



February 2023

EpiTect[®] Hi-C Handbook

For performing Hi-C experiments on bulk populations of cells

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Kit Contents

EpiTect Hi-C Kit, Box 1 of 3
Catalog no.
Number of preps

(6)
59971
6

Component	Tube cap color	Quantity
Hi-C Digestion Buffer	Blue	25 µL
Hi-C Digestion Enzyme	Blue	25 µL
Hi-C End Labeling Mix	Yellow	36 µL
Hi-C End Labeling Enzyme	Yellow	10 µL
Hi-C Ligation Buffer	Orange	2 x 1.25 mL
Ultralow Input Ligase	Orange	2 x 65 µL
ER/A-Tailing Enzyme Mix	Red	60 µL
ER/A-Tailing Buffer	Red	30 µL
Adapter Ligation Buffer	Violet	90 µL
Illumina® Adapters 1	Violet	10 µL
Illumina Adapters 2	Violet	10 µL
Illumina Adapters 3	Violet	10 µL
Illumina Adapters 4	Violet	10 µL
Illumina Adapters 5	Violet	10 µL
Illumina Adapters 6	Violet	10 µL
2x HiFi PCR Master Mix	Green	1.25 mL
Primer Mix Illumina Library Amp	Clear	95 µL

EpiTect Hi-C Kit, Box 2 of 3
Catalog no.
Number of preps

(6)
59971
6

Component	Tube cap color	Quantity
MinElute® DNA Spin Columns	Clear	12 columns
Buffer PE	Clear	10 mL
Buffer EB	Clear	2 x 1 mL
Buffer SB1	Clear	5 mL
Bead Wash Buffer 1	Clear	2 x 1.8 mL
Bead Wash Buffer 2	Clear	2 x 1.8 mL
Bead Resuspension Buffer	Clear	500 µL
RNase-Free Water	Clear	10 mL

EpiTect Hi-C Kit, Box 3 of 3
Catalog no.
Number of preps

(6)
59971
6

Component	Tube cap color	Quantity
Proteinase K	Green	250 µL
QIAseq® Beads	Clear	2.5 mL
Streptavidin Beads	Black	150 µL
Buffer C1	White	500 µL

Storage

The contents of the EpiTect Hi-C Kit, Box 1, should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer. The contents of Box 2 should be stored at room temperature (15 – 25°C). The contents of Box 3 should be stored at 2 – 8°C . When the kits are stored under these conditions and handled correctly, performance is guaranteed until the expiration date printed on the box.

Intended Use

The EpiTect Hi-C Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

The EpiTect Hi-C Kit is not intended to be used for metagenomics.

All due care and attention should be exercised in the handling of the products.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in a convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the EpiTect Hi-C kit is tested against predetermined specifications to ensure consistent product quality.

Product Information

Component	Description
Hi-C Digestion Buffer + Enzyme	Optimized combination of endonuclease and buffer used to digest crosslinked chromatin at GATC sites
Hi-C End Labeling Enzyme + Mix	Optimized combination of an ultrapure DNA polymerase and a cocktail of ultrapure biotinylated and unmodified nucleotides used to fill in and label GATC overhangs with biotin
Hi-C Ligation Buffer	Buffer optimized for ligating blunt, biotinylated DNA ends
Ultralow Input Ligase	Highly concentrated DNA ligase, either for ligating blunt, biotinylated DNA ends together or for ligating Illumina Adapters to Hi-C fragments
ER/A-Tailing Enzyme Mix + Buffer	Unique cocktail of enzymes and buffer for the simultaneous end-repair, A-tailing, and phosphorylation of fragmented DNA
Adapter Ligation Buffer	For ligating Illumina Adapters to Hi-C fragments
Illumina Adapters 1–6	Barcoded oligonucleotides containing sequences essential for binding dual-barcoded libraries to a flow cell for sequencing and binding standard Illumina sequencing primers
2x HiFi PCR Master Mix	Proprietary high-efficiency, high-fidelity, and low-bias PCR master mix for NGS library amplification
Primer Mix Illumina Library Amp	Primer mix for the amplification of Illumina NGS libraries
MinElute DNA Spin Columns	Spin columns with selective binding properties of a unique silica membrane, designed to give high end concentrations of purified DNA fragments in low elution volumes
Buffer PE	Wash buffer for MinElute column-bound DNA
Buffer EB	Ultrapure Tris-based elution buffer
Buffer SB1	Proprietary buffer to select for DNA fragments >150 bp in size
Bead Resuspension Buffer	Used to resuspend Streptavidin Beads
Bead Wash Buffers 1 and 2	Used to wash Streptavidin Beads
RNase-Free Water	Ultrapure-quality, PCR-grade water
Proteinase K	Subtilisin-type protease suitable for short digestion times
QIAseq Beads	Paramagnetic beads for purifying NGS libraries after library PCR
Streptavidin Beads	Paramagnetic beads for capturing biotinylated Hi-C fragments
Buffer C1	Optimized buffer for lysing crosslinked cells

Introduction and Principle

First described in 2009, Hi-C is a technology based on the original yet remarkably simple idea that digestion and religation of fixed chromatin in cells, followed by the quantification of ligation junctions, allows for the determination of DNA contact frequencies and, thereby, chromosome topology (1). Over the past decade, Hi-C experiments have revealed that our genomes are organized into spatial hierarchies of increasing resolution, beginning with chromosomal territories and extending down to chromosomal compartments, topologically associating domains (TADs), and chromatin loops. Importantly, these conformational features contribute to the global regulation of gene expression. Aberrant chromosome topology can lead to developmental defects and disease states.

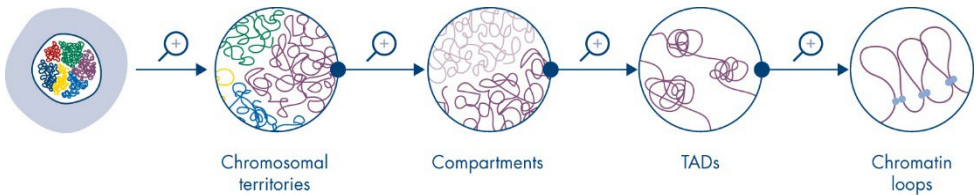


Figure 1. Levels of chromatin organization.

In recent years, the utility of Hi-C has been extended to de novo assembly of genomes, haplotype phasing, and identification of chromosomal rearrangements. Early Hi-C datasets revealed that individual chromosomes are physically separated into discrete territories and, therefore, primarily interact in cis. A significant portion of these cis interactions is long range, occurring between loci separated by millions of bases of DNA. In the case of de novo genome assembly, the power of Hi-C data can be exploited to assign, order, and orient shotgun sequencing reads into chromosomes, without the need of a reference genome. Similarly, Hi-C interaction maps can be employed to assign genetic variants to the correct homologous chromosome (2–4) and identify chromosomal rearrangements (5).

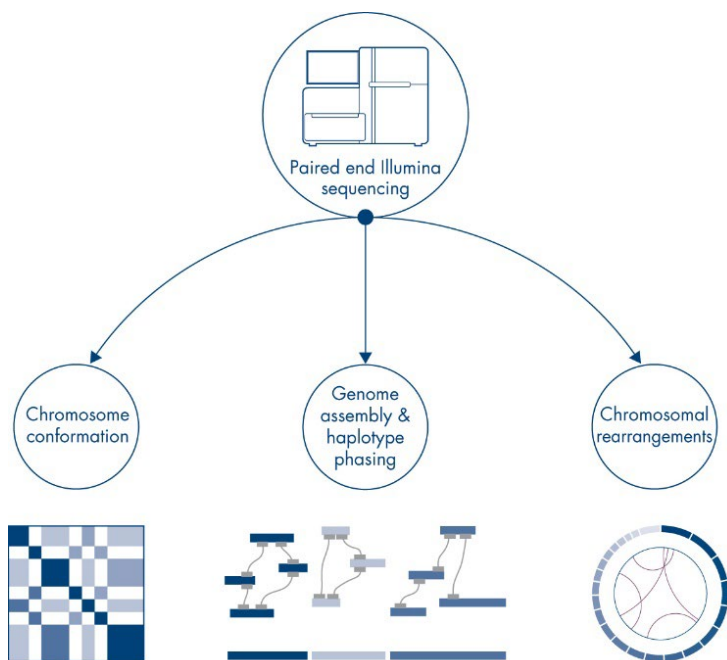


Figure 2. Downstream applications of Hi-C sequencing data.

