3C-digital PCR for quantification of chromatin interactions

Meijun Du and Liang Wang*

Abstract

Background: Chromosome conformation capture (3C) is a powerful and widely used technique for detecting the physical interactions between chromatin regions in vivo. The principle of 3C is to convert physical chromatin interactions into specific DNA ligation products, which are then detected by quantitative polymerase chain reaction (qPCR). However, 3C-qPCR assays are often complicated by the necessity of normalization controls to correct for amplification biases. In addition, qPCR is often limited to a certain cycle number, making it difficult to detect fragment ligation with low frequency. Recently, digital PCR (dPCR) technology has become available, which allows for highly sensitive nucleic acid quantification. Main advantage of dPCR is its high precision of absolute nucleic acid quantification without requirement of normalization controls.

Results: To demonstrate the utility of dPCR in quantifying chromatin interactions, we examined two prostate cancer risk loci at 8q24 and 2p11.2 for their interaction target genes MYC and CAPG in LNCaP cell line. We designed anchor and testing primers at known regulatory element fragments and target gene regions, respectively. dPCR results showed that interaction frequency between the regulatory element and MYC gene promoter was 0.7 (95% CI 0.40–1.10) copies per 1000 genome copies while other regions showed relatively low ligation frequencies. The dPCR results also showed that the ligation frequencies between the regulatory element and two EcoRI fragments containing CAPG gene promoter were 1.9 copies (95% CI 1.41–2.47) and 1.3 copies per 1000 genome copies (95% CI 0.76–1.92), respectively, while the interaction signals were reduced on either side of the promoter region of CAPG gene. Additionally, we observed comparable results from 3C-dPCR and 3C-qPCR at 2p11.2 in another cell line (DU145).

Conclusions: Compared to traditional 3C-qPCR, our results show that 3C-dPCR is much simpler and more sensitive to detect weak chromatin interactions. It may eliminate multiple and complex normalization controls and provide accurate calculation of proximity-based fragment ligation frequency. Therefore, we recommend 3C-dPCR as a preferred method for sensitive detection of low frequency chromatin interactions.

Keywords: Chromatin interaction, Chromosome conformation capture, Digital PCR, Quantitative PCR
usually detects amplification signals at high cycle threshold (Ct) (such as Ct ≥ 35), which significantly reduces the assay’s sensitivity. In addition, current 3C-qPCR is complex because randomly ligated control is needed to normalize the amplification efficiency of different primer pairs.

Recently, digital PCR (dPCR) has been emerged as a powerful tool for nucleic acid quantification, in particular, for rare molecule detection [6]. The technology detects number of targeted nucleic acids for absolute quantification by molecular counting. During dPCR, DNA samples are partitioned into thousands or millions of individual PCR reactions. Due to significant dilution, each reaction partition contains zero or one target molecule, sometimes multiple copies if dilution is not sufficient. After PCR amplification, each independent reaction can be defined as positive or negative for the target molecule by intensity of its recorded fluorescence signal [6]. Characterized by high sensitivity and specificity, the dPCR is increasingly being used for various applications such as absolute nucleic acid quantification, rare mutation detection, and copy number variation [7–9]. Here we reported a 3C-dPCR assay by incorporating dPCR technology into 3C assay [10]. We tested this assay at two prostate cancer risk regions of 8q24 and 2p11.2 for their interaction target genes MYC and CAPG [11, 12]. Our results show that 3C-dPCR is easier to use and more sensitive in determining chromatin interactions. The 3C-dPCR is likely to offer a valuable alternative method for accurate quantification of low frequency chromatin interactions.

**Result**

**3C-dPCR workflow**

To identify a chromatin interaction through looping structure, it is necessary to show that the two interaction fragments have higher contact frequency than randomly ligated fragments. The first step of the procedure is to design primer and probe. In principle, a series of primers covering both pre-defined regions should be selected. In this study, we examined one fixed anchor primer (interaction hot spot) in combination with a series of test primers (covering target region). TaqMan probe was located downstream of the anchor primer (Fig. 1A). The second step is to build a 3C library including chromatin crosslinking, restriction enzyme digestion and intramolecular fragment ligation (Fig. 1B). The third step is to measure interaction (ligation) frequency using primers specific for the restriction fragments of interest. After PCR amplification in a digital PCR system, positive and negative reactions were determined by the fluorescence signal intensity. The number of the concentration of ligation product was reported as copies/μL (Fig. 1C).

