C-HiC: A High-Resolution Method for Unbiased Chromatin Conformation Capture Targeting Small Locus

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Chapter 6

C-HiC: A High-Resolution Method for Unbiased Chromatin Conformation Capture Targeting Small Locus

Jérôme D. Robin

Abstract

Within the nucleus, precise DNA folding and organization is mandatory for a tight control of gene expression. In the past 20 years, a wealth of molecular approaches has unraveled the existence of DNA territories. With the emergence of affordable deep-sequencing approaches, "Cs" techniques such as 4C, 5C, and HiC, to name a few, are now routinely performed by the scientific community in a large number of model systems. We have modified the HiC approach to a capture probe-based version named C-HiC. This updated assay has resulted in an improved throughput analysis, reduced input material, and good repeatability. The protocol described below details our procedure and notes for a C-HiC approach, designed to target only specific portion of a given genome.

Key words Higher-order organization, HiC, Chromatin conformation capture, Chromatin looping, Chromatin domain

1 Introduction

Higher order organization of chromatin allows DNA to be compacted into separated compartments (e.g., A and B) and territories. This organization sets the chromatin into defined structured loops and discrete domains allowing precise gene expression patterns that can be modulated by protein complexes controlling long-distance interactions between promoters and *cis*-regulatory regions [1–3].

First investigated by contrast microscopy [4] then, more recently by chromatin conformation capture (i.e., 3C) [5], chromosomal territories and corresponding 3D organization constitute a wide field of investigation that aims at understanding the relation between chromatin states, dynamics, and influence on gene expression in various contexts (i.e., pathologies, cancers, differentiation). The rise of techniques used to monitor chromatin landscapes has established the existence of major chromosomal domains of different scales. Topologically associated domains (TADs) [6] are the

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most studied and represent domains of 500Kb to few Mbs. Associations between similar TADs (based on dynamic gene expression) form the higher scale of organization, dividing the DNA into chromosomal compartments (A and B). A being the open transcriptionally active chromatin (e.g., euchromatin), found mostly internally, whereas B compartments are associated with silenced chromatin (e.g., heterochromatin) and localized at the nuclear periphery [7, 8]. At smaller scales, other domains have been detailed such as those associated with nuclear organelles known as lamina-associated domains (LADs) [9] and nucleolus-associated domains (NADs) [1, 10]. The formation of chromosomal domains is thought to be driven by a combination of sequence specificity, genomic localization, and epigenetic states. Our understanding of the 3D structure of DNA arises from a continuum of complementary techniques, performed over the past 30 years, that have set our views on DNA folding from yeast to human nuclei. Indeed, the most recent studies in yeast revealed that the sequences per se seem to hold the code of this organization rather than its genomic localization [11, 12].

The complete folding of the human genome was released a decade ago, thanks to a "C-like" technique named HiC (for high-resolution capture) that was adapted to a deep sequencing design [7]. Since then, the description of the higher-order organization of the human genome has been explored in different normal and pathological contexts [13–15]. Importantly, the core of C-based techniques relies on the determination of frequency of interactions detected by PCR or sequencing. If the average of 3D structures can be drawn from these data, it is not clear yet how one can detect discrete *per cell* information, underlying the need of controls (*see* Subheading 3.12). Additionally, the lack of resolution (e.g., data are limited to 500 kb to 1 Mb scale) and the requirement of large amounts of materials to achieve appropriate depth of sequencing have led to innovative approaches combining already existing techniques.

Among many, tethered HiC (T-HiC) [16, 17] and captured-based HiC (C-HiC) [18–22] seem to be the easiest on-hand assays. The first one allows for a protein-centered vision of the 3D chromatin, using an immunoprecipitation step (i.e., emphasis on *trans*-regulatory interactions). The second uses a probe capture step, hence focusing on a specific region of the genome (5–10 Mb), leading to a higher resolution otherwise not solved by classical HiC [19–21]. Importantly, C-HiC techniques with additional steps not only filter out regions that are not of interest but also increase the depth of sequencing of the targeted region, decreasing at the same time the complexity of the data to be handled. Last, those approaches require less material, an important factor when dealing with rare samples (e.g., biopsies or pathological specimen) [21, 22]. The C-HiC aims to provide regulatory information

(in *cis*) of targeted loci relevant to specific clinical diseases [19, 22], ultimately reducing cost of sequencing and increasing the potential treatment of side-by-side samples (patients analyzed per flow cell).

The protocol provided below describes all materials, reagents, and steps needed to perform a complete C-HiC assay along with important controls and notes that one should consider when designing such experiments (Fig. 1).

2 Materials

Prepare all solutions following appropriate safety rules (i.e., fume hoods, gloves). Set up all amplification and PCR steps using typical PCR precautions.

2.1 Equipment

Rocking shaker.

Dounce homogenizer.

