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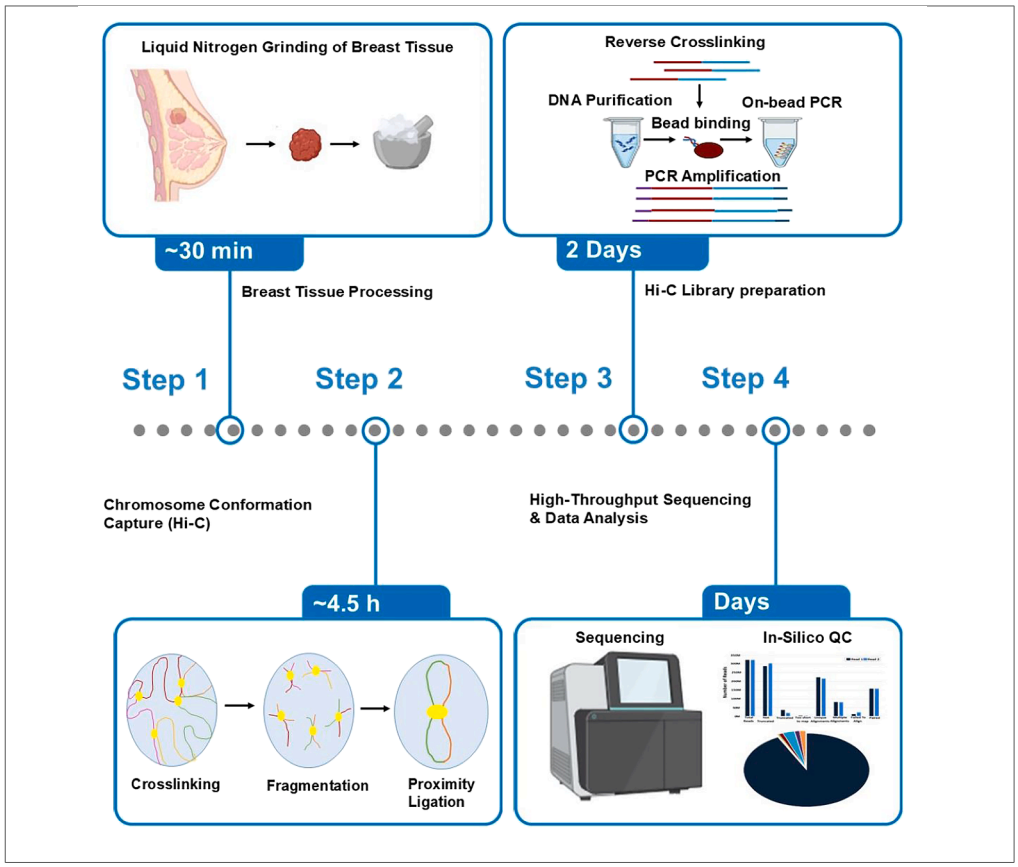
High-resolution Hi-C profiling of human breast tissues using an optimized protocol for clinical samples



Here, we present an optimized high-resolution Hi-C protocol for human breast tissues using the Phase Genomics Proximo Hi-C Kit. We describe steps for liquid nitrogen tissue processing, crosslinking, and quenching, followed by cell lysis and controlled chromatin fragmentation. Proximity ligation captures three-dimensional interactions, followed by reverse crosslinking, DNA purification, and streptavidin bead enrichment. Libraries are prepared on beads, amplified, and cleaned with size selection, producing high-quality material for Illumina sequencing and genome-wide 3D chromatin analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Lavanya Choppavarapu, Kun Fang, Stephen Eacker, Kyle Langford, Janet S. Rader, Victor X. Jin

vjin@mcw.edu

Highlights

An optimized protocol for Hi-C library preparation from human breast tissues

Preserves native chromatin interactions via controlled tissue processing

Enzymatic fragmentation and proximity ligation to capture spatial chromatin organization

Quality control to ensure high-quality Hi-C libraries suitable for sequencing

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High-resolution Hi-C profiling of human breast tissues using an optimized protocol for clinical samples

Lavanya Choppavarapu,[1,2,5] Kun Fang,[1,2] Stephen Eacker,[3] Kyle Langford,[3] Janet S. Rader,[2,4] and Victor X. Jin[1,2,6,*]

[1]Divison of Biostatistics, Data Science Institute, Medical College of Wisconsin, Milwaukee, WI 53226, USA

[2]MCW Cancer Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA

[3]Phase Genomics, Seattle, WA 98109, USA

[4]Department of Obstetrics and Gynecology, Medical College of Wisconsin, Milwaukee, WI 53226, USA

[5]Technical contact

[6]Lead contact

*Correspondence: vjin@mcw.edu
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SUMMARY

Here, we present an optimized high-resolution Hi-C protocol for human breast tissues using the Phase Genomics Proximo Hi-C Kit. We describe steps for liquid nitrogen tissue processing, crosslinking, and quenching, followed by cell lysis and controlled chromatin fragmentation. Proximity ligation captures three-dimensional interactions, followed by reverse crosslinking, DNA purification, and streptavidin bead enrichment. Libraries are prepared on beads, amplified, and cleaned with size selection, producing high-quality material for Illumina sequencing and genome-wide 3D chromatin analysis.

For complete details on the use and execution of this protocol, please refer to Choppavarapu et al.[1]

BEFORE YOU BEGIN

Prepare the mortar and pestle

1. Clean the mortar and pestle thoroughly with 70% ethanol and allow them to dry completely.
2. Pre-cool the mortar by filling it with liquid nitrogen and allowing it to evaporate. Repeat this cooling step 2–3 times to ensure full cryogenic conditioning.

Set up the cryogenic workstation

3. Prepare a stable work surface designated for liquid nitrogen grinding.
4. Place a Styrofoam container filled with liquid nitrogen within easy reach.
5. Keep dry ice accessible in an insulated container.
6. Pre-chill microcentrifuge tubes and a tube rack by placing them on dry ice.

Prepare the magnetic rack

7. Ensure the magnetic rack is clean and free of residual beads.
8. Position the magnetic rack in an easily accessible location for rapid sample transitions during the protocol.

Innovation

Achieving high-quality Hi-C data from solid human tissues is challenging due to difficulties in efficient dissociation, preservation of nuclear integrity, and prevention of chromatin damage. Here,

we present a key innovation: a pre-crosslinking liquid nitrogen grinding step that enables uniform and gentle pulverization of dense tissues such as human breast samples. This overcomes limitations of conventional homogenization, which often causes incomplete lysis, excessive shearing, or variable sample quality. Integrating this cryogenic disruption into the Phase Genomics Proximo Hi-C workflow improves nuclei recovery, enhances proximity ligation efficiency, and supports high-yield, high-complexity Hi-C libraries for reliable 3D chromatin mapping.

Chromosome Conformation Capture (3C) technologies, particularly Hi-C, have transformed our understanding of genome organization by identifying physically interacting DNA loci. Hi-C provides genome-wide insights into chromatin architecture using high-throughput sequencing.[2,3] The Phase Genomics Proximo Hi-C (Human) Kit (Phase Genomics, Seattle, WA) offers a robust and streamlined workflow for preparing dual-indexed Hi-C libraries from human tissues, from initial processing to sequencing-ready constructs.