The EpiTect Hi-C procedure is a version of the in situ Hi-C method (6), which maintains nuclear integrity from cell lysis until the ligation of digested and end-labeled chromosomes. This is crucially important because intact nuclei minimize random ligation events between chromatin fragments that are not topologically associated, thereby reducing the frequency of uninformative ligation events.

The protocol provided in this handbook has been developed to work on bulk populations of cells to generate 25 μl of a 5–10 nM DNA sequencing library that is compatible with Illumina sequencing platforms.

EpiTect Hi-C Kit workflow

Processing fixed cells into a Hi-C library

Fixed cells, in which the native structure of chromatin has been preserved by formaldehyde crosslinking, are first gently lysed such that intact nuclei are recovered. Crosslinked chromatin is then digested with an endonuclease that cuts at GATC sequences to generate 5' overhangs. A DNA polymerase subsequently fills in and labels 5' overhangs with biotinylated nucleotides. Spatially proximal, labeled DNA ends are then joined by DNA ligase. Proteinase K and high temperature are employed to eliminate protein complexes and reverse DNA crosslinks.

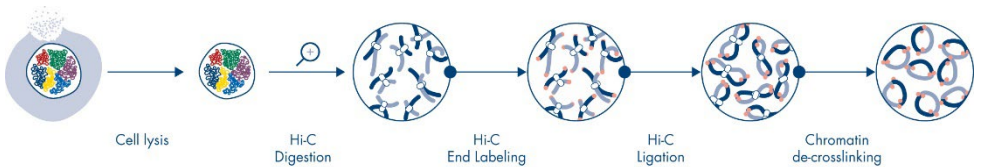


Figure 3. Workflow for processing fixed cells into a Hi-C library.

Processing a Hi-C library into an NGS sequencing library

Following de-crosslinking, ligated DNA is fragmented to a median size of 400–600 bp. Fragmented DNA is incubated with Streptavidin Beads to enrich for biotin-labeled Hi-C fragments. Hi-C fragments bound to Streptavidin Beads are processed to generate a DNA library for Illumina sequencing platforms.



Figure 4. Workflow for processing a Hi-C library into an NGS sequencing library.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

For “Crosslinking Chromatin in Cultured Cells” protocol

- Cell culture plasticware
- Cell culture medium
- Trypsin EDTA
- Sterile phosphate buffered saline (PBS)
- Cell scraper
- Centrifuge
- 37% formaldehyde
- Sterile 3 M Tris, pH 7.5
- 15 or 50 mL conical tubes
- Automated cell counter or hemocytometer
- Nuclease-free microcentrifuge tubes

For “Preparing collagenase I and II solutions for tissue crosslinking procedures”

- Collagenase I (C0130, Sigma-Aldrich)
- Collagenase II (C6885, Sigma-Aldrich)
- Nuclease-free microcentrifuge tubes
- Nuclease-free water

For “Crosslinking Chromatin in Vertebrate Tissue” protocol

- Hanks’ Balanced Salt Solution (HBSS) with calcium, magnesium and sodium bicarbonate and without phenol red (14025092, ThermoFisher Scientific; 55037C, Sigma-Aldrich)
- BioMasher II (EOG-sterilized) tube (320103, Nippi Inc.)
- 37% formaldehyde
- Sterile 3 M Tris, pH 7.5
- Thermal mixer capable of reaching 37°C (e.g., ThermoMixer®)
- Collagenase I (C0130, Sigma-Aldrich) in solution at 40 mg/ml
- Collagenase II (C6885, Sigma-Aldrich) in solution at 40 mg/ml
- 10% SDS
- Qubit™ dsDNA BR or Qubit™ dsDNA HS Assay kit (Thermo Fisher Scientific)
- Qubit Fluorometer (Thermo Fisher Scientific)
- Nuclease-free microcentrifuge tubes
- Microcentrifuge
- Liquid nitrogen

For “Crosslinking Chromatin in Insect Tissue” protocol

- Hanks’ Balanced Salt Solution (HBSS) with calcium, magnesium and sodium bicarbonate and without phenol red (14025092, ThermoFisher Scientific; 55037C, Sigma-Aldrich)
- BioMasher II (EOG-sterilized) tube (320103, Nippi Inc.)
- 37% formaldehyde
- Sterile 3 M Tris, pH 7.5
- Thermal mixer capable of reaching 37°C (e.g., ThermoMixer)
- Collagenase I (C0130, Sigma-Aldrich) in solution at 40 mg/ml
- Collagenase II (C6885, Sigma-Aldrich) in solution at 40 mg/ml
- 10% SDS
- Qubit dsDNA BR or Qubit™ dsDNA HS Assay kit (Thermo Fisher Scientific)

- Qubit Fluorometer (Thermo Fisher Scientific)
- Nuclease-free microcentrifuge tubes
- Microcentrifuge
- Liquid nitrogen

For “Preparing Aliquots of Low Input Crosslinked Tissue Samples” protocol

- Nuclease-free microcentrifuge tubes
- Microcentrifuge
- Liquid nitrogen

For “Hi-C Part 1” and “Hi-C Part 2” protocols

- 1% SDS
- 10% Triton X-100
- 100% isopropanol
- 80% ethanol
- 3 M sodium acetate, pH 5.2
- Nuclease-free 0.2 mL PCR tubes, PCR strips, or PCR plates (and sealing foils for PCR plates)
- Pipettes
- Nuclease-free aerosol-barrier pipette tips
- Thermal cycler
- Thermal mixer capable of reaching 37°C (e.g., ThermoMixer)
- Heating block or water bath capable of reaching 16–80°C
- Vortexer
- Microcentrifuge and plate centrifuge
- Magnetic rack compatible with microcentrifuge tubes
- Capillary electrophoresis device (e.g., QIAxcel®)

- Mechanical DNA fragmentation device (e.g., sonicator)
- Nuclease-free microcentrifuge tubes
- Nuclease-free, DNA low binding microcentrifuge tubes

Important Notes

Sample input

The EpiTect Hi-C protocol has been optimized for the use of 5×10^5 human or mouse cells (or the equivalent of 3 μg of DNA) per sample. However, as few as 5000 and as many as 2.5×10^6 human or mouse cells (or the equivalent of 0.03–15 μg DNA) per replicate may also be used. Using input amounts outside of this range, particularly at the upper end, risks affecting the quality of the resulting NGS library, such that it is likely to have higher levels of inward facing (FR) sequence read bias, unligated ends, and read pairs containing contiguous, religated restriction fragments.

It is therefore critical to accurately quantify sample input amounts before proceeding with the Hi-C protocol. For cultured cells, an automated cell counter or hemocytometer should be used to assist in distributing the correct amount of cells to each sample. The tissue crosslinking protocols include a procedure for quantifying input amounts.

Sample type

In principle, the EpiTect Hi-C protocol should work for any type of crosslinked eukaryote cells. The following sample types have been validated to work with the EpiTect Hi-C kit:

- Cultured mammalian cells
- Fresh frozen vertebrate tissue (e.g. liver, spleen, lung, heart)
- Fresh insect tissue (whole insects, imaginal discs)

The EpiTect Hi-C protocol will not work with bacterial samples and is not intended to be used for metagenomics.

Good PCR laboratory practices

Recommendations to reduce the risk of contaminating NGS libraries with amplicons from previous PCR reactions and consequently obtaining false results:

- Always wear a clean lab coat. Use separate lab coats for sample preparation and for setting up PCR reactions or handling PCR products.
- Change gloves often, especially if you suspect that they may have been contaminated.
- **Important:** Establish and maintain a designated area for PCR setup that is separate from areas where PCR amplification and PCR product handling are performed.
- Never bring amplified PCR products into the PCR setup area.
- Spin down all reaction and sample tubes before opening. Open and close all reagent and sample tubes carefully; avoid splashing or spraying PCR samples.
- Keep reactions and components capped whenever possible.
- Use filter barrier pipette tips to prevent aerosol-mediated contamination of your pipetting device.
- Clean laboratory benches and equipment regularly.

