April 2019

EpiTect® Hi-C Handbook

For performing Hi-C experiments on bulk populations of cells

Sample to Insight

QIAGEN

Contents

Kit Contents

EpiTect Hi-C Kit, Box 1 of 3	**(6)**	
Catalog no.	**59971**	
Number of reactions	**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	**(6)**	
Catalog no.	**59971**	
Number of reactions	**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	**(6)**	
Catalog no.	**59971**	
Number of reactions	**6**	
Component	**Tube cap color**	**Quantity**
Proteinase K	Clear	250 µl
QIAseq® Beads	Clear	2.5 ml
Streptavidin Beads	Clear	150 µl
Buffer C1	Clear	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 the EpiTect Hi-C Kit, Box 2, should be stored at room temperature (15–25°C). The contents of the EpiTect Hi-C Kit, 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.

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.

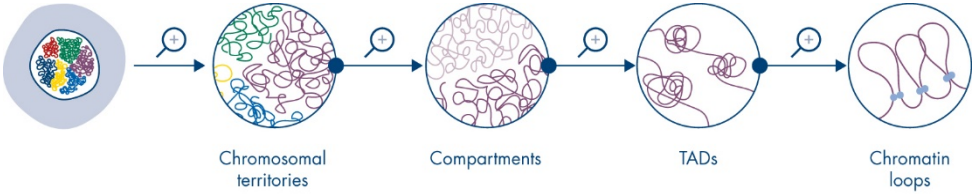

Figure 1. Levels of chromatin organization.

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).

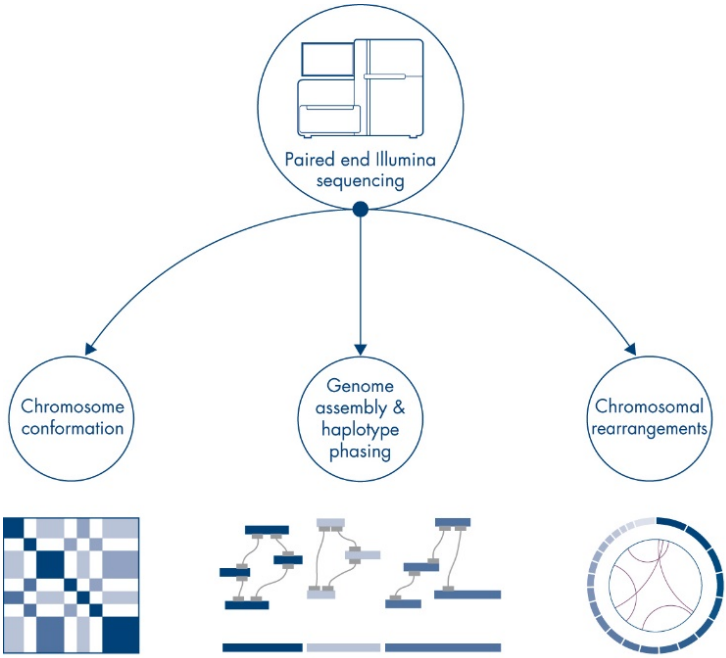

Figure 2. Downstream applications of Hi-C sequencing data. Product description and workflow.

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, 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.

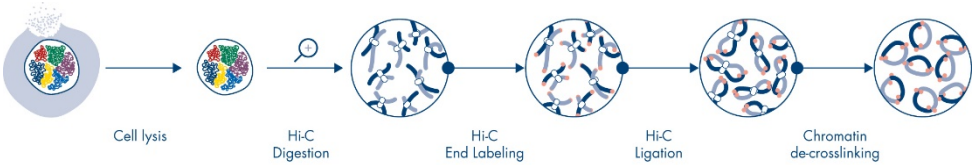

Figure 3. Workflow for processing fixed cells into a Hi-C library.

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.

Important Notes

Sample input

The EpiTect Hi-C protocol has been optimized for the use of 5 x 10^5 human or mouse cells (or the equivalent of 3 µg of DNA) per sample. Input amounts between 2.5 x 10^5 and 2.5 x 10^6 human or mouse cells (or the equivalent of 1.5–15 µg DNA) per replicate may also be used. However, the use of input amounts beyond this range 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* that sample-input amounts are accurately quantified before proceeding with the Hi-C protocol. An automated cell counter or hemocytometer should be used to assist in distributing the correct amount of cells to each sample.

Sample type

In principle, the EpiTect Hi-C protocol should work for any type of crosslinked eukaryote cells, but so far it has only been experimentally validated with cultured human and mouse cells.

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 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.

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 "Hi-C Part 1" and "Hi-C Part 2" protocols

- 1% SDS
- 10% Triton X-100
- 100% isopropanol
- 80% ethanol
- 3M 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

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

- 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.
- 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 17), before starting.