While the standard Proximo Hi-C workflow works well for dissociable samples like cultured cells or blood, solid tissues such as human breast present challenges.[4] Dense cellular composition and extracellular matrix can compromise dissociation and chromatin integrity, limiting downstream ligation. To overcome this, we optimized the protocol (Figure 1) with a controlled liquid nitrogen grinding step[5] before crosslinking, ensuring uniform tissue pulverization, preserved nuclei, and minimal DNA shearing. This approach reliably generates high-quality Hi-C libraries for mapping 3D genome architecture, detecting structural variants, and analyzing epigenetic landscapes in both normal and diseased tissues.[1,6–8]

Institutional review board approval

Ensure that appropriate Institutional Review Board (IRB) approval has been obtained for the collection and use of human breast tissue samples. All procedures must comply with institutional and national guidelines for human tissue research.

Tissue collection planning

Coordinate with the surgical teams to minimize the time between tissue excision and processing. Tissue can be either processed immediately or flash-frozen in liquid nitrogen as soon as possible after collection to preserve chromatin structure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kits		
Proximo Hi-C Kit (Human)	Phase Genomics	KT6040B
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Kit components (from Proximo Hi-C Kit)		
Cross Linking Solution	Phase Genomics	KS0008
Quench Solution	Phase Genomics	KS0003
Lysis Buffer 1	Phase Genomics	KB0036
Lysis Buffer 2	Phase Genomics	KB0003
Fragment Buffer	Phase Genomics	KB0038
Fragment Enzyme	Phase Genomics	KB0025
Finishing Enzyme	Phase Genomics	KB0019
Stop Solution	Phase Genomics	KB0010
Ligation Buffer	Phase Genomics	KB0022
Ligation Enzyme	Phase Genomics	–
Rx Enzyme	Phase Genomics	KB0023

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Elution Buffer	Phase Genomics	KB0031
Recovery Beads	Phase Genomics	KR0011
Recovery Wash Buffer	Phase Genomics	KB0035
Strept Beads	Phase Genomics	KR0010
Bead Bind	Phase Genomics	KB0029
Wash Buffer 1	Phase Genomics	KB0023
Wash Buffer 2	Phase Genomics	KB0024
Universal Adapter	Phase Genomics	–
FERAT Buffer	Phase Genomics	–
FERAT Enzyme	Phase Genomics	–
Adapter Ligation	Phase Genomics	–
Hot Start Mix	Phase Genomics	–
Primer	Phase Genomics	KP000N
10XCRB	Phase Genomics	KB0017
Restriction enzymes (from Proximo Hi-C Kit)		
DpnII-GATC	Phase Genomics	–
DdeI-CTNAG	Phase Genomics	–
MseI-TTAA	Phase Genomics	–
HinfI-GANTC	Phase Genomics	–
Chemicals		
95% Ethanol	Fisher BioReagents	BP2818500
RNase/DNase-free water	Invitrogen	10977015
Liquid nitrogen	N/A	N/A
1X PBS, sterile, ice-cold	Gibco	14190144
Dry Ice	N/A	N/A
Equipment/Other		
Mortar and pestle	Fisher Scientific	Cat# 539132
Styrofoam container for liquid nitrogen	Fisher Scientific	N/A
Microcentrifuge tubes (1.5 mL)	Eppendorf	Cat# 022363204
Microcentrifuge tubes (2 mL)	Eppendorf	Cat# 022363352
Microcentrifuge tube rack	Microcentrifuge tube rack	Cat# 05-541-25
Forceps	VWR	Cat# 82027-406
Scalpel handle with blades	Fisher Scientific	Cat# 08-920
Laboratory balance	Sartorius	Cat# BCE124I1S
Spatulas (metal, pre-chilled)	Fisher Scientific	Cat# 21-401-10
Magnetic rack for microcentrifuge tubes	Thermo Fisher Scientific	Cat# 12321D
Thermocycler	Applied Biosystems	Model # 2720
Microcentrifuge	Eppendorf	Cat# 022620601
Vortex mixer	Fisher Scientific	Cat# 75004061
0.2 mL PCR tubes	Axygen	Cat# PCR-0208-C
Pipettes (adjustable, 0.5–1,000 μL)	Thermo Fisher Scientific	N/A
Filtered pipette tips (sterile)	Axygen	N/A
Software and algorithms		
Bowtie2	Johns Hopkins University	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
HiCUP	Babraham Bioinformatics	https://www.bioinformatics.babraham.ac.uk/projects/hicup/