**Characterization of dPCR for detection of 3C product**

dPCR assay provides a convenient and straightforward approach to run up to millions of PCR reactions in parallel. In this study, we applied 3D Digital PCR system and performed duplex dPCR by including both target and genome copy control. Figure 2a and b displayed the representative plot showing digestion efficiency and self-ligation rate, where we observed 197 copies undigested EcoRI fragments and 44 self-ligated copies per 1000 genome copies. Figure 2c indicated moderate adjacent fragment ligation with 3.6 copies per 1000 genome copies. Figure 2d showed the representative plot of long-range chromatin interaction with 1.7 copies per 1000 genome copies. For each plot, signals in the lower left quadrant were negative (yellow) for both targets, in the lower right quadrant were positive for genome copy number (red), and in the upper left quadrant were positive for target fragment ligation (blue). The green signals between red and blue were positive for both the target and genome copy control. The intensity of fluorescence signals reflected target copy numbers after PCR amplification. The signals were specific to each primer/probe set.

**Detection of chromatin interactions at selected target regions**

To determine the ligation frequency between the two restriction fragments in 3C libraries, we first tested a previous reported interaction between 8q24 region 1 and MYC gene [11], we examined an anchor primer 8L at EcoRI site (chr8: 128537495) paired with five test primers at MYC gene locus (MYC1–5). The anchor primer was also paired with three other test primers at 9, 85 and 92 kb downstream as nearby ligation and random ligation controls (Fig. 3a). The highest ligation frequency (approximately 2%) was observed for the fragment located directly upstream of the anchor fragment (Fig. 3b). The interaction frequency between the regulatory element and MYC gene promoter fragment (MYC3), ~200 kb away from the anchor fragment, was 0.7 copies (95% CI 0.40–1.10) per 1000 genome copies while the regions (~85 and ~92 kb regions) assumed to be looped out from the hub [11] showed ligation frequencies of 0.18 (95% CI 0.08–0.29) and 0.16 (95% CI 0.07–0.30), respectively. Moreover, there are no obvious signals for ddH₂O and random ligated genomic DNA negative control (data not shown).