Thermoshaker.

Sonicator/Covaris/Bioruptor.

DNA LoBind tubes.

Magnet holder.

Rotation wheel.

Thermocycler.

2.2 Chemicals

16% formaldehyde (w/v), methanol-free.

2.5 M glycine.

PBS 1×.

Protease inhibitors.

1% SDS.

10% Triton X-100.

Phenol pH 8.0.

Chloroform.

3 M Na-acetate.

0.5 M EDTA pH 8.0.

100% ethanol.

UltraPure BSA.

0.1 M NaOH.

1 M Tris–HCl pH 7.5.

1 M Tris–HCl pH 8.0.

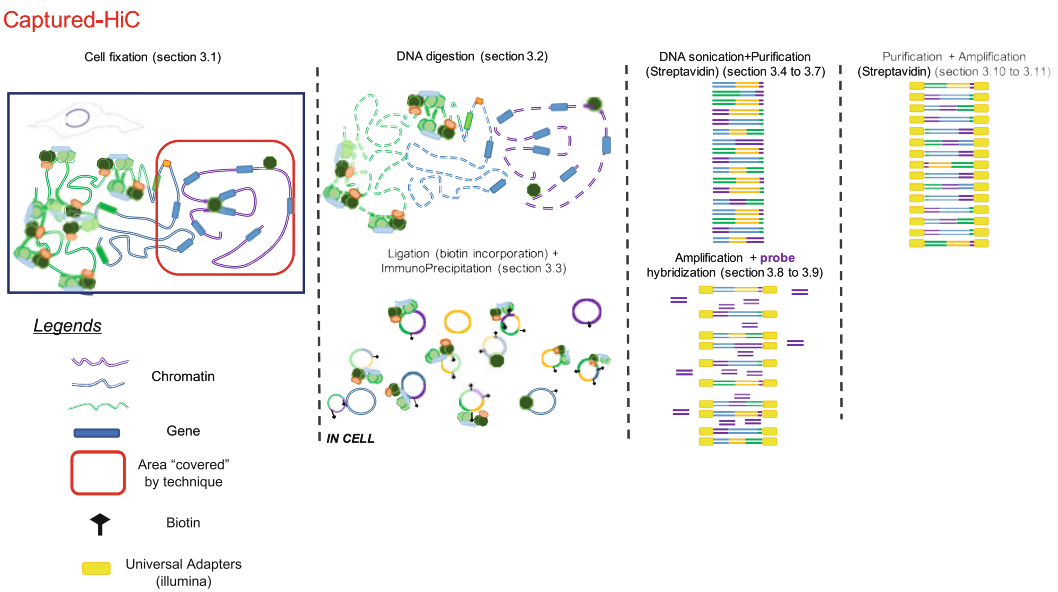


Fig. 1 Captured HiC (C-HiC) overview. Schematic representation of the main steps of a typical C-HiC procedure, where the target locus is represented in purple (red square region). Importantly, all steps up to ligation (Subheading 3.1, **steps 1–9**) are performed in cellulo. Matching steps' numbers are reported accordingly to the protocol provided

2.3 Enzymes

*Hind*III, *Nhe*I restriction enzyme.

T4 DNA ligase.

T4 DNA polymerase.

T4 polynucleotide kinase.

DNA polymerase I large fragment (Klenow).

Klenow (exo-).

Proteinase K.

RNase A.

NEBNext® Ultra™ II Q5® Master Mix (NEB).

2.4 Buffers

Fixation buffer: Cell culture media (depending on cell culture) complemented with formaldehyde (i.e., 10 ml media +0.625 μl formaldehyde; 1% final).

Lysis buffer: 10 mM Tris–HCl pH 8.0, 10 mM NaCl, 0.2% NP40.

Lysis buffer^{+}: 10 mM Tris–HCl pH 8.0, 10 mM NaCl, 0.2% NP40, 1× protease inhibitors.

CutSmart® Buffer 10×: 500 mM potassium acetate, 200 mM Tris-acetate, 100 mM magnesium acetate, 100 μg/ml BSA.

NEBuffer 2 10×: 500 mM NaCl,100 mM Tris–HCl pH 7.9, 100 mM $MgCl_2$, 10 mM DTT.

Ligation buffer 10×: 500 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT.

TE: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.

Tween wash buffer 1× (TWB): 5 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween.

Binding buffer 2× (BB): 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 2 M NaCl.

RNABait wash buffer 1 (RWB1): 1× SSC, 0.1% SDS.

RNABait wash buffer 2 (RWB2): 0.1× SSC, 0.1% SDS.

MySelectHYB#1: 20× SSPE.

MySelectHYB#3: 50× Denhardt's.

2.5 Nucleic Acids, Nucleotides, and Oligos

dNTPs (separated mix; 100 mM each).