STEP-BY-STEP METHOD DETAILS

The detailed protocol in the following 13 stages

Timing: Tissue preparation and liquid nitrogen grinding: 30 min

Timing: Crosslinking: 1 h

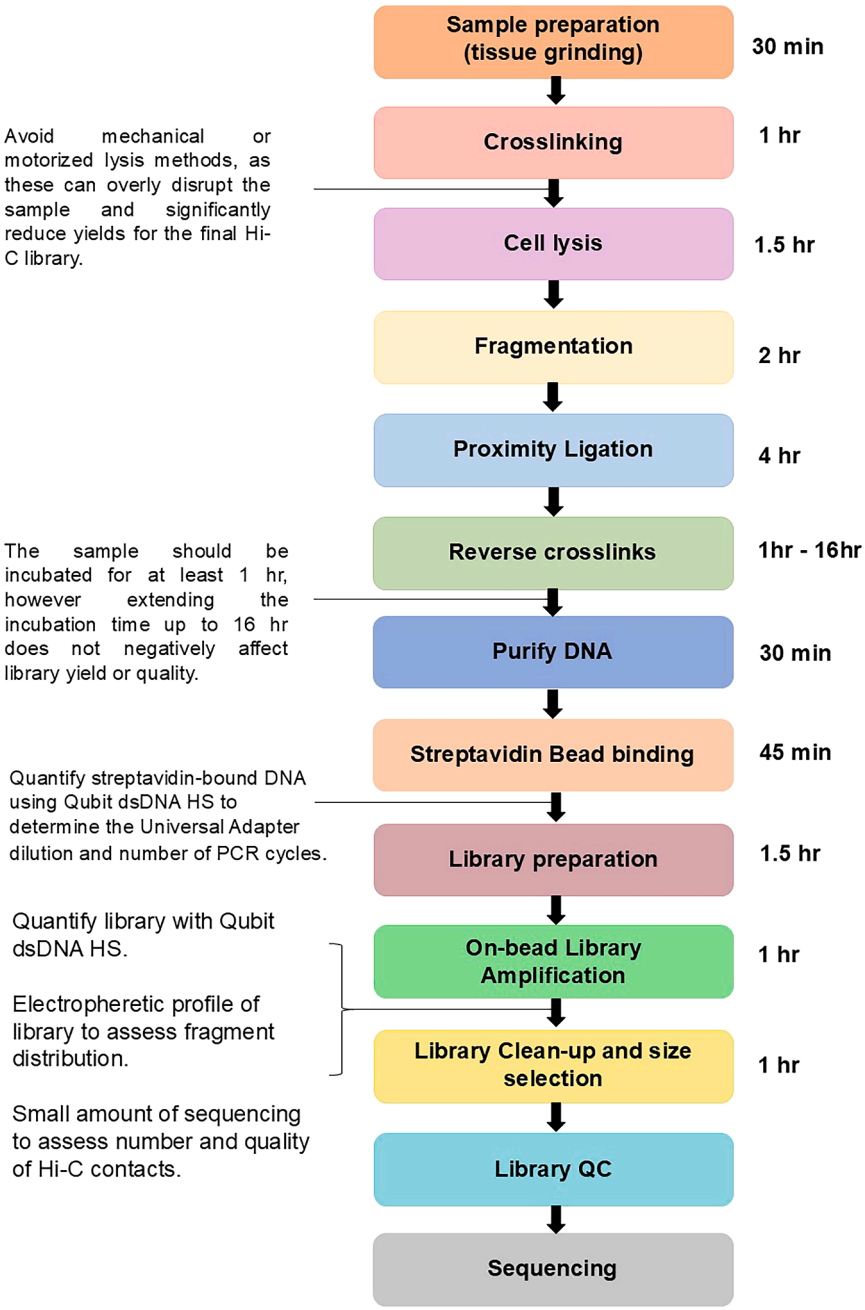

Figure 1. High-Resolution Hi-C Library Preparation Workflow

This flowchart outlines the key experimental stages for generating High-Resolution Chromosome Conformation Capture (Hi-C) libraries from tissue samples. Approximate timing for each step is indicated.

Timing: Cell lysis: 1.5 h

Timing: Fragmentation: 2 h

Timing: Proximity ligation: 4 h

Timing: Reverse crosslinking: 1–16 h (typically overnight)

Timing: DNA purification: 30 min

Timing: Streptavidin bead binding: 45 min

Timing: Library preparation: 1.5 h

Timing: On-bead library amplification: 1 h

Timing: Library clean-up and size selection: 1 h

Liquid nitrogen tissue processing for human breast tissues

Timing: 30 min

This section details the optimized cryogenic workflow required to uniformly pulverize human breast tissue while preserving nuclear and chromatin integrity for high-quality Hi-C library preparation (Figure 2).

1. Sample Collection and Initial Preparation.
   a. Collect fresh human breast tissue samples using appropriate clinical procedures.
   b. Transfer tissue immediately from patient to a sterile empty container.
   c. Transport from OR on ice to sample processing table or liquid nitrogen tank.
   d. Process tissue as soon as possible after collection to preserve chromatin integrity.
   e. If immediate processing is not possible, flash-freeze tissue in liquid nitrogen and
   f. Store at −80°C until processing.

⚠ CRITICAL: Fresh frozen tissue can be stably stored at −80°C for several months (typically up to 6 months) without noticeable loss of chromatin integrity. For best results, we recommend processing within 3 months to maximize Hi-C library quality.

2. Pre-cooling Equipment.
   a. Pre-cool the mortar and pestle by filling the mortar with liquid nitrogen and allowing it to evaporate completely. Repeat this process 2–3 times.
   b. Pre-chill microcentrifuge tubes and tube rack on dry ice.
   c. Pre-cool spatulas by placing them in liquid nitrogen briefly, then allowing excess nitrogen to evaporate.
3. Tissue Dissection and Preparation.
   a. If using frozen tissue, maintain the frozen state by keeping the sample on dry ice until ready for processing.
   b. If using fresh tissue, place the sample in a sterile petri dish on ice.
   c. Using sterile forceps and scalpel, dissect the breast tissue to obtain a representative sample of approximately 50–100 mg (roughly 2–4 mm^3, about the size of a small pea).

Note: Weighing is not required for every piece, but it is recommended to check occasionally to ensure samples fall within the target range.

   d. Briefly rinse the tissue fragment in ice-cold 1X PBS to remove blood if present.
   e. Blot the tissue gently on sterile gauze to remove excess PBS.
   f. Weigh the tissue sample and record the mass.
4. Liquid Nitrogen Grinding.
   a. Pour sufficient amount of liquid nitrogen into the pre-cooled mortar to cover the bottom (approximately 1–2 cm depth).

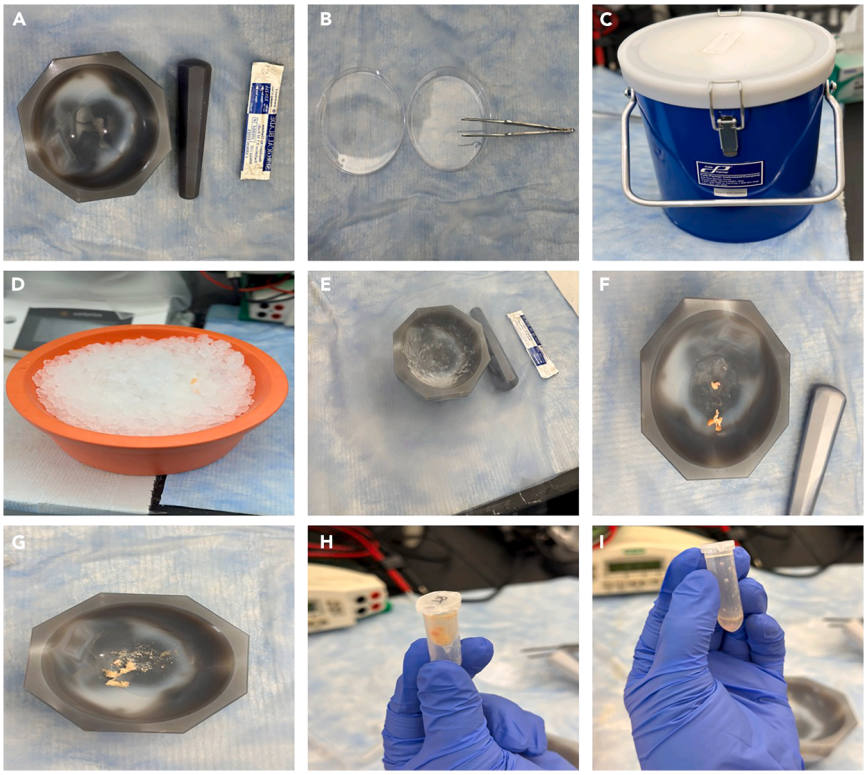

Figure 2. Liquid Nitrogen Tissue Processing for Human Breast Tissue Pulverization

(A) Equipment setup for tissue processing showing mortar and pestle. The mortar and pestle are pre-cooled with liquid nitrogen before use to ensure optimal freezing of the tissue.

(B) Tissue preparation materials including sterile petri dishes and forceps for tissue handling and dissection. These sterile tools are essential for maintaining aseptic conditions.

(C) Liquid nitrogen container. This specialized insulated container is used for safe storage and dispensing of liquid nitrogen, which is crucial for flash-freezing tissue and maintaining extremely low temperatures during the grinding process.

(D) Orange bowl containing ice for pre-chilling microcentrifuge tubes and maintaining the frozen state of the tissue sample. Maintaining cold temperatures throughout the process is critical for sample integrity.

(E) Mortar and pestle with liquid nitrogen. This image shows the pre-cooled mortar, now filled with liquid nitrogen, ready to receive the tissue sample. The liquid nitrogen rapidly freezes the tissue, making it brittle and amenable to pulverization.

(F) Tissue sample in mortar with liquid nitrogen being added. The tissue must become completely frozen and brittle (typically 1-2 minutes) before grinding begins to ensure efficient and uniform pulverization into a fine powder.

