Quantitative analysis of chromosome conformation capture assays (3C-qPCR)

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Chromosome conformation capture (3C) technology is a pioneering methodology that allows *in vivo* genomic organization to be explored at a scale encompassing a few tens to a few hundred kilobase-pairs. Understanding the folding of the genome at this scale is particularly important in mammals where dispersed regulatory elements, like enhancers or insulators, are involved in gene regulation. 3C technology involves formaldehyde fixation of cells, followed by a polymerase chain reaction (PCR)-based analysis of the frequency with which pairs of selected DNA fragments are crosslinked in the population of cells. Accurate measurements of crosslinking frequencies require the best quantification techniques. We recently adapted the real-time TaqMan PCR technology to the analysis of 3C assays, resulting in a method that more accurately determines crosslinking frequencies than current semiquantitative 3C strategies that rely on measuring the intensity of ethidium bromide-stained PCR products separated by gel electrophoresis. Here, we provide a detailed protocol for this method, which we have named 3C-qPCR. Once preliminary controls and optimizations have been performed, the whole procedure (3C assays and quantitative analyses) can be completed in 7–9 days.

INTRODUCTION

Insight into genomic organization is key to understanding gene regulation in mammals. However, owing to technical limitations, we still have little idea about how the mammalian genome is structured *in vivo* at the scale at which long-range physical interactions between genes and dispersed regulatory elements most often take place (1–10^3 kbp). The recent development of the "Tagging and recovery of associated proteins"[1] and 3C (see ref. 2) assays allowed the very first glimpse into this crucial level of organization of the genome[3–5]. However, the RNA-TRAP technique, which is based on the targeting of peroxidase activity to nascent transcripts, is restricted to physical interactions occurring with actively transcribed genes, while 3C assays potentially allow identification of physical interactions between any chromatin segments. 3C technology is particularly suited to identify chromatin loops formed in genomic regions of up to several hundreds of kilobases in size. 5C technology[6,7] offers a robust high-throughput alternative for this analysis, based on large-scale sequencing or microarray analysis. 5C is however more laborious to set up. To identify DNA segments that interact over distances larger than several hundreds of kilobases, we recommend using 4C technology[8–11], which allows for an unbiased genome-wide screen for DNA elements that interact with a genomic site of choice.

The principle of 3C technology[2] (**Fig. 1**) is based on formaldehyde crosslinking of interacting chromatin segments, followed by restriction digestion and intramolecular ligation of crosslinked fragments. Ligation products are subsequently analyzed by PCR using primers specific for the restriction fragments of interest. The mere detection of a ligation product between two segments does not reveal much. To identify a chromatin loop between two segments, it needs to be demonstrated that they interact more frequently with each other than with neighboring DNA fragments. A meaningful 3C analysis therefore critically relies on the accurate quantification of the different ligation products, which in turn requires that measurements are taken when the DNA amplification reaction is in the linear range.

In traditional 3C experiments, a standard PCR protocol, which uses a standard number of PCR cycles and a standard amount of DNA template, is applied to the analysis of all different ligation products. Amounts are then estimated by measuring the intensity of ethidium bromide-stained PCR products separated by gel electrophoresis. This semiquantitative method is prone to provide inaccurate data, as measurements will not always be taken when the DNA amplification reaction is in the linear range.

The TaqMan quantitative PCR technology[12–14] provides more accurate measurements and was recently used to analyze higher order chromatin structure at the mouse HoxB1 (see ref. 10), α-globin[15] and β-globin loci[16] using the protocol described here. TaqMan technology makes use of a locus-specific oligodeoxynucleotide probe modified at its 5′ and 3′ ends by a fluorophore and a quencher, respectively. During the elongation step of the PCR, the 5′–3′ exonuclease activity of the *Taq* DNA polymerase[17] degrades the probe and releases the fluorophore from the quencher (**Fig. 1**), thus generating a fluorescent signal that is quantified at each PCR cycle[12,13]. The latter allows measurements to be always taken during the linear range of amplification. Quantifications correspond to a "threshold cycle" (Ct) that is determined as close as possible to the base of the exponential phase by defining a baseline to eliminate the background found in early cycles of amplification. Here we explain how TaqMan technology can be applied to the quantitative analysis of the ligation products generated by the 3C procedure. We refer to this strategy as 3C-qPCR. In addition, we provide a detailed step-by-step protocol for the preparation of 3C DNA templates prepared from mouse tissues that can subsequently be used for quantitative (or semiquantitative) PCR analysis. Modifications to the method presented below may be required to prepare 3C templates from other sources.

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Experimental design

Preparations before 3C assays, as described below, are the most laborious and time-consuming part of the procedure. It is also a critical part of the procedure, as both the resolution and sensitivity of the technique largely depend on its experimental design.

Restriction enzyme choice

The restriction enzyme should dissect the locus such that it allows for the separate analysis of the relevant regulatory elements (gene bodies, promoters, enhancers, insulators, etc.). Analyzing the topology of small loci (< 10–20 kb) requires the use of frequently cutting restriction enzymes such as *Dpn*II or *Nla*III (4-base cutters). When analyzing larger loci, 6-base cutters such as *Eco*RI, *Bgl*II or *Hin*dIII can also be used. Enzymes that generate cohesive ends are recommended, as blunted ends do not allow efficient ligation (Step 13 of PROCEDURE). Restriction enzymes that are sensitive to DNA methylation should not be used, as they can introduce a bias into the assay owing to differences in the digestion efficiency of the restriction sites analyzed. An additional digestion of the ligation products with a different restriction enzyme may optionally be performed for reliable quantification; this should help to minimize potential PCR biases owing to differences in template accessibility[15] (see **Box 1**). Obviously, the corresponding restriction site (*Eco*RI in the example in **Box 1**) should be absent from the PCR amplicons that will be used for ligation product quantification. Therefore, this additional restriction enzyme should be chosen when designing PCR primers used for quantifications. A prerequisite is thus to possess a reliable genomic sequence of the locus pertaining to the biological samples that will be studied.