0.4 mM biotin-14-dCTP (Life Technologies).

100 mM ATP.

1 μg/ml human *Cot1* DNA (Life Technologies).

1 μg/ml Salmon sperm DNA (Life Technologies).

Dynabeads MyOne Streptavidin C1 beads (Life Technologies).

Biotinylated-RNA capture probe library (MYcroarray-MYselect, MI) or SureSelectXT RNA library (Agilent).

Control-*Hind*III -*1* GTTCATCTTGCTGCCAGAAATGCCGAG CCTG.

Control-*Hind*III-*2* ATCCCAGCTGTCTGTAGCTTTAGAAAG TGGG.

Illumina Paired End adapters.

Illumina PE PCR Primer 1.0.

Illumina PE PCR Primer 2.0.

MYSelect purification Kit (MYcroarray).

Gel extraction kit (Qiagen).

MinElute purification Kit (Qiagen).

dsDNA QuBit assay (Life Technologies).

3 Methods

3.1 Cross-Linking

1. Grow 5 dishes of five million cells in recommended media to 70% of confluence, and wash cells once with fresh media at 37 °C.
2. Fix cells using a fresh fixation buffer (*see* **Note 1**).
3. Incubate at room temperature for 10 min on a rocking platform.
4. Add 580 µl of 2.5 M glycine solution (0.1 M final), and incubate at room temperature for 5 min on a rocker.
5. Incubate dishes on ice for 5 min.
6. Wash cells with ice-cold PBS1×, twice.
7. Using 200 µl of ice-cold PBS1×, scrap cell from dish and transfer them to a 1.5 ml epi tube.
8. Centrifuge at 250 × *g* for 10 min at 4 °C.
9. Discard supernatant.
10. Proceed with next steps or flash freeze cells and store at −80 °C.

3.2 Lysis-Chromatin Digestion

1. Resuspend the pellet by adding 250 µl lysis buffer complemented by 50 µl of protease inhibitors per cross-linked cell pellets.
2. Incubate on ice for at least 15 min.
3. Centrifuge at 2500 × *g* for 10 min at 4 °C, and discard the supernatant.
4. Wash pellets once with ice-cold lysis buffer^{+}.

5. To remove uncross-linked proteins, resuspend pellet in 50 µl lysis buffer^{+} complemented with 0.5% SDS (add 2.5 µl of a 10% SDS solution).
6. Avoid foam formation and incubate 8 min at 62 °C in a thermoshaker.
7. To quench the reaction, add 150 µl of Triton X-100 mixture (20 µl of Triton X-100 in 130 µl water). Mix gently to avoid foam formation.
8. Incubate 15 min at 37 °C—Take at this step an aliquot of 10 µl of each sample. Store and label tubes as undigested control.
9. Add 22.5 µl of CutSmart Buffer 10× and 400 units of *Hind*III.
10. Incubate overnight at 37 °C in a thermoshaker to digest the chromatin—Take an aliquot of 10 µl of each sample. Store and label tubes as digested control.

3.3 DNA Ends Labeling and In-Cell Ligation

1. Inactivate the restriction enzyme at 62 °C for 20 min in a thermoshaker.
2. Centrifuge at 2500 × *g* for 10 min at 4 °C, and discard the supernatant.
3. Resuspend the cells in 300 µl NEBuffer2 1.1×, and split samples in 3 epi tubes.
4. Keep 1 tube on ice as a 3C control.
5. Add 50 µl of fill in mix to each of the two remaining tubes as prepared below:
   1.5 µl of 10 mM dATP.
   1.5 µl of 10 mM dGTP.
   1.5 µl of 10 mM dTTP.
   37.5 µl of 0.4 mM biotin-14-dCTP (Invitrogen).
   8 µl of 5 U/µl Klenow (NEB).
6. Incubate at 37 °C for 45 min in a thermoshaker.
7. Put all tubes on ice, and retrieve 3C control tube.
8. Add 1 ml of complete ligation mix 1× prepared as below:
   130 µl of ligation buffer 10×.
   130 µl of 10% Triton X-100.
   13 µl of 10 mg/ml BSA.
   1.3 µl of 100 mM ATP.
   1 µl of T4 DNA ligase—use 0.1 µl for the 3C control.
   qsp 1 ml water.
9. Incubate at 16 °C for 8 h on a rotating wheel. Add 1.3 µl of 100 mM ATP every 2 h.