Things to do before starting

- Bring the complete cell culture medium (i.e., medium with serum) to room temperature (15–25°C).
- 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 x 10^6 and 1 x 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 conical tube and centrifuge for 10 min at 450 × *g* at room temperature.
5. Remove supernatant and gently resuspend 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 × *g* at 4°C.
12. Resuspend cell pellet in 10 ml cold PBS.
13. Use a hemocytometer or automated cell counter to determine cell density.
14. Centrifuge cells for 5 min at 800 x *g* at 4°C and aspirate supernatant.
15. Resuspend cells in fresh, cold PBS to desired concentration.
16. Distribute 5 x 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 2.5 x 10^5 to 2.5 x 10^6 cells per sample.
17. Centrifuge 5 min at 800 x *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: 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 15). Each Hi-C kit contains reagents sufficient for processing 6 samples.

Important points before starting

- It is *critical* to use a hemocytometer or automated cell counter to assist in distributing the correct amount of cells to each replicate.
- 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 Tables 1 and 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 Tables 1 and 2.

Table 1. Setup for preparing Hi-C Digestion Solution

Component	Volume
Hi-C Digestion Buffer	4 µl
1% SDS	4 µl
RNase-free water	32 µl
Total volume	**40 µl**

Table 2. Setup for preparing Hi-C Ligation Solution

Component	Volume
Hi-C Ligation Buffer	400 µl
10% Triton X-100	80 µl
Ultralow Input Ligase	10 µl
RNase-free water	260 µl
Total volume	**750 µl**

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 x 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 2.5 x 10^5 and 2.5 x 10^6 human or mouse cells (or the equivalent of 1.5–15 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 tube back and forth 5 times.
8. Incubate tube on ice for 10 min.
9. Centrifuge tube for 5 min at 2500 x *g* at 4°C.
10. Carefully aspirate supernatant, leaving the soft nuclear pellet behind.
11. Carefully resuspend nuclei in 500 µl of ice-cold RNase-free water and mix by gently pipetting up and down 3–4 times.
12. Centrifuge tube for 5 min at 2500 x *g* at 4°C.
13. Carefully aspirate and discard the supernatant, leaving the soft nuclear pellet behind.
14. Proceed immediately to "Hi-C digestion" below.

Hi-C digestion

15. Add 40 µl Hi-C Digestion Solution to washed nuclei. Mix by gently pipetting up and down 3–4 times.
16. Incubate tube at 65°C for 10 min.

    Important: Do not shake tube during this incubation.
17. Place tube on ice immediately after incubation.
18. Add 4.4 µl 10% Triton X-100. Mix by gently pipetting up and down 3–4 times.
19. Add 4 µl Hi-C digestion enzyme. Mix by gently pipetting up and down 3–4 times.
20. Incubate tube in a thermal mixer set to 37°C with 600 rpm shaking for 2 h to digest chromatin.
21. Incubate tube at 65°C for 20 min.

    Important: Do not shake tube during this incubation.
22. Place tube on ice and proceed immediately to "Hi-C end labeling", next page.

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

Hi-C end labeling

23. 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.
24. Mix by gently pipetting up and down 3–4 times.
25. Incubate the tube at 37°C for 30 min to label the digested chromatin with biotinylated nucleotides.

    Important: Do not shake tube during this incubation.
26. Place tube on ice and proceed immediately to "Hi-C ligation" below.

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

Hi-C ligation

27. Transfer all of the Hi-C Ligation Solution to tube containing end-labeled chromatin. Mix by gently inverting 5 times.
28. Incubate tube at 16°C for 2 h to ligate spatially proximal DNA fragments.

    Important: Do not shake tube during this incubation.

    Important: Ligation at temperatures >16°C is not recommended.
29. Place tube on ice and proceed immediately to "Chromatin de-crosslinking" below.

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

Chromatin de-crosslinking

30. Add 20 µl Proteinase K solution to ligated chromatin. Mix gently by inverting tube 5 times.
31. Incubate tube at 56°C for 30 min, followed by 80°C for 90 min.
32. Briefly spin the tubes at low speed (4000 x *g*) to collect any condensation from the lid.

    Cool the reaction mixtures to room temperature and proceed directly to "DNA purification following de-crosslinking", next page.

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

DNA purification following de-crosslinking

33. Add 80 µl of 3 M sodium acetate, pH 5.2, to the de-crosslinked DNA. Vortex briefly to mix.
34. Add 560 µl of 100% isopropanol to tube. Vortex briefly to mix.
35. Place a MinElute column, including its provided collection tube, in a rack.
36. To bind DNA, apply one-half of the mixture (725 µl) to the MinElute column, and then centrifuge for 1 min at 17,900 × *g*.
37. Discard flow-through and place the MinElute column back into the same collection tube.
38. Apply the remaining half of the mixture (~725 µl) to the MinElute column, and then centrifuge for 1 min at 17,900 × *g*.

