ORIGINAL RESEARCH ARTICLE

Development and Evaluation of a Droplet Digital PCR Assay for 8p23 β-Defensin Cluster Copy Number Determination

Tingting Wen[1] · Xianghong Zhang[2] · Christoph Lippuner[3] · Marcel Schiff[3] · Frank Stuber[3]

Abstract

Introduction It has been proposed that the copy number (CN) variation (CNV) in β-defensin genes (DEFB) on human chromosome 8p23 determines phenotypic differences in inflammatory diseases. However, no method for accurate and easy DEFB CN quantification is yet available.

Objective Droplet digital polymerase chain reaction (ddPCR) is a novel method for CNV quantification and has been used for genes such as *CCL4L*, *CCL3L1*, *AMY1*, and *HER2*. However, to date, no ddPCR assay has been available for DEFB CN determination. In the present study, we aimed to develop and evaluate such a ddPCR assay.

Methods The assay was designed using *DEFB4* and *RPP30* as the target and the reference gene, respectively. To evaluate the assay, 283 DNA samples with known CNs previously determined using the multiple ligation-dependent probe amplification (MLPA) method, the current gold standard, were used as standards. To discover the optimal DNA template amount, we tested 80 to 2.5 ng DNA by a serial of one to two dilutions of eight samples. To evaluate the reproducibility of the assay, 31 samples were repeated to calculate the intra- and inter-assay variations. To further validate the reliability of the assay, the CNs of all 283 samples were determined using ddPCR. To compare results with those using quantitative PCR (qPCR), DEFB CNs for 48 samples were determined using qPCR with the same primers and probes.

Results In a one-dimensional plot, the positive and negative droplets were clearly separated in both *DEFB4* and *RPP30* detection channels. In a two-dimensional plot, four populations of droplets were observed. The 20 ng template DNA proved optimal, with either high (80 ng) or low (10, 5, 2.5 ng) template input leading to ambiguous or inaccurate results. For the 31 standard samples, DEFB CNs were accurately determined with small intra- and inter-assay variations (coefficient of variation < 0.04 for both). In the validation cohort, ddPCR provided the correct CN for all 283 samples with high confidence. qPCR measurements for the 48 samples produced noisy data with high uncertainty and low accuracy.

Conclusions ddPCR is an accurate, reproducible, easy-to-use, cheap, high-throughput method for DEFB CN determination. ddPCR could be applied to DEFB CN quantification in large-scale case–control studies.

1 Introduction

Copy number variations (CNVs) affect about 10% of the human genomic sequence [1, 2]. Among the many genes

✉ Xianghong Zhang
xianghong_zhang@zju.edu.cn

[1] Department of Anesthesiology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

[2] Department of Cardiac Intensive Care Unit, The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Heath, Hangzhou, Zhejiang, China

[3] Department of Anesthesiology and Pain Medicine, Bern University Hospital, Inselspital, Bern, Switzerland

Key Points

A robust method is needed to accurately measure the copy numbers of β-defensin genes on human chromosome 8p23, which in turn could determine susceptibility to inflammatory disease.

Digital droplet polymerase chain reaction (ddPCR) was used to measure the copy numbers, and results were compared with those obtained using multiple ligation-dependent probe amplification, the current gold standard method.

ddPCR is an accurate, easy-to-use, high-throughput method that delivers reproducible copy number results. It is a good tool for large-scale case–control studies.

with CNVs, β-defensin genes (DEFBs) located in 8p23.1 form a cluster, and the cluster exhibits extensive CNVs ranging from 2 to 12 in Caucasian populations (Fig. 1) [3, 4]. The DEFBs are a group of small cationic peptides with antimicrobial and immune regulatory properties [5, 6]. To date, DEFB CNVs have been reported to impact on *DEFB4* messenger RNA levels and probably also protein levels in many types of tissue [4, 7–10]. Based on functional analysis, genetic association studies have been conducted by different groups and DEFB CNVs found to be associated with many diseases, such as psoriasis [11, 12], chronic obstructive pulmonary disease [9], Crohn's disease [7, 13], and reproductive tract infections [10]. However, most of the association studies cannot be replicated, and the discrepancy is primarily because DEFB CN determination methods are unreliable [14–17].

In our previous work, we used multiple ligation-dependent probe amplification (MLPA), a pseudogene-based paralog ratio test, and quantitative polymerase chain reaction (qPCR) to determine DEFB CN and showed that MLPA was the most reliable method among the three since it targets multiple DEFB and reference genes [16]. Therefore, MLPA is regarded as the gold standard method. However, widespread application of this method is constrained mainly by the amount of work required for experiments and data processing, its requirement for large amounts of DNA, and its high costs.