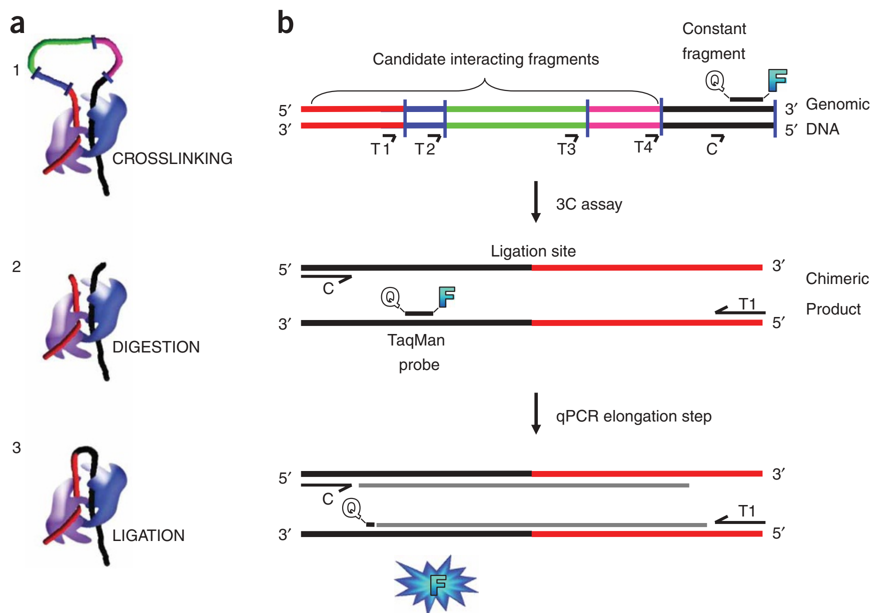

Figure 1 | ** Schematic diagram showing the principles of 3C and qPCR. (a**) Principle of the 3C assays (adapted, with permission, from Dekker *et al.*[2]). 3C assays involve three essential steps: Step 1: interacting chromatin segments are crosslinked by a formaldehyde treatment; Step 2: DNA is digested by an appropriate restriction enzyme; and Step 3: crosslinked fragments undergo intramolecular ligation and give ligation products that will be quantified by qPCR. (**b**) TaqMan probe and qPCR primers design. The locations of the constant fragment (black segment) and candidate interacting fragments (red, blue, green and pink segments) are shown in the upper part of the figure. Restriction sites that will be used in the 3C assay are depicted as small vertical bars in blue. The relative positions of the TaqMan probe (Q–F) and test primers (black arrows T1–T4) that will be used for qPCR quantifications are also depicted. The TaqMan probe should be designed on the strand antisense to the "Constant" primer (black arrow C), but in the same restriction fragment, as depicted in the figure. During the elongation step of the PCR, the 5′–3′ exonuclease activity of the *Taq* DNA polymerase degrades the probe and releases the fluorophore from the quencher, thus generating a fluorescent signal that is strictly specific to the amplification of the relevant ligation products. T, test primer; F, fluorophore; Q, quencher.

TaqMan probe design

From the quantity of genomic DNA required to detect ligation products, we estimate that some locus-specific ligation events may occur in only 1/2,000 to 1/20,000 mammalian cells. Therefore, reliable quantification of ligation products is possible only if large amounts of DNA (i.e., many genome equivalents) are added to each PCR; we routinely use 50–200 ng of 3C template ($\sim 8 \times 10^4$ to 3×10^5 genome equivalents) per PCR. As such large amounts of DNA preclude the use of Syb^{R}Green incorporation as a means to quantify PCR products[4], the highly specific TaqMan chemistry is required for quantifications by real-time PCR. The 3C analysis quantifies ligation products generated by ligation of a "constant fragment" with candidate interacting fragments of the locus. Quantification of these ligation products requires a TaqMan probe and a "constant primer" (C) both located in the "constant fragment", combined with a battery of "test primers" (T) designed in the candidate interacting fragments. For efficient amplification of ligation products, the amplicon needs to be small, and test primers therefore should be designed to hybridize as close as possible to, and preferably within 50 bp of, the restriction sites of interest. The TaqMan probe and the "constant primer" should also be designed as close as possible to the relevant restriction site and, importantly, they should hybridize to opposite strands of the "constant fragment"[16]. This configuration results in a fluorescent signal that is strictly specific to the amplification of the ligation product selected for analysis (see **Fig. 1**). As the TaqMan probe is designed to hybridize within the constant fragment, this probe can be used for quantification of all products ligated to the "constant fragment". Further details on primer and probe design can be found in the REAGENT SETUP section.

Control template/primer efficiency

A control template that contains all ligation products in equal amounts is used to optimize real-time PCRs and to establish the minimal amount of ligation product that can still be quantified in a reliable manner. For the PCR control template, we recommend the use of a single Bacterial Artificial Chromosome (BAC) clone covering the genome segment under study. Alternatively, a set of minimally overlapping BAC clones mixed in equimolar amounts could be used[18,19]. This BAC is then cut with the restriction enzyme of choice (e.g., *Hin*dIII) and religated by T4 DNA ligase. A secondary restriction enzyme (e.g., *Eco*RI) can be used to linearize DNA circles, which may otherwise affect primer hybridization

BOX 1 | OPTIONAL COMPLEMENTARY DIGESTION ● TIMING 3–4 H

This additional digestion of religated DNA can help to minimize potential PCR biases that may result from limited template accessibility[20]. We have used *Eco*RI as an example below, but any suitable restriction enzyme and buffer can be used (see the Experimental design section).
(i) To the sample from Step 34, add 28 μl of dH_2O and 20 μl of 1× *Eco*RI buffer (commercial 10× buffer for *Eco*RI (or any other selected restriction enzyme) diluted in water).
(ii) Place the sample in a 1.5 ml tube and add 2 μl of 50 U μl^{-1} *Eco*RI enzyme (100 U final).
(iii) Incubate for 2 h at 37 °C.
(iv) Add 200 μl of phenol–chloroform, mix vigorously.
(v) Centrifuge for 5 min at 16,100*g* at room temperature (18–22 °C).
(vi) Transfer the supernatant into a new tube and add 20 μl of 2 M sodium acetate pH 5.6, then 0.5 ml of ethanol.
(vii) Mix and place at −80 °C until frozen (about 45 min).
(viii) Centrifuge for 20 min at 16,100*g* at 4 °C.
(ix) Remove the supernatant and add 200 μl of 70% ethanol.
(x) Centrifuge for 4 min at 16,100*g* at room temperature (18–22 °C).
(xi) Remove the supernatant and dry the pellet at room temperature (18–22 °C).
(xii) Resuspend the pellet into 150 μl of 10 mM Tris-HCl pH 7.5.
(xiii) Proceed from Step 35 of the main procedure.

efficiency[20]. It is then necessary to make serial dilutions of this reaction to obtain standard curves that cover the same range of ligation product concentrations as those that will be obtained in the 3C samples. To mimic 3C sample conditions, the final DNA concentration in these dilutions is adjusted to the amount of DNA used in the 3C samples (see Step 38 of PROCEDURE). Thus, depending on the amount of 3C sample used, these dilutions are performed on a 50–200 ng genomic DNA solution.