(G) Grinded tissue with pestle in mortar. Pulverized tissue powder in the mortar after grinding. The tissue should be reduced to a fine, homogeneous powder before collection.

(H) Collection of pulverized tissue in pre-chilled microcentrifuge tube. After collection, the tube is immediately closed and placed on dry ice to prevent thawing and degradation of the sample.

(I) Resuspension of tissue powder in PG Shield buffer. The suspension is gently mixed and incubated at room temperature for 30 minutes with periodic inversion to ensure complete penetration and stabilization of the tissue fragments by the buffer.

⚠ CRITICAL: Always wear appropriate personal protective equipment when handling liquid nitrogen.

b. Using pre-cooled forceps, transfer the tissue sample to the mortar containing liquid nitrogen.
c. Allow the tissue to become completely frozen and brittle (approximately 1–2 minutes).
d. Add more liquid nitrogen as needed to maintain sufficient volume during grinding.
e. Using the pestle, carefully pound the frozen tissue initially to break it into smaller pieces.
f. Once fragmented, grind the tissue with firm, consistent pressure in a circular motion until a fine powder is achieved.

⚠ CRITICAL: Maintain the frozen state throughout grinding to prevent DNA degradation and chromatin loss by adding liquid nitrogen to the mortar as needed.

g. The tissue should be reduced to a fine, homogeneous powder.
h. Continue grinding for 3–5 minutes to ensure complete homogenization.

5. Collection of Pulverized Tissue.
   a. Allow excess liquid nitrogen to evaporate from the mortar but work quickly to prevent sample thawing.
   b. Using a pre-cooled spatula, quickly transfer the pulverized tissue powder to a prechilled 1.5 mL microcentrifuge tube.
   c. Close the tube immediately and place it on dry ice.
   d. If processing multiple samples, clean the mortar and pestle thoroughly between samples.
      i. Rinse with 70% ethanol.
      ii. Allow to dry completely.
      iii. Pre-cool again with liquid nitrogen before processing the next sample.

Crosslinking

Timing: 1 h

This section details the crosslinking of samples, a crucial step for preserving molecular interactions, followed by the quenching process to halt the crosslinking reaction. Unless otherwise noted, all reagents listed are included in the Proximo Human Hi-C kit.

6. Crosslinking.
   a. Add 1ml of Crosslinking Solution to each sample tube.
   b. Gently vortex to ensure thorough mixing.
   c. Incubate samples at room temperature (RT) for 15 minutes, mixing occasionally by inversion or rotation.
7. Quenching.
   a. Add 100 µL of Quenching Solution to each sample tube.
   b. Gently vortex to ensure thorough mixing.
   c. Incubate at RT for 20 minutes, with occasional mixing by inversion or rotation.
   d. Centrifuge samples at **6,000 x** *g* for 5 minutes at RT to pellet all sample material.
   e. Carefully remove and discard the supernatant without disturbing the pellet.
   f. Wash the pellet by adding 1 mL of 1X Chromatin Rinse Buffer (CRB).

Note: To prepare 1X CRB from a 10X stock solution supplied with the Proximo kit, simply dilute the 10X CRB with distilled water at a 1:9 ratio. For example, to make 100 mL of 1X CRB, you would mix 10 mL of 10X CRB with 90 mL of distilled water. Once prepared, 1X CRB is stable and can be stored at room temperature for up to 1 year.

   g. Centrifuge at **6,000 x** *g* for 1 minute to gently compact the cellular material.
   h. Carefully remove and discard the supernatant.

Pause point: Pellet can be stored at −25°C to −15°C.

Cell lysis

Timing: 1.5 h

In this step, cells are lysed to release chromatin, and crosslinked DNA-protein complexes are captured on Recovery Beads to preserve interactions for downstream Hi-C analysis.

8. Vortex Lysis Buffer 1 to ensure any settled particulates are fully resuspended.
9. Resuspend the sample in 700 µL of Lysis Buffer 1.
10. Mix thoroughly by gently vortexing or pipetting up and down.
11. Incubate at room temperature (RT) for 20 minutes, mixing occasionally by inversion or rotation.
12. Centrifuge the sample at **500 x** *g* for 1 minute at RT. The chromatin will be in the supernatant.
13. Carefully transfer the chromatin-containing supernatant to a fresh microcentrifuge tube.

⚠ CRITICAL: While it's important to avoid transferring large amounts of cellular debris, a small amount of tissue debris is generally not a concern.

14. Centrifuge the supernatant at **6,000 x** *g* for 5 minutes at RT. The chromatin will now be in the pellet.
15. Discard the supernatant carefully.
16. Resuspend the chromatin pellet in 500 µL of 1X CRB.
17. Centrifuge at **6,000 x** *g* for 5 minutes at RT.
18. Discard the supernatant.

⏸ **Pause point:** The sample pellet can be stored at −25°C to −15°C for up to 1 month.

19. Resuspend the chromatin pellet in 100 µL of Lysis Buffer 2.
20. Transfer the entire sample to a 0.2 mL PCR tube.
21. Incubate at 65°C for 15 minutes.
22. Allow the sample tube to cool briefly.
23. Thoroughly resuspend the Recovery Beads by vortexing.
24. Add 100 µL of Recovery Beads to the sample tube.
25. Mix well by gently vortexing or pipetting thoroughly.

Note: Chromatin will bind irreversibly to the Recovery Beads. The crosslinked DNA-protein complexes will remain bound to the beads until the reverse crosslinking step is completed.

26. Incubate at RT for 10 minutes.
27. Wash Beads:
    a. Place the sample tube in a magnetic rack or on a magnet.
    b. Once the solution has cleared, carefully remove the supernatant without disturbing the beads.
    c. Remove the tube from the magnet and gently resuspend the beads in 200 µL of 1X CRB.

⏸ **Pause point:** Bead bound sample may be stored in 1XCRB at +2 to +8°C overnight.

Fragmentation

⏱ **Timing: 2 h**

In this stage, bead-bound chromatin is enzymatically fragmented to generate appropriately sized DNA fragments while preserving crosslinked interactions, followed by washes to prepare the sample for proximity ligation

It is recommended that the incubations in this stage are conducted in a thermocycler. Make sure program is setup before beginning.

28. Remove the sample tube from the magnetic rack.
29. Gently resuspend the bead-bound sample in 148 µL of Fragmentation Buffer (Proximo Hi-C Kit).
30. Add 2.5 µL of Fragmentation Enzyme (Proximo Hi-C Kit) to the sample.

31. Mix thoroughly by gentle vortexing or pipetting.
32. Incubate the reaction at 37°C for 60 minutes.
33. Immediately transfer the sample to 4°C and cool for a minimum of 1 minute.
34. Once cooled, add 2.5 µL of Finishing Enzyme (Proximo Hi-C Kit) to the reaction.
35. Mix thoroughly by gentle vortexing or pipetting.
36. Incubate the reaction at 12°C for 30 minutes.

⚠ CRITICAL: Promptly add Stop Solution immediately after the 30 min incubation at 12°C. Extended incubation at 12°C beyond 30 min may compromise library quality.