Droplet digital PCR (ddPCR) is an emerging method for CN determination that is gaining popularity. It is an endpoint PCR method for nucleotide acid quantification, and the PCR reactions take place in oil-wrapped nano-sized droplets. After PCR, the droplets are classified as positive or negative clusters according to the signal intensity. Thereafter, the concentration of nucleotide acid is calculated using Poisson statistics according to the percentage of positive droplets [18–20]. Because ddPCR does not need a standard curve, it has advantages in terms of accuracy, reproducibility, and sensitivity in quantification of a wide variety of genetic materials, such as mitochondrial DNA [21], tumor-cell-free DNA [22], circulating microRNA [23], and virus copies, especially for severe acute respiratory syndrome coronavirus 2 [24], plant DNA [25], low-level somatic mosaicism [26], and even absolute gene copies in a single cell [27].

To date, ddPCR has also been tested for the determination of several gene CNVs, such as *KIR* [28], *CCL4L* [29], *CCL3L1* [30], *AMY1* [31], *HER2* [32], and *BRCA1* [33]. These genes' CNVs are mostly caused by deletion and duplication, so the CNVs range from 0 to 4. In contrast, the DEFB CNVs have a broad dynamic range from 2 to 12, which means it is a substantial challenge for the methodology to distinguish high CNs. Therefore, whether ddPCR can be used to accurately determine DEFB CN is unknown. The aim of the present study was to develop and evaluate a ddPCR assay for DEFB CN determination, which, with careful design and optimization, we successfully completed. In addition, we showed that ddPCR was superior to MLPA for DEFB CN determination.

2 Materials and Methods

2.1 Cohort

A cohort of 283 healthy European blood donors (Red Cross cohort, code RC) with known CNs as previously determined using MLPA was used in this study [16]. Written informed consent that covered the present study was obtained previously. This study was approved by the ethics committee of the Canton of Bern (no. KEK 041/09).

Fig. 1 Design of *DEFB4* primers and probe. Assembly of the human genome region of 8p23 (GRCh38, hg38) is shown. The green arrow depicts the α-defensin cluster. The red arrows depict two copies of the β-defensin cluster. The boxes depict genes located within the β-defensin cluster. The *DEFB4* sequence was referred from GenBank with ID 1673. Yellow shading indicates the second exon of the *DEFB4* gene; green indicates the primer-binding sites; pink indicates the probe-binding site
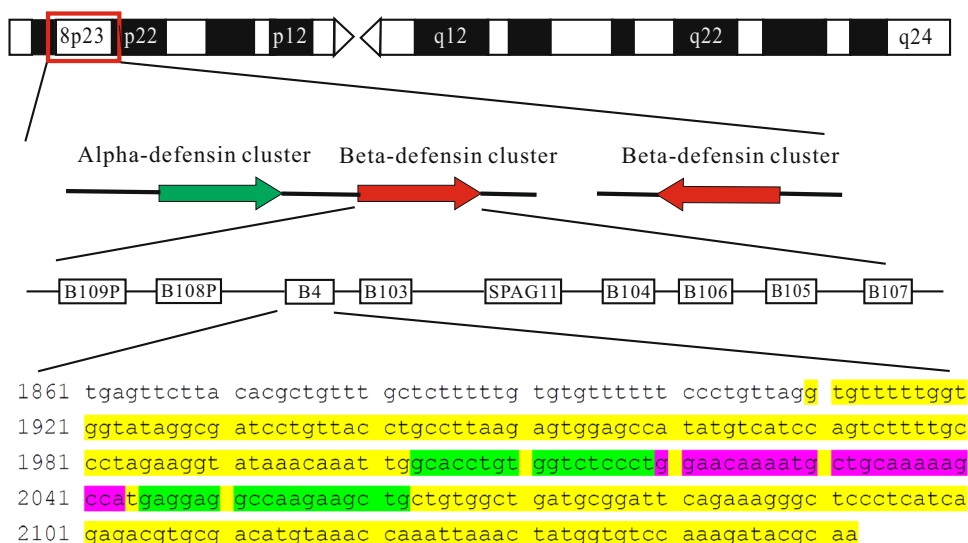
2.2 DNA Digestion

DNA samples were previously isolated and stored in − 20 °C as stock [16]. To generate a single-copy *DEFB4* template, genomic DNA was digested using MseI enzyme (New England Biolabs, Switzerland) in reaction that contains approximately 100 ng of DNA, 2.5 µl 10 × CutSmart buffer, and 2 units of MseI enzyme in a total volume of 25 µl. The digestion was performed at 37 °C for 60 min and then at 65 °C for 20 min.

2.3 Primers and Probes

Specific primers and probes targeting the second exon of the *DEFB4* gene, which is located in the DEFB cluster, were designed and generated (Fig. 1). *RPP30* is a gene without CNVs in the human genome so served as a reference gene. The primers and probes for *RPP30* were commercially available and well-validated (assay ID: dHsaCP1000485, Bio-Rad Laboratories, Hercules, CA, USA). Table 1 lists the sequences for primers and probes.