3.4 Reverse Cross-link-DNA Purification

1. Centrifuge at 2500 × *g* for 10 min at RT, and discard the supernatant (*see* **Note 2**).
2. Resuspend the cells in 200 µl TE.
3. Add 20 µl of proteinase K, and incubate at 60 °C overnight in a thermoshaker.
4. Add an extra 5 µl of proteinase K, and incubate at 60 °C for 1 h in a thermoshaker.
5. Cool the reaction mixture to RT.
6. Extract DNA by standard phenol/chloroform procedure:
   Add 300 µl of phenol (pH 8.0) to each tube.
   Vortex.
   Centrifuge at 20,000 × *g* for 10 min at RT.
   Transfer supernatants to new epi tubes.
   Add 300 µl of phenol/chloroform (1:1 mix) to each tube.
   Vortex.
   Centrifuge at 20,000 × *g* for 10 min at RT.
   Transfer supernatants to new epi tubes.
   Add 300 µl of chloroform to each tube.
   Vortex.
   Centrifuge at 20,000 × *g* for 10 min at RT.
   Transfer sups to new epi tubes.
   Add 300 µl of chloroform to each tube.
   Vortex.
   Centrifuge at 20,000 × *g* for 10 min at RT.
   Transfer supernatants to new epi tubes.
7. Bring the volume of each tubes to 200 µl with TE.
8. Precipitate DNA by adding 20 µl of 3 M Na-acetate to each sample.
9. Mix well and add 500 µl of ice-cold 100% ethanol.
10. Invert tubes several times, and incubate at −80 °C for 1 h.
11. Centrifuge at 20,000 × *g* for 20 min at 4 °C and carefully remove the supernatant.
12. Wash pellets five times with 500 µl 70% ethanol to remove salts.
13. Resuspend the pellets in 50 µl TE.
14. Degrade RNA by adding 1 µl of RNAse A (1 mg/ml) to each tube.
15. Incubate at 37 °C for 25 min.
16. Add 150 µl of TE and extract DNA by standard phenol/chloroform procedure (re-do Subheading 3.4, **steps 6–13**, without the phenol-only step).

17. Resuspend the pellets in 50 µl TE, and quantify DNA using a dsDNA QuBit assay. For now on, only use DNA LoBind tubes (*see* **Note 2**).

3.5 Quality Controls (Fig. 2)

1. Digestion-ligation control: run a 0.8% agarose gel, and load 1 µl of each sample aside with the undigested and digested controls (from Subheading 3.2, **steps 8** and **10**).
2. HiC ligation control (*see* **Note 3**):
   Perform a PCR reaction using 1 µl of each sample (HiC and 3C) as template and Control-*Hind*III primers.
   Run PCR using the following program:

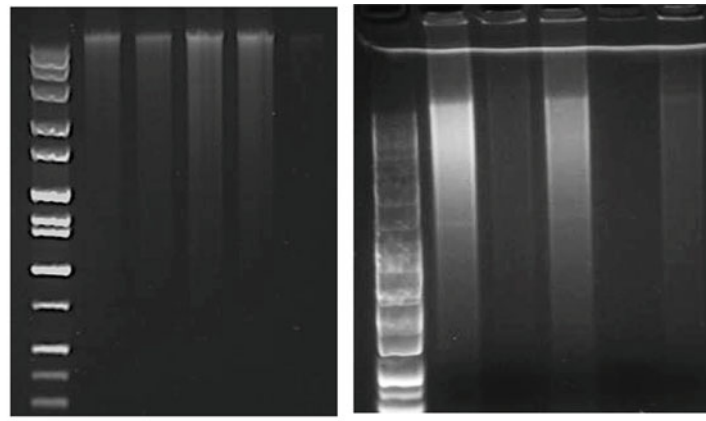
95 °C for 3 min	
40 cycles at:	95 °C for 45 s 60 °C for 45 s 72 °C for 90 s
7 min final extension at 72 °C	

   Digest PCR products by either *Hind*III or *Nhe*I and run products on a 1% agarose gel.
   Efficient procedure is verified by a yield of 70% of digestion by *Nhe*I.
3. Pursue steps only for successful samples; store the 3C control sample at −80 °C.

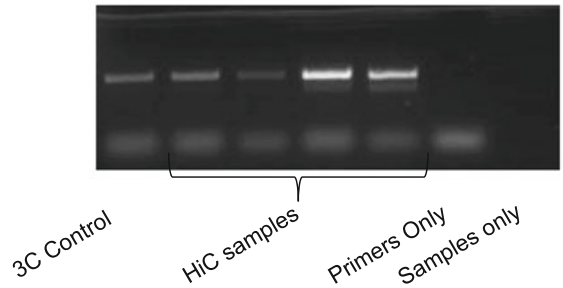
3.6 Biotin-End Removal, DNA Shearing, and Repair