    Note: For maximum recovery, transfer all traces of the sample to the column.
39. Discard flow-through. Place the MinElute column back into the same collection tube.
40. To wash, add 0.75 ml Buffer PE to the MinElute column. Centrifuge for 1 min at 17,900 × *g*.
41. Discard flow-through and place the MinElute column back in the same tube.
42. Centrifuge the MinElute column for an additional 1 min at 17,900 × *g*.
43. Place MinElute column into a new 1.5 ml microcentrifuge tube.
44. To elute DNA, add 35 µl Buffer EB prewarmed to 65°C to the center of the membrane.

    Note: If DNA is to be fragmented in volumes <35 µl, elute DNA in 20 µl prewarmed Buffer EB. Elution in volumes <20 µl will result in lower DNA yields.
45. Incubate for 1 min at room temperature.
46. Centrifuge the column for 1 min at 17,900 × *g*.
47. Store purified DNA at –20°C or proceed with "Protocol: Processing a Hi-C Library Into an NGS Sequencing Library (Hi-C Part 2)", next page.

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, found in Appendix A (page 31).

  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", next page.

   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 32).

DNA purification following mechanical fragmentation

3. Add 4 volumes of Buffer SB1 to 1 volume of fragment 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 x *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 x *g*. Discard the flow-through and place the MinElute spin column back into the same tube. Repeat this step.
8. Centrifuge for 1 min at 17,900 x *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" below, 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 Tables 4–6, next page.

Table 4. ER/A-Tailing Solution

Component	Volume
ER/A-Tailing Buffer	5 µl
ER/A-Tailing Enzyme Mix	10 µl
RNase-free water	35 µl
Total volume	**50 µl**

Table 5. Adapter Ligation Buffer Dilution

Component	Volume
Adapter Ligation Buffer	15 µl
RNase-free water	135 µl
Total volume	**150 µl**

Table 6. Hi-C Sequencing Library Amplification Mix

Component	Volume
HiFi PCR Master Mix, 2x	200 µl
Primer Mix Illumina Library Amp	12 µl
RNase-free water	188 µl
Total volume	**400 µl**

13. Mix solutions thoroughly by pipetting, and then place on ice.
14. Proceed to "Streptavidin pulldown of Hi-C fragments" below.

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, next page.

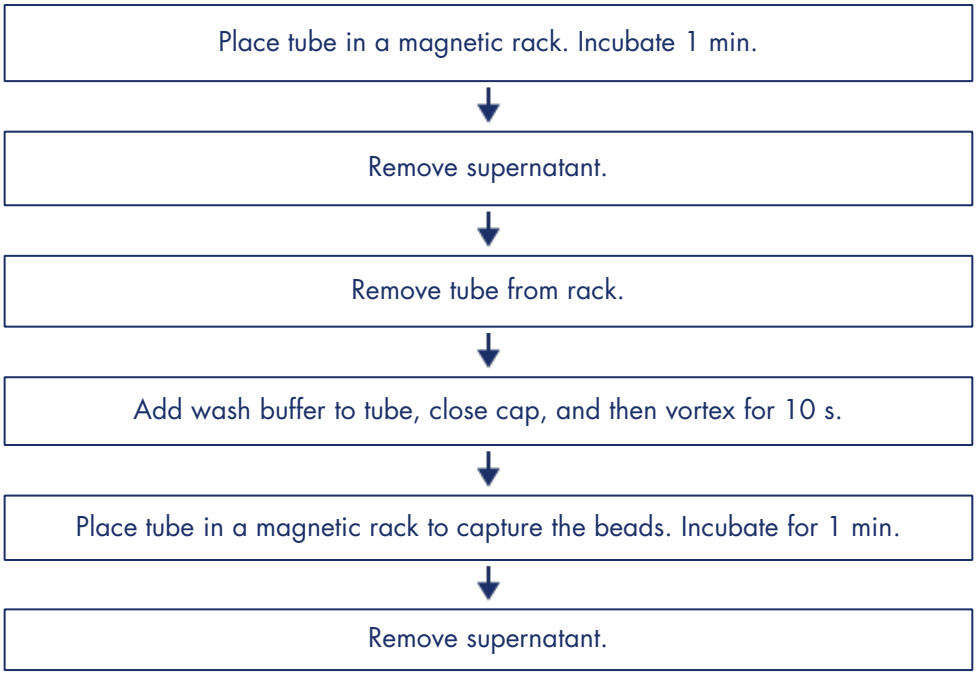

Figure 5. Standard bead-washing process.

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" below.

DNA end-repair/A-tailing/phosphorylation

21. Wash beads once in 100 µl Bead Wash Buffer 2; see steps in Figure 5.
22. Resuspend beads in 50 µl of prepared ER/A-tailing solution.
23. Incubate 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", next page.