Experimental controls

Several controls need to be performed on each 3C sample to obtain meaningful data. Sample purity (Steps 35–37) and digestion efficiency (**Box 2**) should be carefully assessed, as they may impair accurate quantifications. Finally, the level of random collisions needs to be assessed carefully to distinguish between relatively frequent but nonfunctional interactions from physiologically relevant interactions (see the ANTICIPATED RESULTS section). To correct for differences in crosslinking and digestion efficiencies between samples, 3C-qPCR data are normalized toward a set of "control interaction frequencies". For this purpose, we routinely use the locus of the *ERCC3* gene (excision repair cross-complementing rodent repair deficiency, complementation group 3), a ubiquitously expressed gene. As this gene is expressed at very similar levels in all mouse tissues, higher order chromatin architecture at this locus is assumed to be identical and can be used for normalization of 3C-qPCR data (for primer sequences used in combination with *Hin*dIII, see Splinter *et al.*[16]).

BOX 2 | DETERMINATION OF DIGESTION EFFICIENCY ● TIMING 7–8 H

Digestion efficiencies should be carefully assessed for each restriction site involved in the analysis. Indeed, a twofold drop in digestion efficiency of a given site causes a twofold reduction in the available amount of a given restriction end, which would affect the number of ligation products it can form. Therefore, digestion efficiencies should be in the same range for each of the sites of interest.
(i) Add 500 μl of 1× PK buffer and 1 μl of 20 mg ml^{-1} PK (20 μg final) to the control aliquots saved in Steps 12 (UND) and 14 (D).
(ii) Incubate for 30 min at 65 °C (or overnight at 65 °C if performed in parallel to Steps 22–34).
(iii) Equilibrate for a few minutes at 37 °C, then add 1 μl of 1 mg ml^{-1} RNase A (1 μg final) and incubate for 2 h at 37 °C.
(iv) Add 500 μl of phenol–chloroform, mix vigorously.
(v) Centrifuge for 5 min at 16,100*g* at room temperature (18–22 °C).
(vi) Transfer the supernatant into a new tube and add 50 μl of 2 M sodium acetate pH 5.6, then 1.5 ml of ethanol.
(vii) Mix and place at −80 °C until frozen (about 45 min)
(viii) Centrifuge for 20 min at 16,100*g* at 4 °C.
(ix) Remove the supernatant and add 500 μl of 70% ethanol.
(x) Centrifuge for 4 min at 16,100*g* at room temperature (18–22 °C).
(xi) Remove the supernatant and dry the pellet at room temperature (18–22 °C).
(xii) Resuspend the pellet in 60 μl of water.
(xiii) Perform real-time PCR quantifications (Syb^RGreen) on both samples (UND and D) using the qPCR conditions detailed in Steps 38A(ii) and (iii) of the main procedure. To check digestion efficiencies, use primer sets that amplify across each restriction site of interest (R); these should be designed as outlined in the REAGENT SETUP section. To correct for differences in the amount of template added to the PCR, also PCR-amplify control regions not containing the restriction sites of interest (use the "internal" primers used for loading adjustments, Step 38).
(xiv) Use the cycle thresholds (Ct values, i.e., the number of PCR cycles required to generate a threshold amount of PCR product) to calculate the restriction efficiency according to the following formula:
$\%\ restriction = 100 - 100/2\text{^}((Ct_R - Ct_C)D - (Ct_R - Ct_C)UND)$
▲ CRITICAL STEP The efficiency of the restriction enzyme digestion should be above 60–70%, but ideally >80% should be digested. Samples with lower digestion efficiencies should be discarded.

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MATERIALS

REAGENTS

- Mice or appropriate cultured cells ! CAUTION Approved regulations and guidelines for animal work must be adhered to.
- 10% (v/v) FCS/DMEM
- 10% (v/v) FCS/PBS
- 0.25% (w/v) trypsin
- 37% formaldehyde (vol/vol; 47629 Fluka) ! CAUTION Formaldehyde is toxic (see material safety data sheet at http://www.sigmaaldrich.com/catalog/search/ProductDetail/FLUKA/47629).
- Glycine, 1 M
- Lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 5 mM $MgCl_2$; 0.1 mM EGTA; 1× complete protease inhibitor; 11836145001 Roche) ▲ CRITICAL For some cell types, a more stringent lysis buffer is required. See REAGENT SETUP for additional information.
- 2.5% (w/v) collagenase type 1 (Sigma C0130)
- 20% (w/v) sodium dodecyl sulfate (SDS) (EU0460B Euromedex)
- Triton X-100 (2327140 BDH Chemicals)
- High-concentration restriction enzyme (40 U μl^{-1})
- T4 DNA ligase, high concentration (20 U μl^{-1}; Promega)
- 10× ligation buffer (660 mM Tris-HCl, pH 7.5; 50 mM DTT; 50 mM $MgCl_2$; 10 mM ATP)
- Ribonuclease A (RNase A), 1 mg ml^{-1}
- Proteinase K (PK), 10 mg ml^{-1}
- PK buffer (5 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0; 0.5% SDS)
- Control PCR primers for assessing DNA digestion efficiency, user-specific (see REAGENT SETUP)
- Forward and reverse “internal” primers for loading adjustments, user-specific (see REAGENT SETUP)
- Constant and test PCR primers for quantifying 3C reaction, user-specific (see REAGENT SETUP)
- PCR primers for determining digestion efficiency, user-specific (see REAGENT SETUP)
- PCR primers for determining control interaction frequencies, user-specific (see REAGENT SETUP)
- TaqMan probe, user-specific (see REAGENT SETUP and Experimental design)
- QuantiTect Probe PCR Master Mix (204343 Qiagen)
- QuantiTect SYBR Green PCR Master Mix (Qiagen) or as described in ref.14
- TE pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- Agarose (A9539 Sigma)

EQUIPMENT

- Microcentrifuge, for example Eppendorf 5415 D, rotor F45-24-11 (for 1.5 ml tubes)
- Eppendorf centrifuge 5810R (for 15 and 50 ml Falcon tubes).
- LightCycler apparatus (Roche) and LightCycler Software, version 3.5 (TaqMan qPCR analyses were performed in “F1/F2” mode)
- 40 µm cell strainer (352340 Falcon).