We further tested the ligation frequency between prostate cancer risk locus 2p11.2 and gene CAPG, which was described in our previous study [12]. The anchor primer at 2q11.3 was designed at the position (chr2: 85778503) named 2L. Eleven test primers were designed on 2q11.3 covering twelve EcoRI cutting sites from chr2: 85619044

```
Du and Wang BMC Molecular Biol (2016) 17:23

Page 3 of 9

(T1) to chr2: 85679686 (T12), which corresponded to the promoter and nearby region of CAPG (Fig. 4a). We observed strong interaction signals at EcoRI fragments containing primer T7 and T6 with the ligation frequency 1.9 copies (95% CI 1.41–2.47) and 1.3 copies (95% CI 0.76–1.92) per 1000 genome copies, respectively. The interaction signals were reduced on either side of this EcoRI site. Another interaction peak was with primer T11, the interaction frequency was 1.2 copies (95% CI 0.76–1.92) molecule per 1000 genomes. The lowest interaction signal was 0.45 copies (95% CI 0.27–0.72) per 1000 genomes (Fig. 4b). We also examined the frequency of self-ligation in the 3C library by pairing the anchor primer with a primer on the same fragment. A primer pair across the EcoRI site was used to test the enzyme digestion efficiency. We found 44 copies (95% CI 34–58) per 1000 genomes for the frequency of self-ligation and 197 copies (95% CI 172–226) per 1000 genomes for the undigested EcoRI fragments (Fig. 2a, b).

Comparison of 3C-dPCR with 3C-qPCR

To investigate the potential precision of 3C-dPCR and compare it with the established technique of 3C-qPCR, a comparison of dPCR and qPCR was performed to detect the interaction frequency between 2p11.2 and the cluster of EcoRI fragments on CAPG locus in another 3C library made from cell line DU145. The 3C product was run separately by both dPCR and standard TaqMan qPCR to directly compare the interaction frequency of different primer pairs. Figure 5a shows strong signals between

---

**Fig. 1** 3C-dPCR workflow. 

A. TaqMan probe and primer design. The locations of the two possible interaction fragments (a and b) are shown (black rectangle). Restriction sites used in the 3C assay are depicted as small vertical bars in black. The relative positions of anchor primer (black arrow), the TaqMan probe (F-Z-Q) and test primers (grey arrows) are also depicted. F fluorophore, Z internal quencher, Q quencher. 

B. Three essential steps of 3C assays: 1. Interacting chromatin segments are cross-linked by formaldehyde. 2. Cross-linked chromatin fragments are digested by a selected restriction enzyme. 3. Cross-linked fragments undergo intra-molecular ligation. 

C. Principle of 3C-dPCR. The reaction mixture containing 3C DNA is prepared and partitioned into thousands of reaction wells. Due to significant dilution, each reaction well receives 0–1 target ligation products. After PCR amplification, the fluorescence signals are imaged and copy numbers of target ligations are reported as copies/μL. In the 3C-dPCR reaction and partition steps, curved lines in blue, red, black, grey and purple curve in the circle represent the different DNA molecules, including ligation products in 3C libraries. In the amplification step, the blue dot (well) shows target amplification signal; the red dots (wells) indicate the genome copy number signal; the green dot (well) displays the overlap of target and genome copy number signals.
the EcoRI fragments covering primer T7, T6, T11 and anchor primer with 1.7 (95% CI 1.26–2.32), 1.2 (95% CI 0.82–1.66) and 1.0 copy (95% CI 0.66–1.46) molecules per 1000 genomes, respectively. However, fragments near this interaction were 2–5 folds lower than the active interaction fragments. Figure 5b showed the corresponding 3C-qPCR results. Although the interaction peak was slightly different between dPCR and qPCR, the overall trend from two results were highly consistent.

**Discussion**

To identify a chromatin interaction, it is necessary to demonstrate higher ligation frequency between two restriction fragments than randomly ligated fragments.
Because ligation frequency is generally low between any two non-adjacent fragments [1, 5], a meaningful 3C analysis critically relies on the accurate quantification of different ligation products. In this study, we evaluated dPCR, the latest DNA quantification technology, for sensitive detection of chromatin interactions. Our results show that the 3C-dPCR is user-friendly and able to detect all previously reported interactions. Its simplicity and accuracy make it ideal for low copy number analysis such as low ligation frequency between chromatin interactions.