Protocol: Crosslinking Chromatin in Cultured Cells

This protocol describes the procedure for crosslinking chromatin in cultured cells by treatment with formaldehyde.

Important points before starting

- This procedure has been developed for cells grown in 25 or 75 cm² culture flasks. If using smaller cell culture volumes, users may reduce the volumes of reagents described in the procedure for convenience. However, the ratio of each reagent must remain unchanged
- Autoclave solutions of PBS and 3 M Tris pH 7.5 before using.
- Do not use formaldehyde solutions in which white precipitates are visible.
- Always work under a hood when working with formaldehyde.
- Do not use cell cultures contaminated with bacteria or fungi.
- Bring cell culture medium to room temperature before proceeding to ensure reproducible crosslinking conditions.
- It is critical to use a hemocytometer or automated cell counter to assist in distributing the correct amount of cells to each aliquot.
- Crosslinked cells can be stored for up to 6 months at –80°C before proceeding with the Hi-C protocol.
- When proceeding immediately from crosslinking to the Hi-C protocols, be sure to review this protocol (i.e., “Protocol: Crosslinking Chromatin in Cultured Cells”), as well as “Protocol: Processing Fixed Cells Into a Hi-C Library (Hi-C Part 1)” (page 30) before starting.

Things to do before starting

- Bring the complete cell culture medium (i.e., medium with serum) to room temperature.
- Chill 50 mL of PBS on ice at least 30 min before beginning.
- Obtain 1.5 mL microcentrifuge tubes.
- Obtain liquid nitrogen to snap freeze samples if they are to be stored at -80°C before proceeding with the Hi-C protocol.

Procedure

1. Grow cells in an appropriate culture medium to the desired density.

Note: Cells 10 μm in length grown to confluency in 25 or 75 cm^2 culture flasks will generally provide sufficient material (between 3×10^6 and 1×10^7 cells) for multiple Hi-C sample replicates. However, cell numbers will vary, because animal cells can range in length from 10 to 100 μm .

2. If working with suspension cell lines, proceed to step 4.
3. If working with adherent cell lines, gently trypsinize cells according to standard procedures, and then proceed to step 4.
4. Transfer cells to a 50 mL conical tube and centrifuge for 10 min at $450 \times g$ at room temperature.
5. Remove supernatant and gently resuspend the cell pellet in 20 mL fresh medium.
6. Add 540 μL of 37% formaldehyde (1% final) and mix thoroughly by inverting 5 times.
Important: Formaldehyde should be handled in a chemical fume hood. Wear protective gloves and glasses. Follow guidelines for waste disposal.
7. Incubate for 10 min at room temperature.
8. To quench the formaldehyde, add 10 mL of 3 M Tris pH 7.5.
9. Mix thoroughly by inverting 5 times.
10. Incubate for 5 min at room temperature, and then on ice for at least 15 min.

11. Centrifuge cells for 10 min at $800 \times g$ at 4°C .
12. Resuspend the cell pellet in 10 mL cold PBS.
13. Use a hemocytometer or an automated cell counter to determine cell density.
14. Centrifuge cells for 5 min at $800 \times g$ at 4°C and aspirate supernatant.
15. Resuspend cells in fresh, cold PBS to desired concentration.
16. Distribute 5×10^5 cells into fresh 1.5 mL microcentrifuge tubes on ice. Each tube represents a single Hi-C sample.

Note: Sample input may range from 5×10^3 to 2.5×10^6 cells (30 ng DNA to 15 μg DNA) per sample.

17. Centrifuge for 5 min at $800 \times g$ at 4°C and aspirate PBS.
18. Proceed immediately with the "Hi-C Part 1" protocol, or snap freeze tubes in liquid nitrogen and store at -80°C .

Protocol: Preparing collagenase I and II solutions for tissue crosslinking procedures

This protocol describes the procedure for preparing solutions of collagenase for use in tissue crosslinking procedures.

Important points before starting

- It is critical that the dry collagenase is fully dissolved before preparing the aliquots.

Things to do before starting

- Obtain 1.5 mL microcentrifuge tubes and nuclease-free water.

Procedure: Preparing collagenase I and II solutions

1. To each of the dry collagenases, add enough nuclease-free water to arrive at a final concentration of 40 mg/mL.
2. Gently mix up and down with a pipette to fully dissolve.
3. For each collagenase, prepare 25 μ L aliquots and store at -20°C .

Protocol: Crosslinking Chromatin in Vertebrate Tissue

This protocol describes the procedure for crosslinking chromatin in vertebrate tissue by treatment with formaldehyde.

Important points before starting

- This protocol has been developed to work with freshly frozen tissue.
- Do not thaw tissue before crosslinking.
- The total mass of tissue per crosslinking tube should be between 20 and 50 mg.
- Autoclave 3 M Tris pH 7.5 before using.
- Do not use formaldehyde solutions in which white precipitates are visible.
- Always work under a hood when handling formaldehyde.
- Crosslinked tissue samples can be stored for up to 6 months at -80°C before proceeding with the Hi-C protocol.
- If proceeding immediately from crosslinking to the Hi-C protocol, be sure to review this protocol (i.e., For “Crosslinking Chromatin in Vertebrate Tissue” protocol), as well as “Protocol: Processing Fixed Cells Into a Hi-C Library (Hi-C Part 1)” (page 30) before starting.

Things to do before starting

- Obtain 1.5 ml microcentrifuge tubes.
- Obtain liquid nitrogen to snap freeze samples if they are to be stored at -80°C before proceeding with the Hi-C protocol.

Procedure: Crosslinking Chromatin in Vertebrate Tissue

1. Weigh out 25 mg of fresh frozen tissue in a BioMasher II tube.
Note: Thin and small pieces of tissue work the best.
2. Immediately add 200 μL of room temperature Hanks' Balanced Salt Solution.
3. Using the supplied pestle, thoroughly homogenize the tissue sample, until no large pieces are visible.
4. Immediately add 10.8 μL 37% formaldehyde (2% final) to the tube.
Important: Formaldehyde should be handled in a chemical fume hood. Wear protective gloves and glasses. Follow guidelines for chemical waste disposal.
5. Mix contents of tube thoroughly by inverting the tube five times.
6. Incubate the tube for 20 min at room temperature without rotation.
7. To quench the formaldehyde, add 100 μL of 3 M Tris-Cl pH 7.5 to the tube.
8. Mix contents of the tube thoroughly by inverting the tube five times.
9. Incubate the tube for 5 min at room temperature without rotation.
10. Add 650 μL of room temperature Hanks' Balanced Salt Solution to the tube.
11. Add 25 μL of Collagenase I (40 mg/mL) and 25 μL of Collagenase II (40 mg/mL) and mix by inverting the tube five times.
12. Incubate tube in a thermal mixer set to 37°C with 600 RPM shaking for 1 h.
13. Remove tube from thermal mixer and mix the contents of the tube thoroughly by gently pipetting.
14. Immediately aliquot 20 μL (5%) of the sample into a fresh microcentrifuge tube.
15. Place the tube containing the rest of the sample on ice.
16. Add 2 μL of 10% SDS to the 20 μL aliquot and mix thoroughly by pipetting.
17. Measure 2 μL of the aliquot with the Qubit dsDNA BR or Qubit dsDNA HS Assay Kit to determine the DNA concentration of your sample.

18. Based on the determined DNA concentration of your sample, prepare aliquots by pipetting the volume of sample equivalent to 3 μg of DNA into a fresh microcentrifuge tube.

Note: Optimal sample input is 3 μg DNA, but input amounts may range from 30 ng to 15 μg of DNA per sample.

Note: If the aliquots will contain less than 600 ng of total DNA, immediately proceed to "Protocol: Preparing Aliquots of Low Input Tissue Samples, a protocol for low input samples that prevents sample loss.

19. Centrifuge tube for 5 min at 800 $\times g$ at 4°C and carefully aspirate the supernatant.

Note: The cell pellets are soft.