1. Remove biotin from unligated ends by preparing 100 µl reactions as follows:
   5 µg of HiC sample.
   1 µl of 10 mg/ml BSA.
   10 µl of 10× T4 DNA polymerase buffer.
   1 µl 10 mM dATP.
   2 U of T4 DNA polymerase.
2. Incubate at 20 °C for 1 h in a thermoshaker.
3. Stop reaction by adding 2 µl of 0.5 M EDTA pH 8.0.
4. Pool tubes and sonicate DNA to a 300–500 bp size range.
5. Using a Bioruptor: 30/90 s cycles on/off for five cycles for a 250 µl sample (*see* **Note 4**).
6. Control sonication by running 1 µl aliquots on a 1% agarose gel. Adjust conditions accordingly.
7. Purify DNA using a MinElute kit. Elute DNA in 2× 50 µl TE and quantify DNA by dsDNA QuBit assay.
8. Repair DNA ends by adding to a 100 µl sample:

A- *Chromatin Digestion*



B- *HiC Library Control: PCR amplification*



C- *HiC Library Control: PCR digestion*

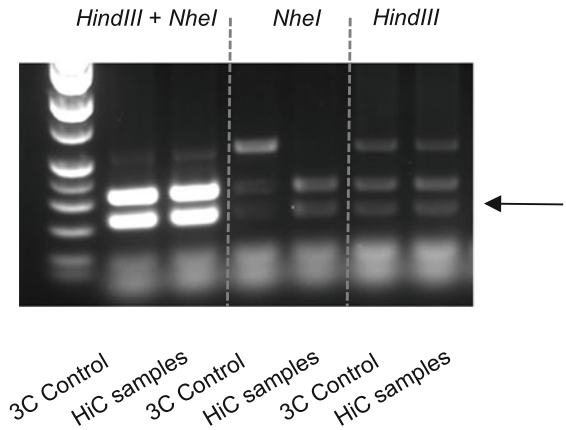


Fig. 2 C-HiC controls. Representative agarose gel (2%) of a typical HiC procedure. (**a**) Enzymatic restriction of chromatin. Failed digestion (left) results in a non-migrating DNA stuck at the top of the gel along with a very

14 μl of 10× ligation buffer.

2 μl of 10 mM dNTPs.

2 U T4 DNA polymerase.

2 U T4 polynucleotide kinase.

1 μl Klenow DNA polymerase.

9. Incubate for 30 min at 20 °C in a thermoshaker.
10. Purify DNA using a MinElute kit. Elute DNA in 2× 15 μl TE.
11. Add an "A" to the 3′ end using Klenow (exo-) mix in a 50 μl final solution:
    5 μl of 10x Klenow buffer.

    1 μl of 10 mM dATP.

    2U Klenow (exo-).
12. Incubate at 37 °C for 30 min in a thermoshaker, and inactivate at 65 °C for 20 min.

3.7 First Biotin Pull-Down

1. Prepare the Dynabeads MyOne Streptavin C1 beads, for each sample:
   Add 400 μl TWB to 6 μl of beads.

   Retain beads by holding tubes against a magnet for 1 min, and remove supernatant.

   Repeat the wash for a total of two TWB washes.

   Resuspend beads in 200 μl BB 2×, and use fresh tubes.
2. Combine the beads in BB 2× mixture to HiC samples mix in a 400 μl final volume (*see* **Note 5**).
3. Incubate at RT for 15 min on a rotation wheel.
4. Hold sample against a magnet and resuspend in 400 μl 1× BB.
5. Transfer to new tubes, hold against magnet, and resuspend in 100 μl ligation buffer.
6. Transfer to new tubes, hold against magnet, and resuspend in 50 μl ligation buffer.

3.8 PE Adapter Ligation: HiC Library

1. Complement each tube with 6 pmol of PE adapters (can be indexed) per μg of DNA (measured in Subheading 3.6, **step 7**).
2. To each tube, add 1 μl of 10 mM ATP and 1U of T4 DNA ligase.

Fig. 2 (continued) faint smear. Successful digestion (right) results in a bright smear, with an ideal average size at 4 kb. (**b**) PCR amplification of a known loop, used to control for biotin's integration (Subheading 3.5, **step 2**). (**c**) Digestion of the PCR product. Successful HiC preparation (biotin integration) will translate into a PCR product that is digested by *Nhe*I (Subheading 3.5, **step 3**). A 3C control is shown as control of digestion. Digested products are indicated by the arrow

3. Incubate at 16 °C overnight on a rotation wheel.
4. Add 400 μl of TWB, hold sample against a magnet for 3 min, remove supernatant, and resuspend beads in 400 μl of TWB.
5. Transfer samples to new tubes, hold against magnet, remove supernatant, and resuspend in 200 μl TWB.
6. Transfer samples to new tubes, hold against magnet, remove supernatant, and resuspend in 200 μl BB 1×.
7. Transfer samples to new tubes, hold against magnet, remove supernatant, and resuspend in 200 μl NEB 2.
8. Transfer samples to new tubes, hold against magnet, remove supernatant, and resuspend in 50 μl NEB 2.
9. Repeat Subheading 3.8, **step 8**.
10. Amplify the library by PCRs, and assess the appropriate number of amplification cycles:
    Set a series of PCR library mixture (5 points):
    1.5 pm of PE PCR primers 1.0.
    1.5 pm of PE PCR primers 2.0.
    10 μl NEBNext® Ultra™ II Q5® Master Mix.
    0.6 μl HiC DNA-bound beads.
11. Run the PCR using the following program:

98 °C for 1 min	
Do *X* cycles of:	98 °C for 10 s 65 °C for 30 s 72 °C for 30 s
Final extension at 72 °C for 7 min	

    where $X = 5, 8, 10, 12$, and 18 cycles.
12. Run PCR products on a 2% agarose gel (Fig. 3).
13. Amplify the library accordingly by large-scale PCRs, in at least five separate tubes to maximize amplification heterogeneity.
14. Clear the DNA from the beads, heat mixture to 98 °C, hold against magnet, and transfer supernatant to a fresh tube. Rinse the beads once with 50 μl TE, hold against a magnet, and retrieve the supernatant. Discard beads.
15. Bring volume to 250 μl with TE.
16. Purify library with AMPure XP beads following Illumina's recommendations.
17. Add 175 μl of RT AMPure beads to the 250 μl library, mix well, and incubate at RT for 15 min.

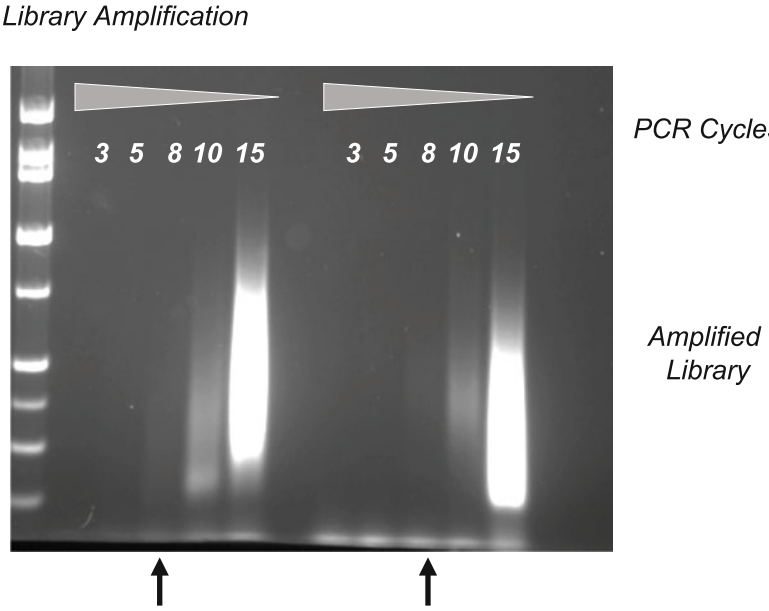


Fig. 3 Library amplification. PCR amplification of the HiC and C-HiC libraries (Subheadings 3.8, **step 12** and 3.11, **step 2**, respectively) using the PE primers and PCR amplification profile according to manufacturers'/sequencing core instructions (Illumina). From 0.6 to 1 μl of library are used for the assessment of the minimal cycles' number needed for amplification. Hence, appropriate number is determined by a visible smear on a 2% agarose gel (black arrow)

18. Hold sample against a magnet, and remove the supernatant.
19. Keep the sample against the magnet and wash twice with 700 μl of freshly made 70% ethanol. Do not mix.
20. Dry the beads by leaving the tube open at RT for 5 min.
21. Add 25 μl of TE to elute DNA. Mix by pipetting.
22. Incubate at RT for 5 min, hold sample against a magnet, and transfer supernatant to a fresh tube. Quantify DNA by dsDNA QuBit assay.

3.9 RNA Probe Capture (Fig. 4)

Use between 50 and 250 ng of HiC library for each capture reaction. Do all capture in triplicate to ensure good reproducibility.

1. Set the following profile on a thermocycler that can hold 0.5 ml epi tubes.
   95 °C for 5 min.
   65 °C for 3 min.
   65 °C for 2 min.
   65 °C for 48 h.