Currently, the most commonly used qPCR-based 3C assay has its own limitations. First, the assay requires preparation of randomly ligated control template DNA to normalize the amplification efficiency differences among different primer pairs [13]. Second, this assay generates relative quantification of ligated fragments [4, 6]. Third, the assay may not be sensitive enough to detect low frequency ligation products. Low concentration of ligation products in standard 3C library often leads to high Ct value, sometimes beyond the limitation of qPCR detection. In contrast, by sub-dividing a reaction mix into thousands of individual replicates, the dPCR assay significantly reduces the total number (hence diversity) of DNA molecules in any given partition effectively enriched for the sequences of interest and diluted out other background sequences. Therefore, dPCR assay is more sensitive and more specific than qPCR assay [14]. It also effectively overcomes qPCR biases due to primer amplification efficiency differences. In addition, the 3C-dPCR is able to generate absolute numbers of ligated target fragments and genome copies in one reaction without requirement of normalization controls. Therefore, the 3C-dPCR is simpler and more sensitive in determining low interaction frequency at the target regions of interest.

The dPCR may also simplify quality control during 3C library preparation. For example, dPCR can be used to determine efficiency of restriction enzyme digestion and proximity-based ligation. In current 3C protocol, internal control primer pair is required to accurately calculate percentage of digested fragments and ligated fragments among all available genome copies. The dPCR, however, does not have amplification bias and can accurately calculate digestion and ligation efficiency. For the low frequency ligations that are close to the lower limit

---

Fig. 3 Interactions between prostate cancer risk region 1 and MYC gene locus at 8q24. a The anchor primer 8L, TaqMan probe; five target test primers (MYC1–MYC5) and three control test primers (9, 85, and 92 kb) are designed for the detection of the cis-acting interactions. Small vertical bars in black represent EcoRI digestion sites. Black and grey arrows show the anchor primer and test primers, respectively. The TaqMan probe is depicted as grey bar. b The copy number of ligation products at each selected restriction site. The highest interaction is at MYC-3 fragment, which contains the MYC promoter region. The y-axis displays the ligation copy numbers at each EcoRI site per 1000 genome. The x-axis is the genomic position of each EcoRI site. The error bars represent 95% CIs.
of detection, dPCR system allows increasing the 3C DNA concentration in the PCR mix to provide more target liguations available for detection. The system also allows running a larger volume of the same sample on multiple chips and pooling the data into one larger “virtual” chip for low frequency ligation detection.

**Conclusion**

Over the years 3C-based technologies have been evolved from single PCR assay to massive parallel sequencing assay [15–17]. Although the sequencing assays have significantly extended the scope of chromatin loop mediated long distance interaction and facilitated understanding biological mechanisms underlying gene regulation, most studies still rely on PCR-based assay to evaluate interactions at pre-defined genomic regions. By introducing dPCR into 3C assay, we show that this digital technology not only eliminates the potential variations of PCR amplification efficiency but also provides more accurate measurement of proximity-based fragment ligation frequency. The 3C-dPCR is a preferred method for sensitive and specific quantification of chromatin interactions.

**Methods**

**Selection of chromatin interaction loci and primers/probes design**

Previous study showed that prostate cancer risk loci at 8q24 were interacted with MYC region [11]. To test the feasibility of dPCR in detection of such chromatin interactions, we designed an anchor primer that was located upstream of the EcoRI site at chr8:128537496 on 8q24 named as 8L. This site was shown to have an interaction peak with MYC in a previous report [11, 18]. Five test primers were selected downstream of each EcoRI site from chr8:128521424 to chr8:128537496 (named as MYC1 to MYC5, respectively). MYC gene promoter was in MYC3 fragment (Fig. 3a). One test primer, 9 kb downstream from the anchor primer, was used as positive control (nearby ligation). Two other test primers, 85 and 92 kb away from the anchor primer were used as long-distance random ligation (negative) controls. We named the corresponding primers as 9, 85 and 92 kb accordingly. Each of these test primers was paired with the anchor primer. One pair of primer within EcoRI fragment (Chr8:128521424–128537496) was used to normalize genome copy number.