20. Proceed immediately with the Hi-C Part 1 protocol or snap freeze tubes in liquid nitrogen and store at -80°C.

Protocol: Crosslinking Chromatin in Insect Tissue

This protocol describes the procedure for crosslinking chromatin in insects by treatment with formaldehyde.

Important points before starting

- This protocol has been developed to work with freshly frozen insects or freshly frozen insect tissue.
- Do not thaw tissue before crosslinking.
- Autoclave 3 M Tris pH 7.5 before using.
- Do not use formaldehyde solutions in which white precipitates are visible.
- Always work under a hood when handling formaldehyde.
- Crosslinked tissue samples can be stored for up to 6 months at -80°C before proceeding with the Hi-C protocol.
- If proceeding immediately from crosslinking to the Hi-C protocol, be sure to review this protocol (i.e., “Crosslinking Chromatin in Insect Tissue”), as well as “Protocol: Processing Fixed Cells Into a Hi-C Library (Hi-C Part 1)” (page 30) before starting.

Things to do before starting

- Obtain 1.5 ml microcentrifuge tubes.
- Obtain liquid nitrogen to snap freeze samples if they are to be stored at -80°C before proceeding with the Hi-C protocol.

Procedure: Crosslinking Chromatin in Insect Tissue

1. Place freshly frozen insect sample in a BioMasher II tube.

Note: If the insect sample does not fit into the BioMasher II tube, perform step 2 in a clean mortar and pestle and transfer the ground sample into the BioMasher II Tube before proceeding with step 3.

2. Immediately add liquid nitrogen to the BioMasher II tube and use the supplied pestle to coarsely grind the insect.

Note: This step is required to break open the insect exoskeleton in order to allow the crosslinking reagents to penetrate the insect tissue.

3. Immediately add 200 μL of room temperature Hanks' Balanced Salt Solution and 10.8 μL 37% formaldehyde (2% final) to the tube.

Important: Formaldehyde should be handled in a chemical fume hood. Wear protective gloves and glasses. Follow guidelines for chemical waste disposal.

4. Mix contents of tube thoroughly by inverting the tube five times.
5. Incubate the tube for 20 min at room temperature without rotation.
6. To quench the formaldehyde, add 100 μL of 3M Tris-Cl pH 7.5 to the tube.
7. Mix contents of the tube thoroughly by inverting the tube five times.
8. Incubate the tube for 5 min at room temperature without rotation.
9. Using the supplied pestle, thoroughly homogenize the tissue sample by hand, until no large pieces are visible.
10. Add 650 μL of room temperature Hanks' Balanced Salt Solution to the tube and mix thoroughly by inverting five times.
11. Add 25 μL of Collagenase I (40 mg/ml) and 25 μL of Collagenase II (40 mg/ml) to the tube containing 950 μL of sample and mix by inverting the tube five times.
12. Incubate tube in a thermal mixer set to 37°C with 600 RPM shaking for 1 h.
13. Remove tube from thermal mixer and mix the contents of the tube thoroughly by gently pipetting.
14. Immediately aliquot 20 μL (5%) of the sample into a fresh microcentrifuge tube.
15. Place the tube containing the rest of the sample on ice.

16. Add 2 μL of 10% SDS to the 20 μL aliquot and mix thoroughly by pipetting.
17. Measure 2 μL of the aliquot with the Qubit dsDNA BR or Qubit dsDNA HS Assay Kit to determine the DNA concentration of your sample.
18. Based on the determined DNA concentration of your sample, prepare aliquots by pipetting the volume of sample equivalent to 3 μg of DNA into a fresh microcentrifuge tube.
Note: Optimal sample input is 3 μg DNA, but input amounts may range from 30 ng to 15 μg of DNA per sample.
Note: If the aliquots will contain less than 600 ng of total DNA, immediately proceed to “Protocol: Preparing Aliquots of Low Input Tissue Samples”, a protocol for low input samples that prevents sample loss.
19. Centrifuge tube for 5 min at 800 $\times g$ at 4°C and carefully aspirate the supernatant.
Note: The cell pellets are soft.
20. Proceed immediately with the Hi-C Part 1 protocol or snap freeze tubes in liquid nitrogen and store at -80°C.

Protocol: Preparing Aliquots of Low Input Tissue Samples

The EpiTect Hi-C procedure can accommodate samples ranging from 30 ng to 15 ug of total DNA. However, low input tissue samples are prone to be lost during handling. Therefore, we have developed a dedicated protocol using QIAseq Beads to efficiently capture and irreversibly bind low input tissue samples prior to proceeding with “Protocol: Processing Fixed Cells Into a Hi-C Library (Hi-C Part 1)” (page 30). This procedure will only work with tissue samples that have been crosslinked and dissociated with collagenase according to procedures described in this handbook.

Important points before starting

- If the total DNA content of your crosslinked sample lies between 30 and 600 ng, follow this procedure to minimize sample loss.
- When using this protocol, users must directly proceed with the “Protocol: Processing Fixed Cells Into a Hi-C Library (Hi-C Part 1)” (page 30).

Things to do before starting

- Obtain 1.5 mL microcentrifuge tubes.

Procedure: Preparing Aliquots of Low Input Samples

1. Centrifuge the sample for 5 min at $800 \times g$ at 4°C .
2. Carefully aspirate the supernatant, leaving $\sim 250 \mu\text{L}$ of solution in the tube.
3. Add $250 \mu\text{L}$ of QIAseq Beads, equilibrated to room temperature, to the sample.
4. Mix by gently inverting and let stand at room temperature for 10 min.
5. Place tube in a magnetic rack for 1 min and remove supernatant.

Proceed immediately with the Hi-C procedure, beginning at step 10 of the Hi-C Part 1 protocol, where the tube containing the sample bound to QIAseq beads is placed in a magnetic rack (omitting the steps 1 through 9).

Note: Following collagenase treatment, crosslinked tissues separate into single cells with partially or completely disrupted cell membranes, which allows enzymes required for the Hi-C procedure to readily enter the nuclei. Therefore, the cell lysis procedure from the Hi-C Part 1 protocol is not required.

Protocol: Processing Fixed Cells Into a Hi-C Library (Hi-C Part 1)

This protocol entails the first half of the procedure for performing Hi-C:

- Cell lysis
- Chromatin digestion
- Hi-C end labeling and ligation
- Chromatin de-crosslinking, followed by DNA purification

The following procedure describes the processing of a single sample of cells that have been fixed with formaldehyde (see “Protocol: Crosslinking Chromatin in Cultured Cells” (page 18), “Protocol: Crosslinking Chromatin in Vertebrate Tissue” (page 22), and “Protocol: Crosslinking Chromatin in Insect Tissue” (page 25). Each Hi-C kit contains reagents sufficient for processing 6 samples.

Important points before starting

- Keep enzymes and thawed reaction buffers on ice at all times.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Use standard 1.5 or 2 mL microcentrifuge tubes throughout the protocol.
- Unless otherwise indicated, centrifugation is performed at room temperature.
- Gentle handling of nuclei throughout the cell lysis, Hi-C digestion, Hi-C end labeling, and Hi-C ligation steps is critical to a successful Hi-C experiment. Rupturing of nuclei during these steps will increase the frequency of random, biologically irrelevant interchromosomal (trans) interactions. Care must be taken to pipette samples gently and to not shake them during the various incubation steps unless specifically stated.

Things to do before starting

- Chill PBS, RNase-Free Water, and Buffer C1 on ice for 30 min before using.
- Thaw frozen reagents on ice.
- Prepare Hi-C Digestion and Ligation Solutions according to Table 1 and Table 2.

Preparing solutions for Hi-C digestion and Hi-C ligation

1. Prepare the Hi-C Digestion and Hi-C Ligation Solutions in 1.5 mL microcentrifuge tubes according to Table 1 and Table 2.