37. Add 6 µL of Stop Solution (Proximo Hi-C Kit) to terminate the reaction.
38. Mix thoroughly by gentle vortexing or pipetting.
39. Wash the beads:
    a. Place the sample tube in a magnetic rack.
    b. Allow the beads to clear (typically 2–3 minute).
    c. Carefully remove the supernatant without disrupting the bead pellet.
    d. Remove the tube from the magnet.
    e. Gently resuspend the beads in 200 µL of 1XCRB.
    f. Repeat the bead wash steps one more time with 200ul of 1xCRB per wash, for a total of two washes.

Pause point: The bead-bound sample can be stored in 200 µL of 1X CRB at 2°C–8°C overnight.

Proximity ligation

Timing: 4–5 h

This section details the proximity ligation reaction, a critical step ligating DNA ends that are in close proximity mediated by crosslinked protein complexes. This step creates chimeric DNA molecules that preserve 3D DNA sequence interaction information.

40. If your bead-bound sample was stored in 1X CRB, carefully remove the buffer before proceeding.
41. Add 98 µL of Ligation Buffer to the bead-bound sample.
42. Add 2 µL of Ligation Enzyme.
43. Mix thoroughly by pipetting.
44. Incubate the sample using the following thermal cycler program:

Step	Temperature (°C)	Time
Ligation	25	4 hours
Enzyme Inactivation	65	10 minutes
Final hold	4	Hold

Pause point: Store sample at 2°C–8°C overnight.

Reverse crosslinks

Timing: 1–16 h

This step reverses formaldehyde crosslinks, releasing DNA from protein complexes into solution for downstream purification and Hi-C library preparation.

45. Preheat a thermocycler to 65°C.
46. Add 5μL of Rx Enzyme to the ligation reaction.
47. Mix thoroughly by vortexing or pipetting.
48. Incubate at 65°C for at least 1 hour (up to 16 hours).

Note: After this incubation, the sample is no longer bound to the beads and has been released into solution.

Pause point: After the 1-h incubation at 65°C, the reaction can be incubated at 65°C overnight or stored at 2°C to 8°C overnight.

Purify DNA

Timing: 30 min

This section describes the purification of proximity-ligated DNA from bead-bound complexes, removing contaminants and recovering clean DNA for downstream Hi-C library preparation.

49. Prepare Recovery Wash Buffer by adding 8 mL of 95% ethanol (not included in kit) to the bottle containing 1.6 mL of provided concentrate. Mix by inverting several times.
50. Allow sample tube to cool to room temperature.
51. Resuspend Recovery Beads thoroughly by vortexing and add 100 μL to the sample tube.
52. Mix thoroughly by vortexing or pipetting and incubate at room temperature for 10 minutes.
53. Place the sample tube in a magnetic rack until the solution clears.
54. Carefully remove the supernatant without disrupting the beads.
55. While keeping the beads on the magnet, gently rinse the beads with 200 μL of prepared Recovery Wash Buffer. Let sit for 30 sec–1 minute. Repeat for a total of two rinses.
56. Air dry the beads at a room temperature for 5–15 minutes on the magnet with the tube cap open.

Note: Over-drying is not problematic for recovery beads.

57. Remove the sample tube from the magnet. Resuspend beads in 100 μL of Elution Buffer.
58. Incubate at room temperature for 5 minutes to elute the DNA.
59. Place the sample tube on the magnetic until the solution clears.
60. Once the solution has cleared, recover the DNA-containing supernatant and transfer to a fresh tube. Discard the beads.

Pause point: Purified, proximity-ligated DNA may be stored at −25 to −15°C indefinitely.

Streptavidin bead binding

Timing: 45 min

In this stage, purified DNA is captured on Streptavidin beads via biotin-streptavidin binding, enabling selective enrichment of proximity-ligated fragments and preparing the sample for library construction and quantification.

61. Streptavidin Bead Preparation.
    a. Thoroughly resuspend the Streptavidin Beads by vortexing.

b. Transfer 20μL of the resuspended beads into a new microcentrifuge tube (or 0.2 mL PCR tube).
c. Wash the beads:
   i. Place the tube on a magnetic tube rack or magnet.
   ii. Once the solution has cleared, carefully remove the supernatant without disturbing the beads.
   iii. Remove the tube from the magnet and gently resuspend the beads in 200 μL of Wash Buffer 1.
   iv. Repeat this bead wash step one more time with 200 μL of Wash Buffer 1 for a total of two washes.

Note: Do not combine these prepared beads with the DNA recovered in Stage 7 yet.

d. Remove the beads from the magnet and resuspend them in 100 μL of Bead Binding Buffer.

62. Bind DNA to Streptavidin beads.
    a. Transfer 100 μL of the purified DNA from Stage 7 to the microcentrifuge tube containing the washed Streptavidin Beads.
    b. Mix well by gently vortexing or pipetting thoroughly.
    c. Incubate at room temperature for 10 minutes, mixing occasionally by gentle vortexing or inversion.
    d. Wash the beads:
       i. Place the tube on a magnetic tube rack or magnet.
       ii. Once the solution has cleared, carefully remove the supernatant without disturbing the beads.
       iii. Remove the tube from the magnet and gently resuspend the beads in 200 μL of Wash Buffer 2.
       iv. Repeat this bead wash step one more time with 200 μL of Wash Buffer 2 for a total of two washes.
       v. Repeat the bead wash steps one more time with 200 μL of Wash Buffer 1.
       vi. Repeat the bead wash steps one more time with 200 μL of molecular biology-grade water (not included in the Proximo kit).
63. Quantify Bead-Bound DNA.

With your bead bound sample suspended in 200 μL of water, measure the concentration of DNA (while still bound to the streptavidin beads) using a Qubit ™ dsDNA HS assay kit or similar fluorometric assay.

⚠ CRITICAL: Thoroughly resuspend the beads in molecular biology-grade water and vortex immediately before measurement. Accurate quantification ensures sufficient yield for downstream steps and guides adapter dilution and PCR cycles.

⚠ CRITICAL: Beads will interfere with spectrophotometric quantification of bound DNA. Use of fluorometric assay is a requirement.

Library preparation

Timing: 1.5 h

This section describes the processing of bead-bound DNA through fragmentation, end-repair, A-tailing, and adapter ligation to generate sequencing-ready Hi-C libraries while preserving the integrity of proximity-ligated DNA fragments.

64. Prepare for Fragmentation, End-Repair, and A-Tailing.

a. Pre-cool a thermocycler to 4°C.
b. Transfer no more than 200 ng of streptavidin bead-bound DNA to a fresh microcentrifuge tube.
c. Place the sample tube in a magnetic rack or on a magnet.
d. Once the solution clears, carefully remove and discard the supernatant without disturbing the beads.
e. Remove the tube from the magnet and resuspend the beads in 40 μL of molecular biology-grade water.
f. Place the sample in the pre-cooled thermocycler.

65. Perform Fragmentation, End-Repair, and A-Tailing.
    a. Add 4 μL of Frag, Repair, A-Tail Buffer to the sample.
    b. Add 6 μL of Frag, Repair, & A-Tail Enzyme and mix by gently vortexing or pipetting thoroughly.
    c. Proceed with the following thermal cycler program for fragmentation, end-repair, and A-tailing:

Step	Temperature (°C)	Time (minutes)
Lid Temperature	105	
Fragmentation, end-repair, and A-tailing	30	5
	65	30
Final hold	4	Hold

66. Prepare Universal Adapter.

Dilute the Universal Adapter (15μM stock) as needed based on your input DNA mass using molecular biology grade water or 10 mM Tris pH 8 as a diluent.

