE(+) samples for the same library side-by-side for easy comparison.

Section 1. (DAY 0) Preparation of cells/tissue

step case

Animal tissue

Sample preparation procedure for an animal tissue of any kind (both fibrous and soft tissue)

2 Dissect and collect tissue in a microtube (1.5 or 2.0 ml) and freeze immediately in liquid nitrogen.

Store the frozen tissue in an ultra-low temperature freezer (e.g., **-80 °C**).

3 Pre-cool a mortar and a pestle in an ultra-low temperature freezer (e.g., **-80 °C**) for at least **01:00:00** .

4 Pour liquid nitrogen into the pre-cooled mortar with a pestle.

step case

Alternative procedure for a soft tissue

Alternative sample preparation procedure for a soft tissue using a frost-mill

2 Dissect and collect tissue in a microtube (1.5 or 2.0 ml) and freeze immediately in liquid nitrogen.

Store the frozen tissue in an ultra-low temperature freezer (e.g., **-80 °C**).

3 Pre-cool the Tokken stainless-steel tubes and bullets in liquid nitrogen.

4 Transfer tissue (up to 4 mm^3 in size) into each stainless-steel tube.

Break a large tissue into small pieces if necessary.
Do not let the tissue thaw during the powderization process.