Table 1. Setup for preparing Hi-C Digestion Solution

Component	Volume (µL)
Hi-C Digestion Buffer	4
1% SDS	4
RNase-Free Water	32
Total volume	40

Table 2. Setup for preparing Hi-C Ligation Solution

Component	Volume (µL)
Hi-C Ligation Buffer	200
10% Triton X-100	40
Ultralow Input Ligase	5
RNase-Free Water	105
Total volume	350

2. Mix solutions thoroughly by pipetting, and then place on ice.
3. Proceed to “Cell lysis” below.

Cell lysis

4. In a microcentrifuge tube, carefully resuspend 5×10^5 crosslinked human or mouse cells (or the equivalent of 3 μg DNA) in 50 μL ice-cold PBS by gently pipetting with a micropipette.

Note: Between 5×10^3 and 2.5×10^6 human or mouse cells (or the equivalent of 0.03–15 μg DNA) is also acceptable.

Note: It is possible to pool multiple tubes of crosslinked cells to obtain the minimum or desired amount of starting material. To do so, resuspend a pellet of crosslinked cells in one tube in 50 μL of ice-cold PBS and transfer the entirety of this mixture to another tube containing crosslinked cells. Repeat until enough or the desired amount of cells are resuspended in PBS.

5. Add 150 μL of ice-cold RNase-Free Water to the tube.
6. Add 50 μL of ice-cold Buffer C1 to the tube.
7. Mix contents by gently inverting the tube back and forth 5 times.
8. Incubate the tube on ice for 10 min.
9. To capture the liberated nuclei, add 250 μL of QIAseq Beads to the sample. Mix by gently inverting and let stand at room temperature for 10 min.
Important: Equilibrate QIAseq Beads to room temperature before use.
10. Place tube in a magnetic rack. Incubate for 1 min.
11. With the tube still in the magnetic rack, carefully remove the supernatant, taking care not to disturb the QIAseq Beads bound to the wall of the tube.
12. Remove tube from rack.
13. To wash the beads, add 500 μL of cold RNase-free water to the sample. Mix by gently inverting.
Note: In contrast to free DNA, nuclei will remain bound to QIAseq Beads following washes with water.
14. Place tube in a magnetic rack. Incubate for 1 min.

15. With the tube still in the magnetic rack, carefully remove the supernatant, taking care not to disturb the QIAseq Beads bound to the wall of the tube.
16. Proceed immediately to “Hi-C digestion” below.

Hi-C digestion

17. Add 40 μL Hi-C Digestion Solution to the washed QIAseq Beads. Completely resuspend the beads gently pipetting up and down 3–4 times.
18. Incubate the tube at 65°C for 10 min.
Important: Do not shake the tube during this incubation.
19. Place the tube on ice immediately after incubation.
20. Add 4.4 μL 10% Triton X-100. Mix by gently pipetting up and down 3–4 times.
21. Add 4 μL Hi-C Digestion Enzyme. Mix by gently pipetting up and down 3–4 times.
22. Incubate the tube in a thermal mixer set at 37°C with 600 rpm shaking for 30 minutes to digest chromatin.
Note: For flexibility, users may incubate the digestion between 30 min and overnight.
23. Incubate the tube at 65°C for 20 min.
Important: Do not shake the tube during this incubation.
24. Place the tube on ice and proceed to “Hi-C end labeling”.
Note: As an optional stopping point, the tube may be stored at –20°C.

Hi-C end labeling

25. While on ice, add 6 μL of Hi-C End Labeling Mix and 1 μL of Hi-C End Labeling Enzyme to the tube containing digested chromatin.
26. Mix by gently pipetting up and down 3–4 times.
27. Incubate the tube at 37°C for 30 min to label the digested chromatin with biotinylated nucleotides.

Important: Do not shake the tube during this incubation.

Important: Adhere to the 30 min incubation time.

28. Place the tube on ice and proceed to “Hi-C ligation”.

Note: As an optional stopping point, the tube may be stored at -20°C .

Hi-C ligation

29. Transfer the entire Hi-C Ligation Solution to a tube containing end-labeled chromatin.

30. Mix by gently inverting 5 times.

31. Incubate the tube at 16°C for 30 min to ligate spatially proximal DNA fragments.

Important: Do not shake the tube during this incubation.

Important: Ligation at temperatures $>16^{\circ}\text{C}$ is not recommended.

Note: For flexibility, users may incubate the ligation between 30 min and overnight.

32. Place the tube on ice and proceed to “Chromatin de-crosslinking”.

Note: As an optional stopping point, the tube may be stored at -20°C .

Chromatin de-crosslinking

33. Add 20 μL Proteinase K solution to the ligated chromatin. Mix gently by inverting the tube 5 times.

34. Incubate the tube at 56°C for 30 min, followed by 80°C for 90 min.

35. Briefly spin the tubes at low speed ($4000 \times g$) to collect any condensation from the lid.

36. Cool the reaction mixtures to room temperature and proceed to “DNA purification following de-crosslinking”.

Note: As an optional stopping point, the tube may be stored at -20°C .

DNA purification following de-crosslinking

37. Add 40 μ L of 3 M sodium acetate, pH 5.2, to the de-crosslinked DNA. Vortex briefly to mix.
38. Add 280 μ L of 100% isopropanol to the tube. Vortex briefly to mix.
39. Place a MinElute column, including its provided collection tube, in a rack.
40. To bind DNA, apply the entire mixture, including the QIAseq Beads, to the MinElute column and then centrifuge for 1 min at $17,900 \times g$.
41. Discard the flow-through and place the MinElute column back into the same collection tube.
42. To wash, add 0.75 mL Buffer PE to the MinElute column. Centrifuge for 1 min at $17,900 \times g$.
43. Discard the flow-through and place the MinElute column back in the same tube.
44. Centrifuge the MinElute column for an additional 1 min at $17,900 \times g$.
45. Place the MinElute column into a new 1.5 mL microcentrifuge tube.
46. To elute DNA, add 35 μ L Buffer EB prewarmed to 65°C to the center of the membrane.
Note: If DNA is to be fragmented in volumes <35 μ L, elute DNA in 20 μ L prewarmed Buffer EB. Elution in volumes <20 μ L will result in lower DNA yields.
47. Incubate for 1 min at room temperature.
48. Centrifuge the column for 1 min at $17,900 \times g$.
Note: Some traces of QIAseq Beads may elute along with the DNA, but they will not interfere with downstream steps of the workflow. If desired, users can remove these traces of beads by placing sample tube into a magnetic rack, incubating for 1 min, and transferring the clear supernatant containing purified DNA into a new 1.5 mL microcentrifuge tube.
49. Store the purified DNA at -20°C or proceed with “Protocol: Processing a Hi-C Library Into an NGS Sequencing Library (Hi-C Part 2)”.

Protocol: Processing a Hi-C Library Into an NGS Sequencing Library (Hi-C Part 2)

This protocol describes the second half of the EpiTect Hi-C procedure, during which mechanical DNA fragmentation is used to prepare Hi-C sequencing libraries:

- Mechanical DNA fragmentation followed by purification
- Hi-C fragment enrichment via streptavidin pulldown
- End-repair, phosphorylation, and A-tailing of bead-bound Hi-C fragments
- Illumina Adapter ligation, library amplification, and purification

Important points before starting

- Prepare 80% ethanol from 96–100% ethanol.
- Enough Buffer SB1 is supplied to purify samples fragmented in volumes ≤ 200 μL . For fragmentation volumes > 200 μL , refer to the protocol for purifying fragmented DNA samples with SPRI beads in Appendix A (page 48).

Note: Users must supply their own SPRI beads for this purification.

- Thoroughly dissolve any white precipitates in the thawed ER/A-tailing buffer by pipetting up and down.
- De-crosslinked DNA must be fragmented to median sizes of 400–600 bp. Before proceeding with the “Hi-C Part 2” protocol, develop a reliable fragmentation protocol using 3 μg of de-crosslinked human or mouse DNA per sample.

Things to do before starting

- Thaw frozen reagents on ice. Keep enzymes, thawed reaction buffers, Illumina Adapters, and PCR primers on ice at all times.
- Mix buffers thoroughly by quickly vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

Mechanical DNA fragmentation

1. Following completion of the “Hi-C Part 1” protocol, mechanically fragment purified DNA samples in Buffer EB such that the median DNA fragment size is 400–600 bp.

Note: The EpiTect Hi-C protocol was developed using the fragmentation protocol outlined in Table 3. If a different mechanical fragmentation method is to be used, users must ensure that the median DNA fragment size is 400–600 bp.