Input Mass(ng)	Adapter concentration
>10	15 μM
1–10	3 μM
<1	0.3 μM

67. Perform Adapter Ligation.
    a. Add 5 μL of diluted Universal Adapter (diluted if necessary) to the sample and mix by gently vortexing or pipetting thoroughly.
    b. Add 20 μL of Adapter Ligation Mix. Mix by pipetting thoroughly.

    Note: Do not vortex Adapter Ligation Mix.

    c. Incubate the sample using the following thermal cycler program:

Step	Temperature (°C)	Time (minutes)
Lid temperature	Off	
Ligation	20	15

68. Wash the beads.
    a. Place the tube on a magnetic tube rack or magnet.

b. Once the solution has cleared, carefully remove the supernatant without disturbing the beads.
c. Remove the tube from the magnet and gently resuspend the beads in 200 µL of Wash Buffer 2.
d. Repeat this bead wash step one more time with 200 µL of Wash Buffer 2 for a total of two washes.
e. Repeat the bead wash steps one more time with 200 µL of Wash Buffer 1.
f. Repeat the bead wash steps one more time with 200 µL of molecular biology-grade water.

On-bead library amplification

Timing: 1 h

This section describes the on-bead PCR amplification of Hi-C libraries, where bead-bound DNA is amplified using indexed primers to generate sufficient material for sequencing while preserving the proximity-ligated fragment structure.

PCR reaction master mix	
Reagent	Amount
Bead-bound DNA (resuspended in water)	20 µL
PCR Primer Mix (unique index)	5 µL
Hot Start PCR Mix	25 µL
Total Reaction Volume	50 µL

Note: The 20 µL of bead-bound DNA includes molecular biology–grade water added to resuspend the beads.

⚠ CRITICAL: Use a different unique index for each sample (four unique indexes are provided with each kit).

PCR cycling conditions			
Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	45	1
Denaturation	98	15	variable*
Annealing	60	30	
Extension	72	30	
Final extension	72	60	1
Hold	12	hold	

Note: Determine the number of PCR cycles using this table:

Mass used as step 9.1 (ng)	Recommended number of PCR cycles (fresh samples or cells)	Recommended number of PCR cycles (FFPE)
<50	10–11	12
50–75	8–9	10–11
75–100	6–7	8–10
100–200	4–5	7–8

Amplify your libraries beyond the above suggested number of cycles can negatively impact the final data quality. This may require you to separate your samples into separate PCR runs.

Pause point: The PCR reaction can be held overnight at +2°C to +8°C or stored at −25°C to −15°C indefinitely.

Library clean-up and double-sided size selection

Timing: 1 h

This section describes the crucial steps for purifying your amplified library and performing a double-sided size selection to enrich for desired fragment sizes, ensuring optimal sequencing results.

Note: Use Recovery wash buffer prepared at step 6.

69. Place the sample tube on a magnetic tube rack or magnet.
70. Once the solution has cleared, transfer the library-containing supernatant to a new microcentrifuge tube.

Note: The used Streptavidin beads can be stored in 1X CRB for troubleshooting if needed; otherwise, they can be discarded.

71. Add 55 µL (1.1X volume) of thoroughly resuspended Recovery Beads to the tube containing the library.

Note: Unwanted high molecular weight fragments will bind to the beads.

72. Incubate at room temperature for 10 minutes.
73. Place the sample tube on a magnetic tube rack or magnet. Your desired library is in the supernatant at this stage; do not discard it.
74. After 2 minutes, or once the solution has cleared, transfer the supernatant (approx. 105 µL) to a new tube containing 17.5 µL of thoroughly resuspended Recovery Beads.

Note: The library is now binding to the beads, leaving unwanted small fragments in the supernatant.

75. Incubate at room temperature for 10 minutes.
76. Wash the beads:
    a. Place the sample tube in a magnetic rack or on a magnet.
    b. Once the solution has cleared, carefully remove the supernatant without disrupting the beads.
    c. Keeping the beads on the magnet, gently rinse the beads with 200 µL of Recovery Wash Buffer without disturbing the beads. Allow the buffer to remain on the beads for 30 seconds to 1 minute between washes.
    d. Repeat this bead rinse step for a total of two rinses with Recovery Wash Buffer.
77. Air dry the beads at room temperature for 10–15 minutes on the magnet with the cap open.

Note: Over-drying is not problematic for Recovery beads. Air dry the beads by leaving the tube on the magnet for 5–15 minutes with the cap open.

78. Remove the sample tube from the magnet and thoroughly resuspend the beads in 30 µL of Elution buffer.
79. Incubate at room temperature for 5 minute to elute the DNA.

80. Place the sample tube on a magnetic tube rack or magnet.
81. Once the solution has cleared, recover the Proximo Hi-C Library -containing supernatant and transfer it to a fresh microcentrifuge tube. Discard the beads.

Library QC

82. Quantify Library Concentration.

Measure the concentration of your DNA library using a Qubit™ dsDNA HS Assay Kit or a similar fluorometric assay.

> ***Note:*** A yield of over 0.5 ng/μL is a strong indication that library preparation has been successful. The prepared library can be stored at −25°C to −15°C indefinitely.

83. Assess Library Fragment Size.

Assess the library fragment size distribution using a BioAnalyzer (Agilent) or similar instrument.

> ***Note:*** Before performing a full sequencing run, it's highly recommended that you perform low-pass sequencing (approximately 1 million read pairs) to assess the quality of your Hi-C library. These data can be analyzed using our open-source Hi-C analysis tools (available from https://github.com/phasegenomic/hic_qc).

Sequencing

Duration: Variable (dependent on the sequencing platform)

Proximo Hi-C libraries are compatible with any Illumina sequencer. The recommended sequencing depth for Proximo Hi-C libraries varies depending on the genome size and targeted chromatin organizations to ensure sufficient data for scaffolding.

Sequencing recommendation
>200 million pairs per 1 Gbp of genome size (2 x75 bp or longer)[9,10]

> ***Note:*** These recommendations serve as general guidelines for the amount of data required.

In silico quality control

To ensure the quality and reliability of the sequencing data from the Proximo Hi-C libraries, in silico quality control is a critical step. Here, we recommend using the HiCUP pipeline,[11] an open-source bioinformatics tool tailored specifically for processing Hi-C data. HiCUP aligns FASTQ data to a reference genome and efficiently filters out common experimental artifacts. The pipeline generates comprehensive summary statistics at each step, aiding in quality assessment and enabling the identification of potential issues, which facilitates optimization of experimental protocols. A critical quality control metric provided by HiCUP is the ratio of valid pairs. Valid Hi-C pairs are defined as ligation products consisting of DNA fragments originating from distinct genomic regions, where typically one fragment is represented by the forward read and the other fragment by the reverse read (Figure 3A).