Illumina adapter ligation

Note: Refer to "Good PCR laboratory practices" (page 12) 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 adaptors have sequence barcodes (see Appendix C, page 33) 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 tube for 45 min at room temperature.
31. Proceed directly to "Hi-C sequencing library amplification" below.

Hi-C sequencing library amplification

32. Wash the beads twice with 100 µl Bead Wash Buffer 1 (see Figure 5).
33. Wash the beads twice with 100 µl Bead Wash Buffer 2 (see Figure 5).
34. Wash the beads once with 100 µl 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 400 µl of Hi-C sequencing library amplification mix to beads. Vortex briefly to completely resuspend the beads.
36. Distribute mixture evenly into 8 PCR tubes, an 8-well PCR strip, or 8 positions of a 96-well PCR plate.
37. Transfer the PCR tubes, strip, or sealed plate into a thermocycler, and cycle according to the program described in Table 7.

    Note: At high concentrations, the paramagnetic streptavidin beads are inhibitory to PCR reactions. For efficient PCR amplification, it is important that ~400 µl of PCR mix is evenly divided into 8 separate reactions.

    Note: To reduce sequencing bias and PCR duplicates, avoid exceeding the recommended 7 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	7
30 s	72°C	
1 min	72°C	1
∞	4°C	Hold

38. Pool the 8 PCR reactions into a single, fresh 1.5 ml DNA low-binding tube.
39. To separate the libraries from streptavidin beads, place the tube into 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", next page, 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 400 µl of QIAseq Beads to the clear supernatant. 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 x *g*, room temperature.
47. Transfer 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 tube from the magnetic stand.
51. Elute DNA library by adding 25 µl of Buffer EB to the beads.

    Pipette up and down until the beads are completely resuspended.

52. Incubate tube for 1 min at room temperature.
53. Place tube in magnetic rack and incubate for 1 min more.
54. Transfer into a new DNA low-binding tube the supernatant containing the purified Hi-C sequencing library.
55. Proceed directly to "Hi-C sequencing library quality control and quantification", next page, or freeze tubes at –20°C before proceeding.

Hi-C sequencing library quality control and quantification

56. Assess the quality of the Hi-C sequencing libraries according to the guidelines in Appendix D (page 34).

57. Determine the concentration of the Hi-C sequencing libraries according to the guidelines in Appendix E (page 36).

58. 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 37).

    Note: The recommended sequencing depth for Hi-C libraries varies, depending on the downstream application. Refer to Appendix G (page 38) for guidance on choosing the appropriate sequencing depth for your samples.

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

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

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 26) twice with 500 µl of 80% ethanol, removing as much excess ethanol as possible after the second wash.
6. Briefly microcentrifuge the tube at 5000 x *g*, room temperature.
7. Transfer 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 tube from the magnetic stand.
11. To elute DNA library, add 50 µl of Buffer EB and pipet beads up and down 3 times.
12. Place tube in magnetic rack and incubate for 1 min.
13. Transfer supernatant into a fresh 1.5 ml microcentrifuge tube.

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 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 75 or 50 nt is required, 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	
		i5 bases			i7 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

* **Note**: 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**: 15 bp
- **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 the size of the fragmented DNA (400–600 bp), plus 120 bp (i.e., 520–720 bp). The 120 bp increase in library length reflects the addition of sequencing adapters to the DNA fragments.

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 36, 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 Using QIAGEN's EpiTect Hi-C Sequencing Data Analysis Portal

At QIAGEN's online GeneGlobe® Data Analysis Center (**www.qiagen.com/DataAnalysisCenter**), Hi-C sequencing results can be analyzed using the EpiTect Hi-C 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**) that is included with each data analysis report.

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 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 topologically associated domains (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).

Chromosomal rearrangements

Using bin sizes of 500 kb – 1 Mb, chromosomal rearrangements can be detected in human and mouse samples with as few as 30 million reads with ~0.6x coverage (5).

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 either 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 2.5 x 10^5 human or mouse cells per Hi-C replicate
b)	Inaccurate quantification of input material	A hemocytometer or automated cell counter must be used to accurately calculate input amounts.
c)	Library amplification PCR was not split into 8 separate reactions	At high concentrations, the paramagnetic streptavidin beads are inhibitory to PCR reactions. For efficient PCR amplification, it is important that the ~400 µl of PCR mix is evenly divided into 8 separate reactions.
d)	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.
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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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
Related products		
For use with Illumina instruments		
QIAseq Library Quant Assay Kit	Laboratory-verified forward and reverse primers for 500 x 25 µl reactions (500 µl); DNA standard (100 µl); 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 electrophoresis devices for analyzing up to 96 samples per run	9001941
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Gel Cartridge, buffers, mineral oil, QX Intensity Calibration Marker, 12-tube strips	929002

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Notes

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