Table 3. Fragmentation protocol used for development of the EpiTect Hi-C protocol

Variable	Detail
Sonicator	Covaris® S220
Sonication tube	microTUBE AFA Fiber Snap-Cap 6x16mm
Sonication tube holder	S-series Holder microTUBE
Sonication volume	130 µL in Buffer EB
Sonicator settings	
Water levels	12
Peak incident power	140 W
Duty factor	10%
Cycles per burst	200
Treatment time	80 s

2. Following DNA fragmentation, tubes can be frozen overnight at -20°C as an optional stopping point or you may proceed directly to “DNA purification following mechanical fragmentation”, below.

Note: To control for DNA fragmentation, analyze 5% of fragmented sample on a capillary electrophoresis device or agarose gel. Samples that are too large should be fragmented further until a median DNA size of 400–600 bp is achieved. Users may proceed with samples with median DNA sizes of 250–400 bp, but the resulting NGS libraries will be of lower quality (see Appendix B, page 50).

DNA purification following mechanical fragmentation

3. Add 4 volumes of Buffer SB1 to 1 volume of fragmented DNA sample. Vortex briefly to mix.
4. Place a MinElute column, including its provided collection tube, in a suitable rack.
5. To bind DNA, apply mixture to the MinElute spin column and centrifuge for 1 min at $17,900 \times g$.

Note: For maximum recovery, transfer all traces of the sample to the column.

6. Discard the flow-through and place the MinElute spin column back into the same tube.
7. To wash, add 700 μL of 80% ethanol to the MinElute column and centrifuge for 1 min at $17,900 \times g$. Discard the flow-through and place the MinElute spin column back into the same tube. Repeat this step once.
8. Centrifuge for 1 min at $17,900 \times g$.
Important: Residual ethanol will not be completely removed unless the flow-through is discarded before this additional centrifugation step.
9. Place the MinElute spin column into a clean 1.5 mL microcentrifuge tube.
10. Add 50 μL of Buffer EB prewarmed to 65°C to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.
11. Proceed directly to “Preparing solutions for on-bead reactions”, or if you choose to make this a stopping point, freeze tubes overnight at -20°C .

Preparing solutions for on-bead reactions

12. Prepare the ER/A-tailing solution, adapter ligation buffer dilution, and Hi-C sequencing library amplification mix in 1.5 mL microcentrifuge tubes according to Table 4–Table 6, below.

Table 4. ER/A-tailing solution

Component	Volume (µL)
ER/A-Tailing Buffer	5
ER/A-Tailing Enzyme Mix	10
RNase-Free Water	35
Total volume	50

Table 5. Adapter ligation buffer dilution

Component	Volume (µL)
Adapter Ligation Buffer	15
RNase-Free Water	135
Total volume	150

Table 6. Hi-C sequencing library amplification mix

Component	Volume (µL)
HiFi PCR Master Mix, 2x	75
Primer Mix Illumina Library Amp	4.5
RNase-Free Water	70.5
Total volume	150

13. Mix solutions thoroughly by pipetting, and then place on ice.
14. Proceed to “Streptavidin pulldown of Hi-C fragments”, next page.

Streptavidin pulldown of Hi-C fragments

15. Thoroughly resuspend Streptavidin Beads by vortexing.
16. Transfer 25 μL of Streptavidin Beads into a new microcentrifuge tube.
17. Wash the beads once with 100 μL Bead Wash Buffer 1, following the steps in Figure 5, below.

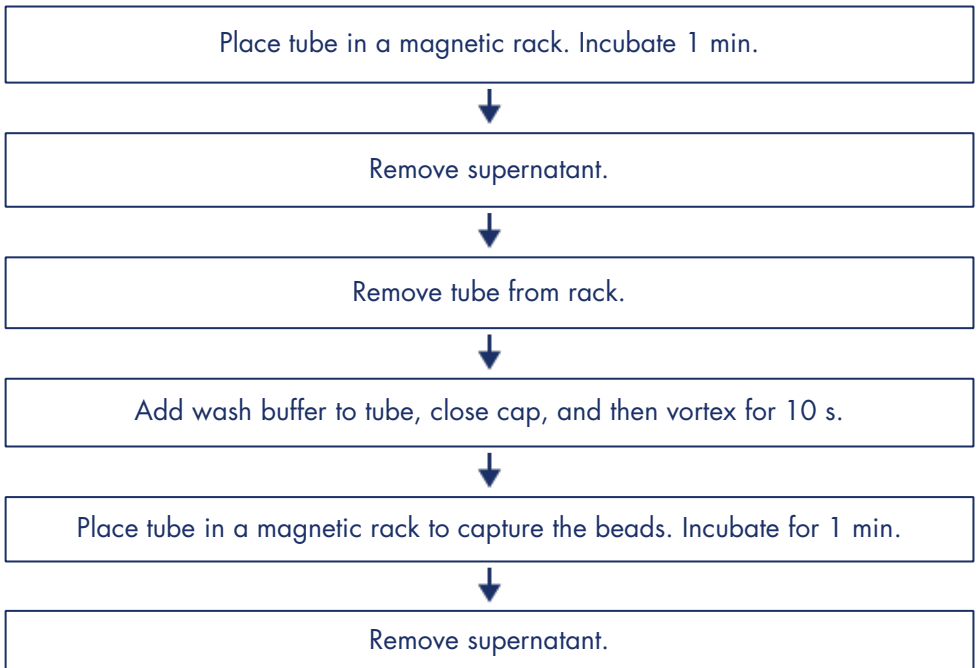


Figure 5. Standard bead-washing process.

18. Resuspend the beads in 50 μL Bead Resuspension Buffer.
19. Add 50 μL of purified DNA sample to the beads.
20. Incubate at room temperature for 15 min in a thermal mixer set at 1000 rpm, and then proceed directly to “DNA end-repair/A-tailing/phosphorylation”.

DNA end-repair/A-tailing/phosphorylation

21. Wash beads once in 100 μL Bead Wash Buffer 2 (see Figure 5).
22. Resuspend beads in 50 μL of prepared ER/A-tailing solution.
23. Incubate the tube for 15 min at 20°C, followed by 15 min at 65°C, to repair, phosphorylate, and A-tail the ends of the captured Hi-C DNA fragments.
24. Proceed directly to “Illumina Adapter ligation”.

Illumina Adapter ligation

Note: Refer to “Good PCR laboratory practices” (page 17) before starting.

25. Wash beads once with 100 μL Bead Wash Buffer 2 (see Figure 5).
26. Wash beads once in 95 μL diluted Adapter Ligation Buffer (see Figure 5).
27. Resuspend beads in 50 μL diluted Adapter Ligation Buffer (see Figure 5).
28. Transfer 5 μL of 1 Illumina Adapter well to a sample tube.

Important: Use only 1 adapter per ligation reaction. Do not reuse adapters. If processing multiple samples in parallel, note which Illumina Adapter is paired with which sample. The adapters have sequence barcodes (see Appendix C, page 51) that allow for multiplex sequencing of up to 6 samples.

Important: When processing multiple samples in parallel, open the Illumina Adapter tubes one at a time. Change gloves between pipetting of different barcoded adapters to avoid cross-contamination.

29. Add 2 μL Ultralow Input Ligase to the sample tube. Mix carefully by pipetting up and down 3–4 times.
30. Incubate the tube for 45 min at room temperature.
31. Proceed directly to “Hi-C sequencing library amplification”.

Hi-C sequencing library amplification

32. Wash the beads twice with 100 μ L Bead Wash Buffer 1 (see Figure 5).
33. Wash the beads twice with 100 μ L Bead Wash Buffer 2 (see Figure 5).
34. Wash the beads once with 100 μ L RNase-Free Water (see Figure 5).

Note: Streptavidin Beads suspended in water are attracted to the magnetic rack more slowly and dislodge more easily once collected on the wall of the tube. Be careful not to lose beads when aspirating the water from the beads.