REAGENT SETUP

Lysis of cells The choice of lysis buffer depends on the tissue or cell type used. For most tissues, the lysis buffer described above will be sufficient. If cells are not properly lysed, a different lysis buffer containing NP-40 can be used (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 0.2% NP-40; 1× complete protease inhibitor (11836145001 Roche)). If this is not sufficient, an additional douncing step can be included as described in troubleshooting Step 5. Staining cells with Methyl-grün-Pyronin and viewing them under a light microscope can confirm cell lysis.

PCR primer design All PCR primers used in this protocol should be typically 20–22-mers with a T_m in the range 55–65 °C with a 2 °C maximum difference between all primers used in a single experiment. Primers could be designed using software available on the internet (e.g., http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR primers used for analyzing digestion efficiency or the 3C assays should be designed close (within 50 bp) to the restriction site used for the 3C assays.

TaqMan probe design The annealing temperature of the probe should be 8–10 °C higher than that of the PCR amplification primers. The probe should be 20–30 nucleotides long and contain more C than G residues. Avoid G in the most 5′ position (which could quench the fluorescent reporter), and stretches of four consecutive identical nucleotides (particularly G). We have used 5′FAM–3′BHQ^R and 5′FAM–3′TAMRA probes[12,13]. TaqMan probes are dissolved in TE pH 8.0 and stored at −20 °C as 1.5 µM aliquots.

PROCEDURE

Single-cell preparation ● TIMING 30 min–1 h

1| Obtain single-cell preparations from mouse tissue (option A), adherent cultured cells (option B) or from cultured cells in suspension (option C).

(A) Single-cell preparations from mouse tissues

(i) Dissect mouse tissues according to approved methods and transfer to a 1.5 ml Eppendorf tube.
! CAUTION Approved regulations and guidelines for animal work must be adhered to.

(ii) For more solid tissues (e.g., fetal brain and most adult tissues), incubate in 10% (v/v) PBS/FCS supplemented with 0.125% (w/v) collagenase at 37 °C with gentle shaking at 300 r.p.m.; the exact duration of the collagenase treatment will need to be optimized for each tissue. For other tissues (e.g., fetal liver), omit the collagenase treatment and add 250 µl of 10% (v/v) FCS/DMEM per 1 × 10^7 cells; resuspend the tissue with a blue tip.
▲ CRITICAL STEP Disrupting more solid tissues by force (e.g., repeated pipetting) will often cause cell death, which may obscure the 3C results.

(iii) Centrifuge the cell suspension for 1 min at 400*g* at room temperature (18–22 °C).

(iv) Discard the supernatant and resuspend the pellet in 500 µl of 10% (v/v) FCS/PBS.

(v) Filter through the 40 µm cell strainer to make a single-cell suspension. The cells are now ready for crosslinking (Step 2).

(B) Single-cell preparations from adherent cultured cells

(i) Remove medium and wash cells twice with PBS.

(ii) Add 1 ml of 0.25% (w/v) trypsin and incubate for 5 min at 37 °C.

(iii) Resuspend cells in 9 ml of 10% (v/v) FCS/DMEM.

(iv) Centrifuge the cell suspension for 1 min at 400*g* at room temperature (18–22 °C).

(v) Discard the supernatant and resuspend the pellet in 500 µl of 10% (v/v) FCS/PBS per 1 × 10^7 cells.

(vi) Filter through the 40 µm cell strainer to make a single-cell suspension. The cells are now ready for crosslinking (Step 2).

(C) Single-cell preparations from suspended cultured cells

(i) Centrifuge the cell suspension for 1 min at 400*g* at room temperature (18–22 °C).

(ii) Discard the supernatant and resuspend the pellet in 500 µl of 10% (v/v) FCS/PBS per 1 × 10^7 cells.

(iii) Filter through the 40 µm cell strainer to make a single-cell suspension. The cells are now ready for crosslinking (Step 2).

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Formaldehyde crosslinking ● TIMING 20–25 min

2| Per 1×10^7 cells, add 9.5 ml of 2% formaldehyde/10% FCS/PBS and incubate, while tumbling, for 10 min at room temperature (18–22 °C). This is the crosslinking step.

▲ CRITICAL STEP 1% formaldehyde is also routinely used. Formaldehyde concentration may affect digestion efficiencies; if digestion efficiency is likely to be a problem, use 1% instead of 2% formaldehyde.

! CAUTION Formaldehyde is toxic (see material safety data sheet at http://www.sigmaaldrich.com/catalog/search/ProductDetail/FLUKA/47629).

3| Transfer the reaction tubes to ice and add 1.425 ml of 1 M glycine (ice cold) to quench the crosslinking reaction.

▲ CRITICAL STEP Glycine will inactivate the excess formaldehyde that did not react with the biological sample. The glycine concentration described here is sufficient for most biological samples. However, if the sample is low in free amino groups, quenching may not be complete; in this case, the glycine concentration should be increased before proceeding immediately to Step 4.

4| Spin for 8 min at 225*g* at 4 °C and carefully remove all the supernatant.

Cell lysis ● TIMING 20–25 min

5| Take up the pellet in 5 ml cold lysis buffer and incubate for 10 min on ice.

▲ CRITICAL STEP It is important to obtain a homogeneous preparation of nuclei, which may be facilitated by gently pipetting the mixture up and down. Depending on the cell type, more stringent lysis buffers may be needed to prepare nuclei (see REAGENT SETUP).

? TROUBLESHOOTING

6| Centrifuge for 5 min at 400*g* at 4 °C and remove the supernatant.

? TROUBLESHOOTING

■ PAUSE POINT The pelleted nuclei can be frozen in liquid nitrogen and stored at –80 °C for several months.

Digestion ● TIMING 18–20 h

7| Take up the nuclei in 0.5 ml of 1.2× restriction enzyme buffer and transfer to a safe-lock tube.

8| Place the tube at 37 °C and add 7.5 µl of 20% (w/v) SDS (final: 0.3% SDS).

9| Incubate for 1 h at 37 °C while shaking at 900 r.p.m.

? TROUBLESHOOTING

10| Add 50 µl of 20% (v/v) Triton X-100 (final: 2% Triton X-100).

11| Incubate for 1 h at 37 °C while shaking at 900 r.p.m.