TaqMan probes were located downstream of the anchor primer and labeled with 5′ FAM (targets) or HEX (copy number control) (Fig. 1A). The primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA). TaqMan probes were dissolved in TE pH 8.0 and stored at −20 °C as 2.5 μM aliquots. The sequences of the primers and probes were listed in Additional file 1: Table S1.

For the interaction between 2q11.3 and gene CAPG, primers and probe were designed as previously reported [12]. In brief, the anchor primer at 2q11.3 was designed near the cutting site chr2: 85778503 named 2L. Eleven test primers were spread twelve EcoRI cutting sites from chr2: 85619044 (T1) to chr2: 85679686 (T12), which covered the promoter and nearby region of gene CAPG. Adjacent ligation primer was designed on the fragment next to 2L. Each test primer was paired with the anchor primer. Self-ligation primer was designed on the same fragment with anchor primer and paired with the anchor primer to test self-ligated DNA circles. Undigested control primer was across the EcoRI site 2L and paired with the anchor primer (Fig. 4a). The sequences of the primers are listed in Additional file 1: Table S1.

**3C library preparation**

3C libraries were prepared as previously described [4]. Briefly, 1 × 10⁷ cells were cross-linked with 1% formaldehyde for 10 min, and quenched with a final concentration of 0.125 mM glycine for 5 min at room temperature. Cells were counted and placed into aliquots 5 × 10⁶ cells. Each aliquot of cells was lysed with 500 μL 1× cold lysis buffer (10 Mm Tris–HCl Ph 8.0, 10 Mm NaCl, 0.2% Igepal CA630) including 1× protease inhibitor (Roche, Indianapolis, IN, USA) for at least 15 min on ice. Cell nuclei were pelleted, washed twice with 500 μL ice cold 1× EcoRI buffer (NEB, Ipswich, MA, USA), and then re-suspended in 500 μL 1× EcoRI buffer with 0.3% SDS and incubated for 1 h at 37 °C, followed by adding 1% Triton X-100 and incubated for another 1 h to sequester the SDS. Each sample was digested overnight with 600 U restriction enzyme at 37 °C. To stop the restriction digestion, 1.6% SDS (final concentration) was added, and samples were incubated at 65 °C for 20 min. Ligation were performed at 16 °C for 4 h in 15 mL tubes containing 745 μL 10× T4 ligase Buffer, 10% Triton-X 100, 80 μL 10 mg/mL BSA, 6 mL water, 575 μL of cell lysate, 10 μL 1U/μL T4 ligase (Invitrogen, Grand Island, NY, USA). The

![Fig. 5 Comparison of 3C-qPCR with 3C-dPCR](image-url)
crosslinks were reversed with Proteinase K (Invitrogen) at 65 °C overnight. 3C samples were then purified using phenol–chloroform extraction and quantified by Qubit dsDNA HS Assay (Life Technologies).

Digital PCR
QuantStudio 3D Digital PCR System (Life Technology, Carlsbad, CA, USA) was used for the dPCR. For each Chip, reactions were performed in 18ul volume using 9 μL of 2× 3D Digital PCR master mix, 500 nM of target primer pairs, 250 nM of probes, 80 ng of 3C template DNA examining long-distance interaction efficiency and self-ligation copy numbers. A copy number control primer/probe mix was added in the same concentration as target primer/probe mix for duplex dPCR for genome copy number determination. To exclude the false positive result caused by high level non-specific background signal from PCR amplification, ddH2O and random ligation control genomic LNCaP DNA after EcoRI digestion and T4 ligation were used as dPCR negative controls. Reactions were the average of at least two independent experiments.

Real-time quantitative PCR
To confirm the dPCR data, TaqMan qPCR technology was used to quantify the ligation frequency of 2p11.2 risk locus and the cluster of EcoRI fragments on CAPG locus. All PCR reactions were performed using Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, Cat# 4440038). Each 10 μL reaction consisted of 1× Taqman Universal MasterMix II, 1 μL 5uM anchor primer, 1 μL test primer, 1 μL Taqman probe (2.5 μM), and 100 ng 3C DNA. PCR cycles were as follows: an initial step 2 min at 50 °C, 10 min at 95 °C, 50 cycles of 15 s at 95 °C, 60 s at 58–60 °C. Each PCR reaction was performed in triplicate, and the data presented were the average of at least two independent experiment results for all PCR reactions. The contact frequency of each interaction pair was normalized using a 3C-control library prepared from pooled PCR products that contained 16 EcoRI-digested and T4 ligase-ligated fragments covering target EcoRI cutting sites and primer binding sites [12]. Adjacent fragment ligation frequency was used to normalize the different loading, fixation and ligation efficiencies between different cell lines.