35. Add 150 μ L of Hi-C sequencing library amplification mix to beads. Vortex briefly to completely resuspend the beads.
36. Pipette the mixture into a single PCR tube, a single well of an 8-well PCR strip, or 1 position of a 96-well PCR plate.
37. Transfer the PCR tube, strip, or sealed plate into a thermocycler, and cycle according to the program described in Table 7.

Note: To reduce sequencing bias and PCR duplicates, avoid exceeding the recommended number of PCR cycles.

Table 7. Cycling program for library amplification

Time	Temperature	Number of cycles
2 min	98°C	1
20 s	98°C	
30 s	60°C	12 (>5 \times 10 ⁴ human cells/sample; >300 ng DNA/sample)
30 s	72°C	16 (<5 \times 10 ⁴ human cells/sample; <300 ng DNA/sample)
1 min	72°C	1
∞	4°C	Hold

38. Transfer the PCR reaction into a single, fresh 1.5 mL DNA low-binding tube.
39. To separate the libraries from Streptavidin Beads, place the tube into a magnetic rack and incubate for 1 min.

40. Transfer the clear supernatant into a new microcentrifuge tube.
41. Proceed directly to “Hi-C sequencing library purification”, below, or freeze tubes overnight at -20°C before proceeding.

Hi-C sequencing library purification

42. Vortex QIAseq Beads briefly to thoroughly resuspend.

Important: Equilibrate QIAseq Beads to room temperature before use.

43. Add 150 μL of QIAseq Beads to the clear supernatant containing the amplified NGS library. Vortex briefly to mix.
44. Incubate the mixture for 5 min at room temperature.
45. Wash the beads twice (see Figure 5) with 500 μL of 80% ethanol, removing as much excess ethanol as possible after the second wash.
46. Briefly microcentrifuge the tube at 5000 $\times g$, room temperature.
47. Transfer the tube back to the magnetic stand and incubate for 30 s.
48. Remove as much of the ethanol from the bottom of the tube as possible.
49. With its lid open, incubate the tube in the magnetic rack for an additional 2–5 min or until the beads are dry.
Important: Look for the first signs of cracking or loss of glossiness. Do not excessively dry the beads, as this may result in lower DNA recovery.
50. Remove the tube from the magnetic stand.
51. Elute the DNA library by adding 25 μL of Buffer EB to the beads.
52. Pipette up and down until the beads are completely resuspended.
53. Incubate the tube for 1 min at room temperature.
54. Place the tube in a magnetic rack and incubate for 1 min more.
55. Transfer the supernatant containing the purified Hi-C sequencing library into a new DNA low-binding tube.

56. Proceed directly to “Hi-C sequencing library quality control and quantification”, or freeze tubes at -20°C before proceeding.

Hi-C sequencing library quality control and quantification

57. Assess the quality of the Hi-C sequencing libraries according to the guidelines in Appendix D (page 52).
58. Determine the concentration of the Hi-C sequencing libraries according to the guidelines in Appendix E (page 54).
59. The purified library can be safely stored at -20°C in a DNA low-binding tube until ready to use for sequencing or other applications.

Note: Prior to deep sequencing, users are strongly advised to sequence samples at a low depth (<1 million reads) for quality control purposes. Low-depth sequencing data can be analyzed online with the EpiTect Hi-C Data Analysis Portal, which generates a sequencing report for each sample (see Appendix F, page 55).

Note: The recommended sequencing depth for Hi-C libraries varies, depending on the downstream application. Refer to Appendix G (page 56) for guidance on choosing the appropriate sequencing depth for your samples.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit www.qiagen.com).

Comments and suggestions

Low library yields

- | | |
|--|---|
| a) Insufficient amount of starting material | Make sure you use at least 5×10^3 human or mouse cells (30 ng DNA) per Hi C replicate. |
| b) Inaccurate quantification of input material | A hemocytometer or automated cell counter must be used to accurately calculate input amounts for cultured cells. The tissue crosslinking protocols described in this handbook include a DNA quantification step. |
| c) Improper end-repair, A-tailing, and phosphorylation of Hi-C fragments | The on-bead incubation with the ER/A-tailing mix and buffer is a critical step for generating sequencing libraries. Be sure to fully dissolve any white precipitates in the ER/A-tailing buffer before use. Adhere to the 15 minute time limit for the incubations at 20°C and at 65°C. |

Unexpected signal peaks in capillary electrophoresis device traces

- | | |
|--|--|
| a) Presence of shorter peaks between 60 and 120 bp | These peaks represent library adapters and adapter dimers that occur when there is no, or insufficient, adapter depletion after library preparation. As adapter dimers can form clusters on the flow cell and will be sequenced, this will reduce the capacity of the flow cell for the library fragments, even though a low ratio of adapter-dimers versus library will not be a problem. Repeating library purification with SPRI beads (e.g., QIAseq Beads) should remove any residual adapter dimers, as well as free adapter molecules. |
| b) Presence of larger library fragments after library enrichment | If the fragment population shifts higher than expected after adapter ligation and PCR enrichment (e.g., more than the expected 120 bp shift), this can be a PCR artifact due to overamplification of the DNA library. Library overamplification should not affect sequencing results. However, reducing the number of amplification cycles in the future will help avoid this effect. |

Comments and suggestions

- c) Incorrect DNA fragment size prior to adapter ligation
- The wrong DNA fragment size prior to adapter ligation can be due to the wrong conditions used for DNA fragmentation. Refer to the Covaris DNA fragmentation protocol described in “Hi-C Part 2” protocol. If using a different instrument, be sure to develop a protocol that reliably fragments de-crosslinked DNA to median sizes of 400–600 bp prior to proceeding with the “Hi-C Part 2” protocol.

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Appendix A: SPRI Bead Purification of DNA Following Mechanical Fragmentation in Volumes Greater than 200 μ L

Enough Buffer SB1 is supplied to purify samples fragmented in volumes ≤ 200 μ L. For fragmentation volumes >200 μ L, refer to the protocol below:

Note: Users must supply their own SPRI beads for this purification.

1. Vortex SPRI beads briefly to thoroughly resuspend.

Important: Equilibrate SPRI beads to room temperature before using.

2. Transfer fragmented DNA to a fresh 1.5 mL microcentrifuge tube.
3. Add 1 volume of SPRI beads to 1 volume of fragmented DNA. Vortex briefly to mix.
4. Incubate the mixture for 5 min at room temperature.
5. Wash the beads as described in Figure 5 (page 40) twice with 500 μ L of 80% ethanol, removing as much excess ethanol as possible after the second wash.
6. Briefly microcentrifuge the tube at 5000 $\times g$, room temperature.
7. Transfer the tube back to the magnetic stand and incubate for 30 s.
8. Remove as much of the ethanol as possible.
9. With the tube lid open, incubate beads for 2–5 min or until the beads are dry.

Important: Look for the first signs of cracking or loss of glossiness. Do not excessively dry the beads, as this may result in lower DNA recovery.
10. Remove the tube from the magnetic stand.
11. To elute the DNA, add 50 μ L of Buffer EB and pipet beads up and down 3 times.
12. Place the tube in magnetic rack and incubate for 1 min.
13. Transfer the supernatant containing the purified DNA into a fresh 1.5 mL microcentrifuge tube.

14. Proceed directly to step 12 of “Processing a Hi-C Library Into an NGS Sequencing Library (Hi-C Part 2)”, or if you choose to make this a stopping point, freeze tubes overnight at -20°C .

Appendix B: Recommended Read Length for Sequencing of Hi-C NGS Libraries

With libraries based on median DNA fragment sizes of 400–600 bp, we recommend a sequencing read length of 75 to 150 nt. Libraries can be made from smaller DNA fragments with a medium size of 250–400 nt. In this case, however, a shorter read length of 50 nt is recommended, and this will result in a lower percentage of mapped reads compared to that obtained with 150 nt read lengths.

Appendix C: Barcodes of Illumina Adapters in EpiTect Hi-C Kit

The barcode sequences for the adapters supplied with the EpiTect Hi-C Kit are listed in Table 8. Indices 501 and 701–706 correspond to the respective Illumina Adapter barcodes.