12| Take a 5 µl aliquot of the sample and label as an undigested genomic DNA control (UND). This sample may be stored at −20 °C until it is needed to determine the digestion efficiency (see Step 14 and **Box 2**).

13| Add 400 U of the selected restriction enzyme to the remaining sample and incubate overnight at 37 °C while shaking at 900 r.p.m.

14| Take a 5 µl aliquot of the sample and label as a digested genomic DNA control (D). To process the remaining sample, proceed to Step 15. To determine the digestion efficiency, analyze the control aliquots from Steps 12 and 14 as described in **Box 2**. This analysis can be carried out in parallel with Steps 22–34.

? TROUBLESHOOTING

Ligation ● TIMING 8–9 h

15| Add 40 µl of 20% (w/v) SDS (final 1.6%) to the remaining sample from Step 14.

16| Incubate for 20–25 min at 65 °C; shake at 900 r.p.m.

17| Transfer the digested nuclei to a 50 ml falcon tube.

18| Add 6.125 ml of 1.15× ligation buffer.

19| Add 375 µl of 20% (v/v) Triton X-100 (final 1% Triton X-100).

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20| Incubate for 1 h at 37 °C while shaking gently.

21| Add 5 µl ligase (100 U total) and incubate for 4 h at 16 °C followed by 30 min at room temperature (18–22 °C).

22| Add 30 µl of 10 mg ml^{-1} PK (300 µg final).

23| Incubate at 65 °C overnight to de-crosslink the sample.

DNA purification ● TIMING 5–6 h

24| Add 30 µl of 10 mg ml^{-1} RNase (300 µg final).

25| Incubate for 30–45 min at 37 °C.

26| Add 7 ml of phenol–chloroform and mix vigorously.

27| Centrifuge for 15 min at 2,200*g* at room temperature (18–22 °C).
? TROUBLESHOOTING

28| Transfer the supernatant into a new 50 ml tube and add 7 ml of distilled water, 1.5 ml of 2 M sodium acetate pH 5.6 followed by 35 ml of ethanol.
▲ CRITICAL STEP Increasing the volume before precipitation will dilute the DTT present in the ligation buffer and prevent it from precipitating (white pellet).

29| Mix and place at −80° C for approximately 1 h.

30| Centrifuge for 45 min at 2,200*g* at 4 °C.

31| Remove the supernatant and add 10 ml of 70% (v/v) ethanol.

32| Centrifuge for 15 min at 2,200*g* at 4 °C.

33| Remove the supernatant and briefly dry the pellet at room temperature (18–22 °C).

34| Dissolve the DNA pellet in 150 µl of 10 mM Tris pH 7.5. The 3C template is now ready for qPCR analysis. If an additional digestion step is required to maximize template accessibility, follow the procedure in **Box 1** before continuing with Step 35 and qPCR analysis.
? TROUBLESHOOTING
■ PAUSE POINT 3C template may be kept at −20 °C for several months.

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Assessment of sample purity ● TIMING 2–4 h

35| Dilute an aliquot of the 3C sample (from Step 34) two- to tenfold. Add genomic DNA to the diluted reaction samples such that the total amount of DNA in each reaction is constant and between 50 and 200 ng.

36| Set up 10 µl qPCRs as detailed in the table below. Any 3C test primer can be used.
Perform a standard curve in the same run using serial dilutions of the control template (see Experimental design) according to the instructions of the manufacturer of the qPCR apparatus.

Stock component	Amount	Final
H_2O	2 µl	
Test primer (10 µM)	0.5 µl (5 pmol)	0.5 µM
Constant primer (10 µM)	0.5 µl (5 pmol)	0.5 µM
QuantiTech Probe PCR Master Mix	5 µl	
TaqMan probe (1.5 µM)	1 µl (1.5 pmol)	0.15 µM
3C sample (from Step 35)	1 µl	

37| Run the qPCR using the conditions tabulated below. Check that real-time PCR quantifications are reduced according to the dilution factors (see Example in the ANTICIPATED RESULTS section). If this is not the case, sample purity is not appropriate and the sample should be discarded.

Cycle number	Denature	Anneal and extend
1	15 min at 95 °C	
2–46	10 s at 95 °C	1 min at 60 °C

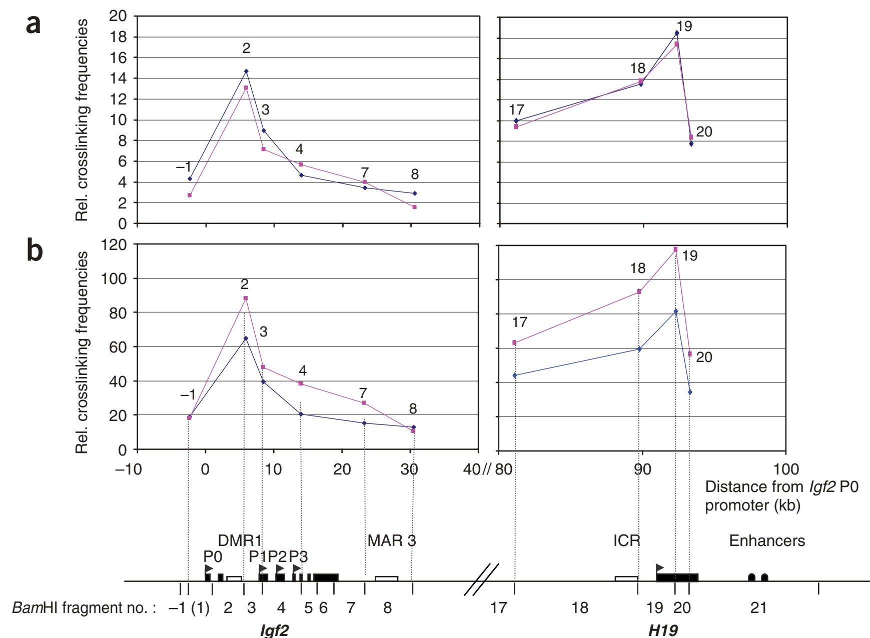

Figure 2 | ** 3C-qPCR analysis of long-distance interactions at the mouse imprinted *Igf2/H19* locus. The relative level of each ligation product (fragments −1 to 8 and 17 to 20) has been plotted according to its distance (in kb) from the *Igf2* promoter P0 (see map below graphs). The "constant" primer and the TaqMan probe were designed as described in **Figure 1 (also see PCR primer design in the REAGENT SETUP section) in a *Bam*HI fragment (fragment 21) containing the *H19* endodermic enhancers (see **Tables 2** and **5**). Two independent 3C-qPCR experiments (A1, blue curve; A2, pink curve) were performed on the same biological samples. The data were normalized either to a *Gapdh* loading control (**a**) or to *Pdhb* "control interaction frequencies" (**b**). Below the graphs, the *Bam*HI restriction fragments are indicated. *Bam*HI fragments are numbered from fragment −1 to 21. Arrows P0–P3 indicate the four *Igf2* promoters. The locations of the imprinting-control region (ICR, white rectangle in fragment 18), *Igf2* and *H19* genes (black rectangle in fragment 19), the endodermic enhancers (black circles in fragment 21), the matrix attachment region 3 (MAR3, white rectangle in fragment 8) and the differentially methylated region 1 (DMR1, white rectangle in fragment 2) are also indicated.