84. Build configuration files.

Run the following command to generate a template configuration file.

```
>hicup --example
```

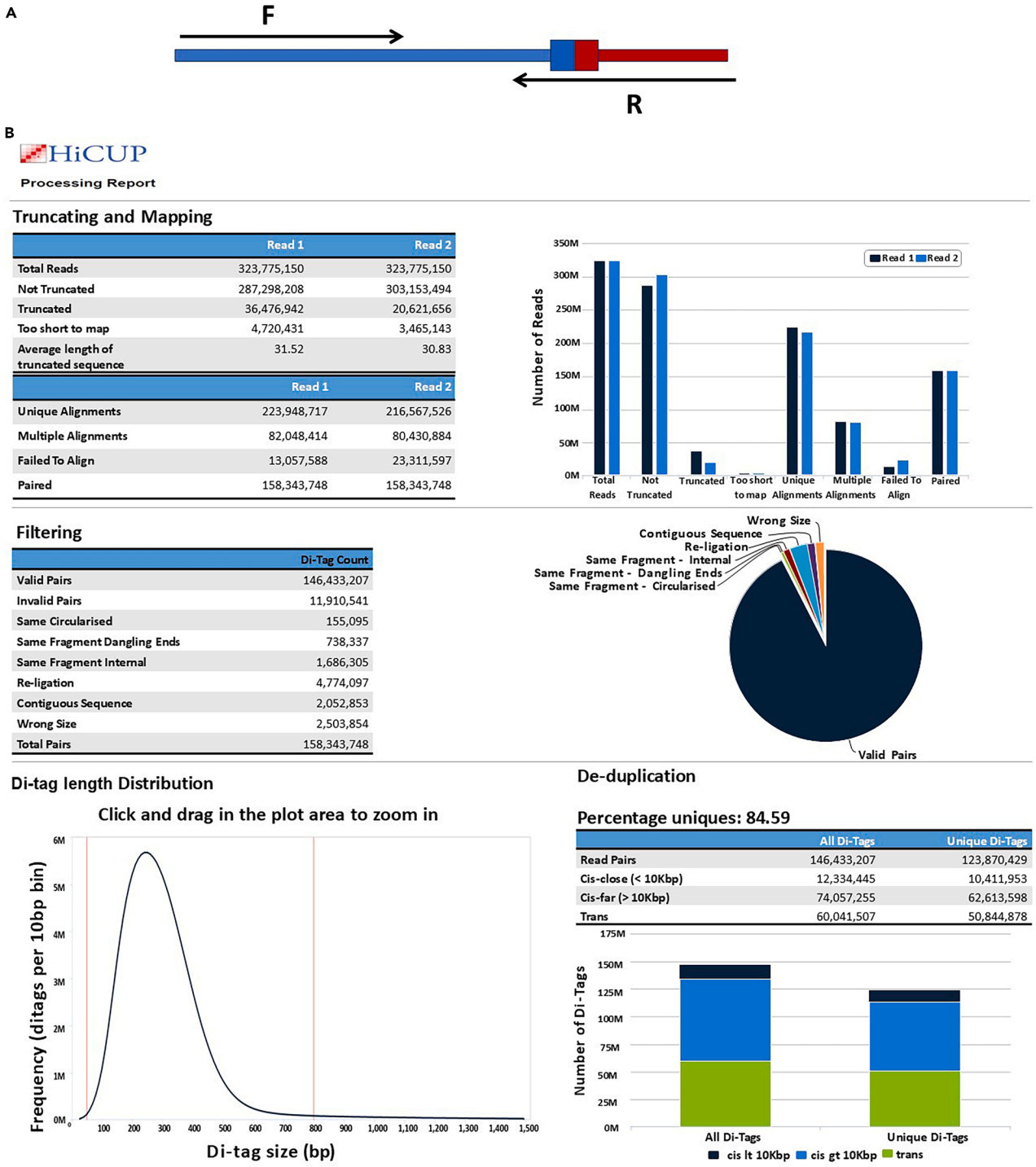

Figure 3. HiCUP quality control metrics presenting various quality control metrics generated by the HiCUP pipeline which illustrates the high-quality Hi-C experimental outcomes

(A) Conceptual diagram illustrating a 'valid pair' in Hi-C data. A valid pair represents two sequenced reads that originate from two distinct restriction fragments which have been successfully ligated together. These pairs are indicative of true chromatin contacts and form the basis for constructing chromosome conformation maps.

(B) HiCUP quality control metrics from a high-quality Hi-C experiment. This panel displays a successful Hi-C run characterized by efficient processing at each stage.

This command creates a file named hicup_example.conf which you must edit to reflect your specific experimental setup.

```
Outdir: work_dir

Threads: 4

Quiet:0

Keep:0

Zip:1

Bowtie2: /usr/local/bowtie2/bowtie2

Digest: Hg38_genome_HindIII_None.txt

#Maximum di-tag length (optional parameter)

Longest: 800

#Minimum di-tag length (optional parameter)

Shortest: 50

# Input FASTQ files

Sample1_1_sequence.fq.gz

Sample1_2_sequence.fq.gz

Sample2_1_sequence.fq.gz

Sample2_2_sequence.fq.gz
```

85. run HiCUP.

Execute the pipeline using your configuration file.

```
>hicup --config hicup_example.conf
```

86. Interpret HiCUP Output.

HiCUP generates an HTML summary report for each processed sample and a consolidated text summary file for all samples. The HTML report contains four key sections.

a. Truncating and Mapping: Provides information about the truncation and mapping of reads, useful for verifying if the intended sequencing depth was achieved.
b. Filtering: Summarizes the composition of read pairs, categorizing them as Valid Pairs, Invalid Pairs, Same Circularised, Same Fragment Dangling Ends, Same Fragment Internal, Re-ligation, Contiguous Sequence, and Wrong Size (Defined in the HiCUP documentation under "Rejected paired reads (Hi-C Experimental Artefacts)"). A Valid Pair proportion greater than 75% (Figure 3B) is recommended. If the proportion falls below 40%, the sequencing quality is considered poor, and redoing the experiment is advisable.
c. Di-tag Length Distribution: Provides insights into the size distribution of ligated fragments. A good experiment typically displays a peak between 0–1000 bp.
d. De-duplication: Indicates the level of duplicated read pairs in the dataset, reflecting the complexity and quality of the library preparation. A good-quality experiment should achieve a percentage of unique reads greater than 80%.

EXPECTED OUTCOMES

When performed correctly, this protocol should yield high-quality Hi-C libraries from human breast tissue samples. A typical library preparation produces 10–50 ng of DNA at a concentration of 0.5–

2 ng/μL. Analysis of fragment size distribution using a BioAnalyzer or similar instrument should show a broad peak centered around 300–500 bp, with minimal adapter dimers (<3%) and limited high molecular weight material. Upon sequencing, high-quality libraries generally demonstrate >70% mapping rate to the reference genome, >40% valid Hi-C interactions (pairs mapping to different restriction fragments), <20% duplicate read pairs, <15% self-ligated fragments, and <20% dangling ends. The resulting chromatin interaction maps should reveal clear compartmentalization patterns (A/B compartments), distinct topologically associating domains (TADs), and identifiable chromatin loops between regulatory elements with reproducible contact patterns across biological replicates. Technical replicates are expected to show high correlation (Pearson $r > 0.8$) in interaction frequencies. Incorporation of the liquid nitrogen grinding step significantly improves crosslinking efficiency in breast tissue samples, yielding higher percentages of valid Hi-C contacts compared to protocols that omit this tissue disruption. Taken together, these outcomes indicate that the protocol reliably generates comprehensive and reproducible Hi-C libraries suitable for downstream analysis of 3D genome organization.