Table 8. Adapter barcodes used in the EpiTect Hi-C Kit (6), for entry on sample sheet

EpiTect Hi-C Illumina Adapter	D50X barcode name	MiSeq®, HiSeq® 2000/2500	MiniSeq®, NextSeq®, HiSeq 3000/4000*	D70X barcode name	i7 bases
		i5 bases			
1	D501	TATAGCCT	AGGCTATA	D701	ATTACTCG
2	D501	TATAGCCT	AGGCTATA	D702	TCCGGAGA
3	D501	TATAGCCT	AGGCTATA	D703	CGCTCATT
4	D501	TATAGCCT	AGGCTATA	D704	GAGATTCC
5	D501	TATAGCCT	AGGCTATA	D705	ATTCAGAA
6	D501	TATAGCCT	AGGCTATA	D706	GAATTCGT

* Sequencing on the MiniSeq, NextSeq, and HiSeq 3000/4000 systems follow a different dual-indexing workflow than other Illumina systems; this requires the reverse complement of the i5 index adapter sequence.

Appendix D: Assessing Hi-C Sequencing Libraries with QIAxcel

After the library is constructed and purified, QIAxcel or similar devices can be used to check for various markers of Hi-C sequencing library quality:

- Fragment size and concentration
- Library overamplification
- Residual adapters in libraries
- Library concentration

QIAxcel program

The recommended setting for checking the EpiTect Hi-C sequencing library on QIAxcel:

- **Cartridge:** QIAxcel DNA High Resolution Kit (1200) (cat. no. 929002)
- **Prepare libraries:** 1:5 dilution of libraries, using a 1:1 mix of Nuclease-Free Water and QIAxcel dilution buffer
- **Size marker:** 100 bp – 2.5 kb, 10 ng
- **Alignment marker:** Use the 15 bp QX Intensity Calibration Marker as an alignment marker. Refer to Technical Services for additional support.
- **Method:** OM1700
- Injection time: 10 s
- **Analysis:** Default Smear DNA
- **Peak calling:** 400 bp, with 20% tolerance

Fragment size and concentration

Libraries should appear as a single smooth curve centered around a size of 520–720 bp. These sizes reflect the size of the fragmented DNA (400–600 bp), plus 120 bp length of the Illumina sequencing adapters.

Library overamplification

A hump or second peak larger than the expected median fragment size is usually the result of overamplification. These “larger fragments” are single-stranded library molecules with the correct size but appear larger due to secondary structure. Library overamplification should not affect sequencing results.

Residual adapters in libraries

Shorter peaks between 60 and 120 bp correspond to residual adapters and adapter dimers. These are present when adapter depletion following library amplification failed or was insufficient. As adapter dimers can form clusters on the flow cell and will be sequenced, this will reduce the capacity of the flow cell for the library fragments. However, a low ratio of adapter dimers versus library will usually not be a problem. If residual adapters are present in your sample at high concentrations, purify your sample again with SPRI beads or the GeneRead® Size Selection Kit (cat. no. 180514) to remove residual adapter dimers, as well as free adapter molecules.

Library concentration

Amounts of DNA under the appropriate peaks can be used to quantify library concentration. However, due to the superior sensitivity of qPCR, we recommend quantifying the libraries using the QIAseq Library Quant System, especially when there are overamplified libraries (see Appendix E, page 54, for more information).

Appendix E: Quantifying Hi-C Sequencing Library Concentration with the QIAseq Library Quant System

Due to the superior sensitivity of qPCR, we recommend quantifying Hi-C sequencing libraries with QIAGEN's QIAseq Library Quant Assay Kit (cat. no. 333314). With this assay, the correct dilution of the library can be determined for sequencing. Please refer to the corresponding handbook for library quantification and quality control.

The 25 μL of purified Hi-C sequencing library can be expected to have a concentration of at least 5–10 nM. Libraries with <5 nM in concentration may be concentrated using vacuum centrifugation. Refer to Illumina guidelines on final library concentrations before proceeding with sequencing.

Appendix F: Data Analysis for EpiTect Hi-C Sequencing Data

At QIAGEN's online GeneGlobe® Data Analysis Center (geneglobe.qiagen.com), Hi-C sequencing results can be analyzed using the EpiTect Hi-C Data Analysis Portal. Sequencing reads are first processed through a pipeline based on the open-source HiC-Pro toolset (7) to generate a sequencing report and Hi-C contact matrices. Upon completion of the data analysis, an installation of HiGlass (8) within GeneGlobe can be used to visualize and interact with the generated contact matrices.

For further information, refer to the *EpiTect Hi-C Data Analysis Portal User Guide* (www.qiagen.com/HB-2631).

Note: Prior to costly deep sequencing, users are advised to sequence Hi-C NGS libraries at low depth (<1 million reads) for quality control purposes. Low-depth sequencing data can be processed with the EpiTect Hi-C Data Analysis Portal, and the generated sequencing report can be used to assess the quality of Hi-C libraries. The same EpiTect Hi-C Data Analysis Portal can subsequently be used to analyze deep sequencing data.

Appendix G: Sequencing Depth Guidelines

Depending on the downstream application, Hi-C NGS libraries should be sequenced at various depths. Refer to the information below for guidance on sequencing depth for human and mouse samples.

Chromosome conformation

To capture compartments and TADs at a resolution of 40–100 kb with ~1000x coverage per bin, 100–300 million read pairs are necessary (9).

To capture finer structures like chromatin loops at a resolution of between 5 and 2.5 kb with ~1000x coverage per bin, 600 million to 1.2 billion read pairs are needed. For this application, users are advised against sequencing a single Hi-C sample at great depth. Instead, the sequence data from multiple technical replicates should be pooled to obtain the required number of total reads. For each technical replicate, the maximum sequencing depth should lie between 300 and 600 million read pairs (10).

Genome assembly

For each Gbp of haploid genome, around 100 million read pairs are needed.

Haplotype phasing

Between 300 and 600 million read pairs, giving 20–30x coverage, are needed to generate complete, accurate haplotypes of high resolution (4).

Structural variants and chromosomal rearrangements

Chromosomal rearrangements and structural variants can be detected in human and mouse samples with as few as 30 to 40 million read pairs with $\sim 0.6x$ coverage (5).

Ordering Information

Product	Contents	Cat. no.
EpiTect Hi-C Kit (6)	For 6 Hi-C reactions: Buffers and reagents for cell lysis, Hi-C digestion, Hi-C end labeling, Hi-C ligation, chromatin de-crosslinking and purification, purification of fragmented DNA, streptavidin pulldown of Hi-C fragments, and NGS library prep (end-repair, A addition, phosphorylation, adapter ligation, and library amplification); for use with Illumina instruments; includes 6 adapters with different barcodes	59971
Relative Products		
For use with Illumina instruments		
QIAseq Library Quant Assay Kit	Laboratory-verified forward and reverse primers for 500 x 25 μ L reactions (500 μ L); DNA standard (100 μ L); dilution buffer (30 mL); (1.35 mL x 5) GeneRead qPCR SYBR [®] Green Mastermix	333314
For assessing NGS library quality		
QIAxcel Advanced Instrument	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel Software, and 1-year warranty on parts and labor; fully automates sensitive, high-resolution capillary	9001941

QIAxcel DNA High Resolution
Kit (1200)

electrophoresis devices for analyzing
up to 96 samples per run

QIAxcel DNA High Resolution Gel
Cartridge, buffers, mineral oil, QX
Intensity Calibration Marker, 12-tube
strips

929002

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
04/2019	Initial revision
10/2020	Added "SPRI beads" in section "Equipment and Reagents to Be Supplied by User" under subsection "For 'Hi-C Part 1' and 'Hi-C Part 2' protocols". Rearranged the sections based on the updated template. Changed the Appendix F title. Added a statement about the computational analytical services offered by Phase Genomics, Inc. Added a license agreement statement.
02/2023	Edited according to new brand template. Added: Protocol: Preparing collagenase I and II solutions for tissue crosslinking procedures Added: Protocol: Crosslinking Chromatin in Vertebrate Tissue Added: Protocol: Crosslinking Chromatin in Insect Tissue Added: Protocol: Preparing Aliquots of Low Input Crosslinked Tissue Samples

Notes

Notes

Limited License Agreement for EpiTect Hi-C Kit

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