Additional file

Additional file 1: Table S1. Table of sequences of the primers and TaqMan probes used for dPCR.

Abbreviations
Abbreviations: 3C: chromosome conformation capture; PCR: polymerase chain reaction; qPCR: quantitative PCR; dPCR: digital PCR; Ct: cycle threshold; CI: confidence interval; AQ: absolute quantity; RQ: relative quantity (RQ).

Authors’ contributions
Conceived and designed the experiments: MD, LW; Performed the experiments: MD; Wrote the manuscript: MD; Read and revised the manuscript: LW. Both authors read and approved the final manuscript.

Acknowledgements
None.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The original data of the real-time PCR and dPCR experiments will be available upon request.

Ethics approval and consent to participate
Cell lines used in this study were purchased from American Type Culture Collection (ATCC) and consisted of prostate cancer cell lines LNCaP (ATCC® CRL-1740™) and DU145 (ATCC® HTB-81™).
Funding
This work was supported by Medical College of Wisconsin Cancer Center Seed Fund [3305738 to LW] and National Institute of Health [R01CA157881 to LW].

Received: 22 February 2016   Accepted: 1 December 2016
Published online: 06 December 2016

References
1. Simonis M, Kooren J, de Laat W. An evaluation of 3C-based methods to capture DNA interactions. Nat Methods. 2007;4(11):895–901.
2. Miele A, Dekker J. Mapping cis- and trans- chromatin interaction networks using chromosome conformation capture (3C). Methods Mol Biol. 2009;464:105–21.
3. Dekker J, Rippe K, Dekker M, Kleckner N. Capturing chromosome conformation. Science. 2002;295(5558):1306–11.
4. Hagege H, Klous P, Braem C, Splinter E, Dekker J, Cathala G, de Laat W, Forne T. Quantitative analysis of chromosome conformation capture assays (3C-qPCR). Nat Protoc. 2007;2(7):1722–33.
5. Gavrilov AA, Golov AK, Razin SV. Actual ligation frequencies in the chromosome conformation capture procedure. PLoS ONE. 2013;8(3):e60403.
6. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright LJ, Lucero MV, Hiddessen AL, Legler TC, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011;83(22):8604–10.
7. Nadauld L, Regan JF, Miotke L, Pai RK, Longacre TA, Kwok SS, Saxornin S, Ford JM, Ji HP. Quantitative and sensitive detection of cancer genome amplifications from formalin fixed paraffin embedded tumors with droplet digital PCR. Transl Med. 2012;2(2).
8. Reid AL, Freeman JB, Millward M, Zirman M, Gray ES. Detection of BRAF-V600E and V600K in melanoma circulating tumour cells by droplet digital PCR. Clin Biochem. 2014.
9. Li N, Ma J, Guarnera MA, Fang H, Cai L, Jiang F. Digital PCR quantification of miRNAs in sputum for diagnosis of lung cancer. J Cancer Res Clin Oncol. 2014;140(1):145–50.
10. Link N, Kurtz P, O’Neal M, Garcia-Hughes G, Abrams JM. A p53 enhancer region regulates target genes through chromatin conformations in cis and in trans. Genes Dev. 2013;27(22):2433–8.
11. Du M, Yuan T, Schilter KF, Dittmar RL, Mackinnon A, Huang X, Tschan mann M, Worth ey E, Jacob H, Xia S, et al. Prostate cancer risk locus at 8q24 as a regulatory hub by physical interactions with multiple genomic loci across the genome. Hum Mol Genet. 2015;24(1):154–66.
12. Du M, Tillmanns L, Gao J, Gao P, Yuan T, Dittmar RL, Song W, Yang Y, Sahr N, Wang T, et al. Chromatin interactions and candidate genes at ten prostate cancer risk loci. Sci Rep. 2016;6:23202.
13. Miele A, Gheldof N, Tabuchi TM, Dostie J, Dekker J. Mapping chromatin interactions by chromosome conformation capture. Curr Protoc Mol Biol. 2006.
14. Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S, Foy CA, Scott DJ. Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res. 2012;40(11):e82.
15. de Wit E, de Laat W. A decade of 3C technologies: insights into nuclear organization. Genes Dev. 2012;26(1):11–24.
16. Dekker J, Marti-Renom MA, Ninio LA. Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data. Nat Rev Genet. 2013;14(6):390–403.
17. Sajan SA, Hawkins RD. Methods for identifying higher-order chromatin structure. Annu Rev Genomics Hum Genet. 2012;13:59–82.
18. Ahmadiyeh N, Pomerantz MM, Grisanzio C, Herman P, Jia L, Almendro V, He HH, Brown M, Liu XS, Davis M, et al. 8q24 prostate, breast, and colon cancer risk loci show tissue-specific long-range interaction with MYC. Proc Natl Acad Sci USA. 2010;107(21):9742–6.
19. Majumdar N, Wessel T, Marks J. Digital PCR modeling for maximal sensitivity, dynamic range and measurement precision. PloS ONE. 2015;10(3):e0118833.