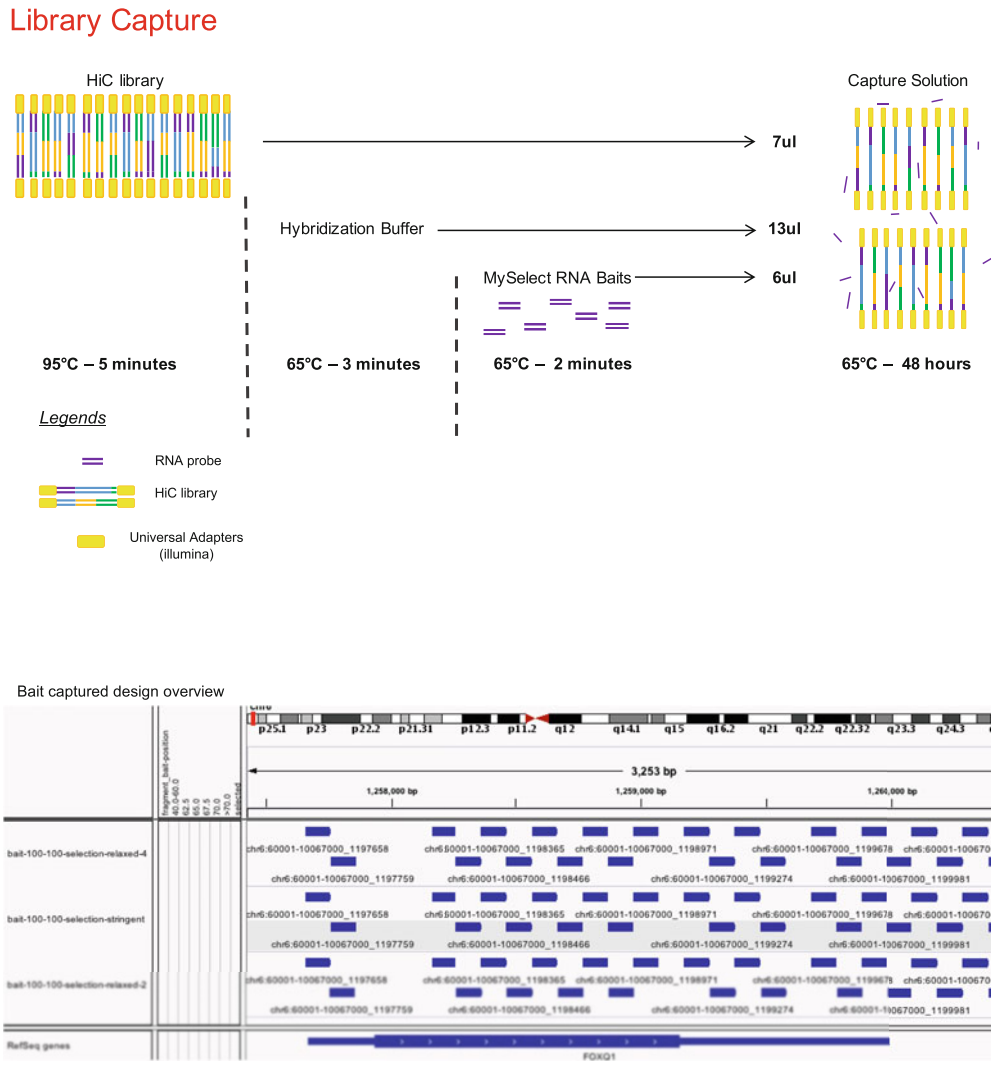


Fig. 4 Locus capture. Schematic representation of the library capture steps, preparation of mixes (top), and localization of baits (bottom). From an amplified Hi-C library, DNA is denatured, mixed with a warmed solution and finally hybridized with biotinylated RNA for 48 h at 65 °C (Subheading 3.9, **steps 1–8**). Baits are designed to overlap the full loci to capture, each bait covering a unique specific sequence. Example is shown for the *FOXQ1* gene, located at the 6p25 locus

2. Prepare the following mix:

Library mix
2.5 μl of 1 μg/μl Cot1 DNA
2.5 μl of 1 μg/μl Salmon sperm DNA
0.6 μl of MySelect blocking buffer
3.4 μl of HiC library

(continued)

Hybridization mix
20 μl of 20× SSPE
0.8 μl 0.5 M EDTA
8 μl of 50× Denhardt's
8 μl of 1% SDS
Capture mix
5 μl of RNA baits (*see* **Note 6**)
1 μl of RNA block

3. Transfer the library mix to the thermocycler, and start program.
4. When reaching **step 2**, briefly put the library mix on ice.
5. Transfer the library mix and hybridization mix to the thermocycler.
6. Add the capture mix to the thermocycler for the last 2 min at 65 °C.
7. Open the thermocycler, leaving all tubes at 65 °C, and add 7 μl of library mix and 13 μl of hybridization mix to the capture mix tube. Briefly mix by pipetting.
8. Hybridize solution for 48 h.