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Performing loading adjustments ● TIMING 2 h

38| Assess the concentration of samples that pass the purity assessment (Steps 35–37). To accurately determine the DNA concentration of a 3C sample, it needs to be compared to a reference sample of genomic DNA of known concentration. This can be done by SybGreen quantitative PCR on 50× dilutions of 3C samples and the reference sample, using "internal" primer sets that do not amplify across sites recognized by any of the restriction enzymes used (option A) (also see Example in the ANTICIPATED RESULTS section). Alternatively, dilutions of 3C samples and reference samples can be run side by side on an agarose gel (option B).

▲ CRITICAL STEP Optical density (OD_{260}) measurements fail to provide an accurate estimate of DNA concentration in 3C samples, probably because of their limited purity.

(A) SybGreen quantitative PCR

(i) Prepare a 50× dilution of an aliquot of the 3C sample (Step 34) and serial dilutions of a reference DNA (10–0.01 ng μl^{-1} of DNA).

(ii) Set up 20 µl qPCRs as follows (LightCycler apparatus, Roche)

Stock component	Amount	Final
H_2O	14 µl	
Forward internal primer (10 µM)	1 µl (10 pmol)	0.5 µM
Reverse internal primer (10 µM)	1 µl (10 pmol)	0.5 µM
qPCR mix[14]	2 µl	
50× dilution of 3C sample (Step 38A(i))	2 µl	

(iii) Carry out qPCR using the conditions tabulated below:

Cycle number	Denature	Anneal and extend
1	3 min at 95 °C	
2–46	1 s at 95 °C	5 s at 70 °C and 15 s at 72 °C
47	Increase temperature to 95 °C at a rate of 0.2 °C s^{-1}	

▲ CRITICAL STEP If using another apparatus than the LightCycler or a different commercial qPCR mix, please follow the instructions of the manufacturers.

(iv) Determine the DNA concentration by comparing the Ct value of the sample to the standard curve.

? TROUBLESHOOTING

(B) Agarose gel analysis

(i) Prepare 1/5, 1/10, 1/20 and 1/40 dilutions of sample and reference sample of known DNA concentration.

(ii) Run corresponding dilutions of sample and reference DNA side by side on an agarose gel (0.8–2%); the ligated 3C samples (and the undigested genomic reference sample) will run as a single high-molecular-weight band.

(iii) Quantify and compare the amount of DNA between the ligated 3C samples and the reference sample by measuring ethidium bromide incorporation using a Typhoon 9200 imager (GE Healthcare). Processed images are analyzed using ImageQuant software (GE Healthcare) to determine the DNA concentration.

39| Adjust the original 3C assays (from Step 34) with H_20 to 50 ng μl^{-1} ±10% and optionally verify this concentration by repeating the measurements as described in Step 38.

Real-time PCR quantifications of ligation products ● TIMING 2–4 days (2–3 h per run; number of runs depends on the number of primer pairs and 3C samples to be analyzed)

40| Perform TaqMan real-time PCR quantifications of ligation products on 1 µl (containing ~50 ng of DNA) of the "adjusted" 3C samples (from Step 39). Use the reaction conditions described in Step 36.

41| For each ligation product, run the qPCR using the PCR parameters tabulated in Step 37. Perform standard curves in each run using serial dilutions of the control template (see Experimental design) according to the instructions of the manufacturer of the qPCR apparatus. Analyze data as described in the ANTICIPATED RESULTS section.

▲ CRITICAL STEP 3C-qPCR data have to be normalized toward a "loading control" ("internal" primers located in the *gapdh* gene in the example experiment below) and a set of "control interaction frequencies". This latter control should be performed on each 3C assay using a pair of test/constant primer localized in a locus that contains a ubiquitously expressed gene. For this purpose, we routinely use primer pairs within the locus of the *ERCC3* (see ref. 16) or *PDHB* genes (see Example in the ANTICIPATED RESULTS section).

? TROUBLESHOOTING

● TIMING

Step 1, preparation of the cells: 30 min–1 h
Steps 2–4, formaldehyde crosslinking: 20–25 min
Steps 5 and 6, cell lysis: 20–25 min
Steps 7–14, digestion: 18–20 h
Steps 15–23, ligation: 8–9 h
Steps 24–34, DNA purification: 5–6 h
Steps 35–37, sample purity: 2–4 h
Steps 38 and 39, loading control: 2 h
Steps 40 and 41, real-time PCR quantification of ligation products: 2–4 days

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
5	Nuclei are aggregated or of a poor quality	Intact nuclei are necessary to perform a valid 3C assay. If nuclei have aggregated, homogenize them by gently pipetting up and down avoiding the formation of bubbles. If cells are not lysed, try different lysis buffers, or include a douncing step to remove the cytoplasmic residue from the nuclei
6	No visible pellet	Low number of nuclei, restart from Step 1
9	The nuclei form aggregates or get stuck on the bottom of the tube	Homogenize the aggregates by gently pipetting up and down, avoiding the formation of bubbles. If the nuclei get stuck on the bottom of the tube, increase the speed of the shaker
14	Inefficient digestion (percentage of digestion should be at least 60%, but preferably >80%)	When using an enzyme other than *Hin*dIII, check that the composition of the recommended restriction buffer is not too different from the one used in the present protocol. Optimizing digestion percentages can also be done by varying the SDS/Triton X-100 amounts before digestion and

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TABLE 1 | Troubleshooting table (continued).