LIMITATIONS

Although this protocol has been optimized for human breast tissue, several limitations should be considered. Breast tissue is inherently heterogeneous, containing varying proportions of epithelial, stromal, and adipose cells, which can influence the uniformity of the Hi-C signal and may require computational approaches to deconvolute cell type-specific interactions. The quality of the starting material is critical, as samples subjected to prolonged ischemia or improper handling may yield suboptimal libraries. The liquid nitrogen grinding step, while improving crosslinking efficiency, can introduce technical variability depending on the consistency of tissue disruption, making careful technique essential for reproducible results. Hi-C provides genome-wide interaction data, but its resolution is inherently limited by sequencing depth, and detecting fine-scale chromatin interactions may require deeper sequencing, particularly in heterogeneous samples. Breast tissue samples with high adipose content may yield lower DNA amounts and require protocol adjustments, such as increased starting material or modified crosslinking times. Additionally, this protocol requires access to specialized equipment, including liquid nitrogen and appropriate safety measures, which may not be available in all laboratory settings. Finally, analysis of Hi-C data requires significant computational resources and specialized bioinformatics expertise, and detection of fine-scale chromatin interactions (<10 kb) may be constrained by both sequencing depth and the inherent resolution limits of the Hi-C method.

TROUBLESHOOTING

Problem 1

Low DNA yield after tissue processing.

Potential causes

- Insufficient tissue input.
- Tissue degradation during collection or processing.
- Inefficient tissue grinding.

Potential solutions

- Increase starting tissue amount to 100–150 mg.
- Ensure tissue is kept frozen throughout collection and processing.
- Extend grinding time to ensure complete tissue homogenization.
- Verify tissue quality using DNA integrity analysis prior to Hi-C library preparation.

Related steps: Stage 1 (Tissue collection and tissue grinding).

Problem 2

Poor crosslinking efficiency.

Potential causes

- Suboptimal crosslinking conditions.
- Delayed processing after tissue collection.
- Inadequate mixing during crosslinking.

Potential solutions

- Ensure crosslinking solution is freshly prepared.
- Optimize crosslinking time (10–20 minutes) based on tissue type.
- Increase mixing frequency during crosslinking incubation.
- Process tissue immediately after collection when possible.

Related steps: Step 2 (crosslinking).

Problem 3

High proportion of dangling ends in sequencing data.

Potential causes

- Inefficient proximity ligation.
- Suboptimal fragmentation.
- Inadequate washing of beads.

Potential solutions

- Extend ligation time to 6 hours.
- Optimize fragmentation enzyme concentration.
- Increase washing stringency during bead purification steps.
- Ensure thorough mixing during ligation reaction.

Related steps: Step 4 (fragmentation), Step 5 (proximity ligation).

Problem 4

Low percentage of valid Hi-C interactions.

Potential causes

- DNA degradation.
- Non-specific ligation events.
- Inefficient biotin incorporation.

Potential solutions

- Verify DNA integrity before library preparation.
- Optimize ligation conditions (temperature, time, buffer composition).
- Increase stringency of streptavidin bead binding and washing.
- Adjust size selection parameters to enrich for fragments with valid interactions.

Related steps: Stage 6 (ligation), Step 9 (purification).

Problem 5

Poor library amplification.

Potential causes

- Inhibitors in the reaction.
- Insufficient adapter ligation.
- Suboptimal PCR conditions.

Potential solutions

- Increase DNA purification stringency before adapter ligation.
- Optimize adapter concentration based on input DNA amount.
- Adjust PCR cycle number based on input amount (12–16 cycles).
- Use fresh PCR reagents and ensure proper thermal cycling conditions.

Related steps: Step 9 (adapter ligation), Step 10 (PCR amplification).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Victor X. Jin (vjin@mcw.edu).

Technical contact

Any technical questions about performing this protocol should be directed to the technical contact, Lavanya Choppavarapu (choppavarapu@mcw.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Raw and processed Hi-C data for breast tissue samples were previously published[1] and deposited in GEO under accession no. GSE261230.

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AUTHOR CONTRIBUTIONS

V.X.J. conceived the project. L.C. conducted and improved the experiments on human breast tissues. K.F. performed the data analyses. S.E. and K.L. provided the input and contributed to the standardization of the protocol. L.C., K.F., and V.X.J. wrote the manuscript with feedback from S.E., K.L., and J.S.R. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.104327.

REFERENCES

1. Choppavarapu, L., Fang, K., Liu, T., Ohihoin, A.G., and Jin, V.X. (2025). Hi-C profiling in tissues reveals 3D chromatin-regulated breast tumor heterogeneity informing a looping-mediated therapeutic avenue. Cell Rep. *44*, 115450. https://doi.org/10.1016/j.celrep.2025.115450.

2. Lieberman-Aiden, E., van Berkum, N.L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., Amit, I., Lajoie, B.R., Sabo, P.J., Dorschner, M.O., et al. (2009). Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science *326*,

289–293. https://doi.org/10.1126/science.1181369.

3. Rao, S.S.P., Huntley, M.H., Durand, N.C., Stamenova, E.K., Bochkov, I.D., Robinson, J.T., Sanborn, A.L., Machol, I., Omer, A.D., Lander, E.S., and Aiden, E.L. (2014). A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. Cell *159*, 1665–1680. https://doi.org/10.1016/j.cell.2014.11.021.

4. Phase Genomics (2024). Proximo Hi-C Kit (Human) Protocol v4.0. https://phasegenomics.com/.

5. Sztachera, M., Wendlandt-Stanek, W., and Piwecka, M. (2025). Protocol for mouse brain tissue processing and downstream analysis of circRNA-protein interactions in vivo. STAR Protoc. *6*, 104036. https://doi.org/10.1016/j.xpro.2025.104036.

6. Belaghzal, H., Dekker, J., and Gibcus, J.H. (2017). Hi-C 2.0: An optimized Hi-C procedure for high-resolution genome-wide mapping of chromosome conformation. Methods *123*, 56–65. https://doi.org/10.1016/j.ymeth.2017.04.004.

7. Schmitt, A.D., Hu, M., and Ren, B. (2016). Genome-wide mapping and analysis of chromosome architecture. Nat. Rev. Mol. Cell Biol. *17*, 743–755. https://doi.org/10.1038/nrm.2016.104.

8. Nagano, T., Lubling, Y., Stevens, T.J., Schoenfelder, S., Yaffe, E., Dean, W., Laue, E.D., Tanay, A., and Fraser, P. (2013). Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature *502*, 59–64. https://doi.org/10.1038/nature12593.

9. Parker, S.M., Davis, E.S., and Phanstiel, D.H. (2023). Guiding the design of well-powered Hi-C experiments to detect differential loops. Preprint at bioRxiv. https://doi.org/10.1101/2023.03.15.532762.

10. Lafontaine, D.L., Yang, L., Dekker, J., and Gibcus, J.H. (2021). Hi-C 3.0: Improved Protocol for Genome-Wide Chromosome Conformation Capture. Curr. Protoc. *1*, e198. https://doi.org/10.1002/cpz1.198.

11. Wingett, S., Ewels, P., Furlan-Magaril, M., Nagano, T., Schoenfelder, S., Fraser, P., and Andrews, S. (2015). HiCUP: pipeline for mapping and processing Hi-C data. F1000Res. *4*, 1310. https://doi.org/10.12688/f1000research.7334.1.