3.10 Second Biotin Pull-Down: C-HiC Sample

1. Prepare Dynabeads MyOne Streptavin C1 beads as described in Subheading 3.7, **step 1**.
2. Hold tubes against a magnet and resuspend in 200 μl BB 1×.
3. Transfer the hybridized solution to the beads.
4. Incubate at RT for 30 min on a rotation wheel.
5. Hold tubes against a magnet, remove supernatant, and resuspend in 400 μl RNABait wash buffer 1 (RWB1).
6. Incubate at RT for 15 min on a rotation wheel.
7. Hold tubes against a magnet, remove supernatant, and resuspend in 400 μl RWB2 (pre-warmed at 65 °C).
8. Incubate at 65 °C for 10 min in a thermoshaker.
9. Repeat Subheading 3.10, **steps 7** and **8**.
10. Hold tubes against a magnet, remove supernatant, and resuspend in 100 μl RWB2 (pre-warmed at 65 °C).
11. Incubate at 65 °C for 10 min in a thermoshaker.
12. Hold tubes against a magnet, remove supernatant, and resuspend in 50 μl of 0.1 M NaOH to degrade the RNA.

13. Vortex and incubate at RT for 10 min.
14. Pellet the beads, and transfer the supernatant to a fresh epi tube labelled C-HiC.
15. Add 70 µl of 1 M Tris–HCl pH 7.5 to neutralize the NaOH.
16. Purify DNA using a MinElute kit. Elute DNA in 2× 15 µl TE.

3.11 C-HiC Libraries

1. Amplify the library by PCR. Assess the number of amplification cycles in order to generate enough material as required by the sequencing core—usually between 8 and 12 pM per lane is required [23]:
   Set a series of PCR library mixture (5 points).
   1.5 pm of PE PCR primers 1.0.
   1.5 pm of PE PCR primers 2.0.
   10 µl NEBNext® Ultra™ II Q5® Master Mix.
   0.6 µl C-HiC.
2. Run the PCR using the following program:

98 °C for 1 min	
Do *X* cycles of:	98 °C for 10 s 65 °C for 30 s 72 °C for 30 s
72 °C for 7 min	

   where $X = 5, 8, 10, 12$, and 18 cycles.
3. Run PCR products on a 2% agarose gel.
4. Amplify the library accordingly by large-scale PCRs, in at least five separate tubes to maximize amplification heterogeneity. This will decrease possible PCR bias and allow the preservation of discrete events.
5. Quantify library, and process with sequencing (*see* **Note 7**).

3.12 Controls

1. Once treated, take advantage of the generated 3C control sample and assess one or two newly found interactions thanks to C-HiC by 3C. As mentioned previously, C-techniques are based on the detection of interactions by de novo ligation after digestion. As frequency interaction/population-based data can mask discrete events, it is important to test the most important interaction by additional approaches such as in situ hybridization. 3D FISH assays should be performed for the same interactions (as 3C) to confirm and consolidate the data.

4 Notes

1. Use fume hood and appropriate handling of formaldehyde. We found more consistent results when cross-linking was done in dish rather than after treatment with trypsin.
2. In case salts were not removed, an agarose dialysis step can be added to the procedure.
   (a) Make an agarose gel in the desired exchange buffer (TE or H_2O). Concentration doesn't really matter. At this point, samples are digested and ligated genomic DNA, hence big structures that will not easily diffuse in the gel.
   (b) Pour agarose in a 24-well plate, 2 ml/well.
   (c) Overlay for >20 min with exchange buffer and let the agarose be saturated with the buffer (this is to prevent the transfer of some of your samples into the gel).
   (d) Discard the buffer from the agarose (shake your wells).
   (e) Overlay the wells with your samples (200 μl), and let stand for 30 min at 4 °C with a smooth shake.
   (f) Retake your DNA samples from the wells; you may lose some of your volumes but *not* your DNA.
3. Confirm that the ligation process worked by taking advantage of the fact that successful ligation creates a *Nhe*I restriction site (AAGCTT) in lieu of *Hind*III (GCTAGC). Use PCR to amplify a ligation product formed by two nearby fragments (use the proximity effect of chromatin conformation capture). Determine that 70% of amplicons can be cut only by *Nhe*I (Fig. 3).
4. We routinely use a Bioruptor (Diagenode). However, any sonication instrument can be used. To note, profile for using a Misonix Sonicator 3000 is two pulses of 30 W for 90s separate by a 90s rest on ice, using a 500 μl sample in TE. For a Covaris setup, settings are as follows: fill level 10, duty cycle 15, PIP 500, and cycles/burst 200 for 60 s.
5. The binding buffer mix can be complemented with 0.1% Triton X-100 + 1% serum to block beads and ensure better stringency.
6. The capture probe library is a set of 200,000 digitally synthesized 120 nt biotinylated RNA probes covering a 10 Mb area.
7. Quality of raw reads is checked using FastQC. Reads are aligned individually to the most recent UCSC hg19 assembly of the human genome reference using Bowtie2, and HOMER (Hypergeometric Optimization of Motif EnRichment) was used for all remaining analysis. Importantly, parameters are set to filter out self-ligation; include only reads within 1000 bp of a *Hind*III restriction site, and ensure a minimum

distance of 20 kb between read pairs. A 100 kb resolution background model was generated using all paired reads, and false discovery rate was controlled using a Benjamini correction.

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