Step	Problem	Solution
		by using 1% instead of 2% formaldehyde (Step 2). If this does not work, reconsider the choice of enzyme
27	After the first extraction, the aqueous phase is still very turbid	Repeat phenol extraction a second time
34	Some precipitates do not resuspend	Dissolve DNA by gently shaking tubes at 37 °C for up to 30 min
38A	Inaccurate qPCR quantifications	If qPCRs are performed in a different thermocycler (than the LightCycler, Roche), the PCR parameters may need to be optimized. When using commercial qPCR kits, follow the instruction of the manufacturer
41	Inaccurate qPCR quantifications	If qPCRs are performed in a different thermocycler (than the LightCycler, Roche), the PCR parameters may need to be optimized. When using commercial qPCR kits, follow the instruction of the manufacturer When preparing the qPCRs, the TaqMan probe has to be added after the PCR Mix. The probe is very sensitive to acidic pH and should always be kept in TE pH 8.0 and protected from light

ANTICIPATED RESULTS

Data analysis

As recently stressed elsewhere[18], for each 3C sample, the level of random collisions needs to be assessed carefully to distinguish relatively frequent but nonfunctional interactions from physiologically relevant interactions. The background of random collisions can be estimated by determining the interaction frequencies between sites separated by increasing genomic distances (see Example of experiment). As mentioned previously, "a specific interaction is detected when a local peak in interaction frequency is observed"[18]. Using the 3C protocol to simply look for an interaction between two sites (e.g., promoter to enhancer) with just a few primer sets can easily result in misinterpretation of the data.

However, some parameters—like the size of the restriction fragments—may influence the quantification of the ligation products[4] and create "artifactual local peaks". Therefore, the comparison of relative crosslinking frequencies between two different samples is the best indication of physiologically relevant interactions.

Example of experiment

3C-qPCR was first validated by Splinter *et al.*[16] at the mouse β-globin locus. To further validate the 3C-qPCR protocol, we analyzed the enhancers/promoter interaction at the imprinted *H19* locus on mouse chromosome 7, which is a mammalian locus recently analyzed by 3C assays using the standard procedure[21,22]. We prepared nuclei from liver samples from 7-day-old mice (when *Igf2* and *H19* are highly expressed)[23] and proceeded until Step 34 of the 3C protocol as described above using *Bam*HI

TABLE 2 | Sequence of qPCR primers used for 3C-qPCR analysis of the mouse imprinted ***Igf2/H19*** locus (see **Fig. 2**).

*Bam*HI fragment number	Forward primer sequence	Constant primer
−1	aatgaccaccagatgtcaagc	C3
2	ctgctccgtgtgagttcctt	C2
3	aggaccgcaaatcagacaag	C1
4	ctgcgtttgtttctccaggt	C3
7	gtggcaaggaaagtgaagga	C1
8	ggcagctcacatacaactcc	C1
17	ccagagcaggatgtgagagg	C2
18	ggcgggagacatagaaactgc	C1
19	gcagggttgccagtaaagac	C1
20	ccttgtcgtagaagccgtctg	C1

Three "constant primers" (C1, C2 and C3) were designed as described in **Figure 1** (also see the REAGENT SETUP section). These three primers are all located within a 35 bp sequence located 129–94 bp upstream of the 3′ *Bam*HI site of fragment 21. Their sequences are as follows (5′ to 3′): C1, aatgtggggagacaaacagc; C2, cccaaacgcttgaccctat; and C3, tgaccctatgcctggaatgt. For each ligation product, the "constant primer" giving the best amplification efficiency was used as indicated in the table. The "ligation product-specific primers" ("forward primers") were designed upstream of the 3′ *Bam*HI site of each restriction fragment (fragments 1–20) (see **Fig. 1**). Their sequences are given in the table. All amplifications were performed as described in the PROCEDURE section (Steps 36 and 37). The sequence of the TaqMan probe used is as follows: 5′FAM-cccaaccagggtcttcacggg-3′BHQ[R]. This probe is located 56 bp upstream of the 3′ *Bam*HI site of fragment 21 (see **Fig. 2**). Loading controls (Step 38) were performed using the following Gapdh primers ("internal" primers, see the REAGENT SETUP section): forward primer (5′ to 3′): acagtccatgccatcactgcc; reverse primer (5′ to 3′): gcctgcttcaccaccttcttg.

TABLE 3 | Sample purity assessment.

Sample name (dilution)		A1	A1 (1/2)	A1 (1/4)	A2	A2 (1/10)	X	X (1/2)	X (1/4)
b (intercept)		33.80	33.80	33.80	30.14	30.14	33.80	33.80	33.80
a (slope)		−3.436	−3.436	−3.436	−3.364	−3.364	−3.436	−3.436	−3.436
Ct		30.07	30.80	32.06	26.97	30.69	31.53	32.06	32.34
Values $10^{(Ct-b)/a}$		12.17	7.47	3.21	8.76	0.686	4.58	3.21	2.66
Dilution factor	Observed	—	1.63	3.79	—	12.8	—	1.43	1.72
	Expected	—	2	4	—	10	—	2	4
		Proceed with Step 38			Proceed with Step 38		Discard this sample		

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(Step 13) and *Eco*RI (**Box 1**) restriction enzymes. Sample purity was then assessed on aliquots of three independent 3C assays (A1, A2 and X) as described above (Steps 35–37) using TaqMan PCR with a constant primer located into the fragment containing the *H19* enhancers and test primer 20 (see **Fig. 2** and **Table 2** for primer sequences). The results of this purity control are given in **Table 3**. The values correspond to the quantifications of the ligation product formed between fragment 20 and the constant fragment (C). These values are calculated using the parameters of the standard curve (*b*: intercept; *a*: slope) as follows: value$=10^{(Ct-b)/a}$.

TABLE 4 | Loading adjustments.

Sample name	A1	A2
Dilution factor of the original 3C sample	5	9
b (intercept)	−3.687	−3.706
a (slope)	17.09	17.06
Mean Ct	16.98	17.03
Values $10^{(Ct-b)/a}$	1.07	1.03
Expected value	1	1
	Proceed with Step 40	Proceed with Step 40

One 3C assay (X) was discarded from the analysis because impurities impaired faithful quantifications (see **Table 3**). The purity of two other 3C samples (A1 and A2) was appropriate and we proceeded with loading adjustments (Step 38), as shown in **Table 4**. SYBR Green quantifications were performed on an aliquot of a 50× dilution of these 3C samples as described in Step 38A. Once the original 3C samples had been appropriately diluted to 50 ng μl^{-1} (i.e., dilution factor of 5 for sample A1 or 9 for sample A2), we verified their concentration on an aliquot of a 50× dilution of these adjusted samples (Step 39). Standard curves were obtained using serial dilutions of a reference sample (see Step 38A), giving a value of 1 for the 1 ng μl^{-1} point. The values were obtained using the "internal" primer set (here we used primers located in the *Gapdh* gene; see **Table 2** for primer sequence). These values are calculated using the parameters of the standard curve (*b*: intercept; *a*: slope) as follows: value$=10^{(Ct-b)/a}$.

Digestion efficiency was controlled as described in **Box 2** (data not shown; for primer sequences see **Table 5**). Quantifications of ligation products were then performed using real-time PCR with a TaqMan probe designed in the fragment that contains the *H19* enhancers (fragment 21; **Fig. 2**; for primer sequences, see **Table 2**). These enhancers are endoderm specific and are necessary for both *Igf2* and *H19* gene expression[24]. As shown in **Figure 2a** (loading control normalization) as well as in **Figure 2b** (normalization to control interaction frequencies, right graphs), for each 3C sample analyzed (A1 and A2), a local interaction peak is found with the restriction fragment that contains the *H19* promoter (fragment 19). Raw data from this experiment are given in **Table 6** as an example of data analysis.

TABLE 5 | Sequence of qPCR primers used to determine the digestion efficiency at each *Bam*HI site used for 3C-qPCR analysis of the mouse imprinted *Igf2/H19* locus; all amplifications were performed as described in PROCEDURE (Step 38A).

Location of *Bam*HI site tested	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)
5′ of fragment −1	agcctccttcagtcagtgga	gctggtctctcccaagagc
3′ of fragment −1	gaatggtggagaagccaatgag	actcagggaagggaagtggt
3′ of fragment 2	ctgctccgtgtgagttcctt	aatgctgaccgattttggag
3′ of fragment 3	aggaccgcaaatcagacaag	gggacaccgtaggagaagtg
3′ of fragment 4	ctgcgtttgtttctccaggt	aagcaccaacatcgacttcc
3′ of fragment 7	gtggcaaggaaagtgaagga	aggtggtctgagggcctatt
3′ of fragment 8	ggcagctcacatacaactcc	tgcccacagaagaggacatt
3′ of fragment 17	ccagagcaggatgtgagagg	gctggtggtgtgactgtgag
3′ of fragment 18	ggcgggagacatagaaactgc	cgctatgctccctcttgcta
3′ of fragment 19	gcagggttgccagtaaagac	tgaccccagcctagaatgcttc
3′ of fragment 20	ccttgtcgtagaagccgtctg	ggggcagaagagaactcacctt
3′ of fragment 21	aatgtggggagacaaacagc	aggaggccaatgttgtgaag

TABLE 6 | Data analysis for interaction between *H19* enhancers and the *H19* gene promoter.

Title of 3C experiment				
H19 enhancers				
		3C assay name	A1	A2
Gapdh		Ct1	17.27	17.61
qPCR run no.	1	Ct2	17.29	17.46
		Ct3	17.24	17.50
Standard curve parameters				
a (slope)	−3.572			
b (intercept)	18.7			
		Mean Ct	17.24	17.50
		Value Gapdh/10	0.26	0.22
Pdhb		Ct1	30.38	31.63
qPCR run no.	2	Ct2	30.22	31.08
		Ct3	30.66	31.29
Standard curve parameters				
a (slope)	−3.558			
b (intercept)	26.02			
		Mean Ct	30.42	31.33
		Value pdhb	0.05798961	0.03211084
Fragment 17		Ct1	33.71	34.66
qPCR run no.	3	Ct2	34.22	34.61
		Ct3	34.79	(33.83)
Standard curve parameters				
a (slope)	−3.946	Mean Ct	34.24	34.64
b (intercept)	35.85	Value	2.56	2.03
		Norm./gapdh	9.98	9.37
		Norm./pdhb	44.12	63.28
Fragment 18		Ct1	33.43	33.55
qPCR run no.	4	Ct2	33.48	33.96
		Ct3	(34.07)	32.94
Standard curve parameters				
a (slope)	−4.608	Mean Ct	33.46	33.76
b (intercept)	35.94	Value	3.46	2.98
		Norm./gapdh	13.51	13.75
		Norm./pdhb	59.69	92.79
Fragment 19		Ct1	32.92	33.40
qPCR run no.	5	Ct2	33.32	33.54
		Ct3	33.18	33.67
Standard curve parameters				
a (slope)	−3.54	Mean Ct	33.12	33.47
b (intercept)	35.51	Value	4.73305164	3.77
		Norm./gapdh	18.4674376	17.39
		Norm./pdhb	81.618958	117.39
Fragment 20		Ct1	33.35	33.71
qPCR run no.	6 and 7	Ct2	33.49	33.75
		Ct3	(34.21)	(33.94)
Standard curve parameters				
aA1 (slope)	−3.501	Mean Ct	33.42	33.73
bA1 (intercept)	34.46	Value	1.98	1.81
aA2 (slope)	−3.853	Norm./gapdh	7.73	8.34
bA2 (intercept)	34.72	Norm./pdhb	34.17	56.27

Ligation products obtained with fragments 17, 18, 19 and 20 (that encompass the *H19* gene and promoter) were quantified from the A1 and A2 3C samples (from 7-day-old mouse livers) as described in Steps 40 and 41 (qPCR run no. 3, 4, 5 and 6/7 respectively). A "loading control" ("internal primers" located in the *gapdh* gene) and a set of "control interaction frequencies" (*PDHB* primers) were performed (qPCR run no. 1 and 2 respectively) (for primer sequences see **Table 2**). For each fragment, we performed a triplicate quantification (Ct1, Ct2, Ct3), we then calculated the mean Ct. However, some of the triplicates (Ct in parentheses) have not been taken into account when calculating the mean Ct because they deviate from two other quantifications. The final value was calculated using the parameters of the standard curve (*b*: intercept; *a*: slope) as follows: value$=10^{(Ct-b)/a}$. These values were finally normalized either to *gapdh* (loading control) (see **Fig. 2a**) or *Pdhb* (control interaction frequency) (see **Fig. 2b**). For fragment 20 (qPCR run no 6/7) two standard curves are given because the A1 and A2 samples were analyzed in two separate runs.

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In the upstream part of the locus, we found that the *H19* enhancers contact the sequences containing the differentially methylated region 1 (DMR1, fragment 2) (**Fig. 2a**,**b**, left graphs). These results confirm the recently described data[21] and thus further validate the 3C-qPCR methodology.

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