2.4 Droplet Digital Polymerase Chain Reaction (ddPCR)

ddPCR was performed using QX200™ Droplet Digital PCR (Bio-Rad Laboratories) according to manufacturer's instructions. In brief, the reaction mixture (20 µl) containing around 20 ng digested template DNA, 10 µl ddPCR supermix, 900 nM of each primer, and 250 nM of each probe (Bio-Rad) was loaded into the sample well in the QX100 Droplet Generator. Then, 70 µl of droplet generation oil (Bio-Rad) was loaded into the oil well. A total of 40 µl of oil–water emulsion containing approximately 12,000–20,000 droplets was generated with the droplet generator and gently transferred into a separate well of a 96-well PCR plate. PCR was performed under the following thermocycling conditions: denaturation at 96 °C for 10 min, amplification of 45 cycles (95 °C for 5 s, 60 °C for 1 min, and 72 °C for 10 s), and extension at 72 °C for 10 min. Annealing at 60 °C was selected after strong gradient tests and was proven optimal (data not shown). Samples with CNs from 2 to 9 as previously determined by MLPA were included in each run as positive controls. After PCR amplification, positive and negative droplets were counted using QuantaSoft software with automated clustering analysis. Nucleotide acid concentration was further calculated with Poisson statistics embedded in QuantaSoft. Raw CN values were two times the ratio of *DEFB4* and *RPP30* concentrations. CN was rounded to the nearest integer of the raw value. Samples with mid-integer raw CN values (those 0.30–0.70 away from an integer number) or confidence intervals wider than 1 or < 10% double-negative droplets were considered unreliable. Re-quantification was performed for

Table 1 Primers and fluorogenic probes in the digital droplet polymerase chain reaction assay

DEFB4	
Forward primer	5′-GCACCTGTGGTCTCCCT-3′
Reverse primer	5′-CAGCTTCTTGGCCTCCTC-3′
Probe	FAM-TGGCTTTTTGCAGCATTTTGTTCC-MGB
RPP30	
Forward primer	5′-AGATTTGGACCTGCGAGCG-3'
Reverse primer	5′-GAGCGGCTGTCTCCACAAGT-3'
Probe	HEX-TTCTGACCTGAAGGCTCTGCGCG-BHQ1

DEFB β-defensin genes

samples from DNA digestion. Two independent runs were conducted for each sample, and CN was determined only when concordant results were obtained from two runs.

2.5 Real-Time Quantitative PCR (qPCR)

Using the same primers and probes as for ddPCR, qPCR was carried out in the same reaction mixture using Roche LightCycler 480 thermocycler under the same reaction conditions as for ddPCR. DEFB CN was inferred using the ΔΔCp method using samples with CNs of 2 as calibrator. Independent replicate runs were also performed for each sample.

2.6 Statistical Analysis

Statistical analyses were conducted in Excel, and plotting was performed using GraphPad Prism 7.0 for Windows (GraphPad Software, San Diego, CA, USA). The coefficient of variation (CV) for intra- and inter-assay variation analysis was calculated using means ± standard deviations.

3 Results

3.1 Performance of ddPCR Assay for β-Defensin Genes (DEFB) Copy Number Determination

In a successful measurement, the positive and negative droplets were clearly separated in both *DEFB4* and *RPP30* detection channels (Fig. 2a–d). Typically, four populations of droplets in a two-dimensional plot were observed in this duplex ddPCR assay (Fig. 2e). *DEFB4* and *RPP30* concentrations were calculated to infer DEFB CN (Fig. 3a). Samples with CNs from 2 to 9 as previously determined with MLPA were used as a preliminary test of the reliability of the ddPCR assay. All eight samples showed concordant CNs (Fig. 3b).

3.2 Optimization of DNA Template Amount for ddPCR

The DNA template amount is critical for the calculation of absolute nucleic acid concentration in a ddPCR assay [34, 35]. To find out the suitable DNA template amount, DNA input were tested from 80 to 2.5 ng by a serial of one to two dilutions of eight samples with CNs from 2 to 9. PCR reaction with 20 ng input DNA was found to be sufficient to obtain the best result (although 40 ng DNA template also provided correct CN results). From our experiment, either high (80 ng) or low (10, 5, and 2.5 ng) amounts of DNA template led to ambiguous (values between 0.3 and 0.7 around the integer number) or incorrect CNs (Fig. 4). High template input (80 ng) resulted in too few negative events for *DEFB4* and/or *RPP30* genes (Fig. 1a in the electronic supplementary material [ESM]), especially for high CN samples. In contrast, low template input (≤ 10 ng) led to ambiguous or discordant CNs for both low and high CN samples because there were too few positive events for target and/or reference genes (Fig. 1b in the ESM).

3.3 Evaluation of Assay Reproducibility

To evaluate the reproducibility of the ddPCR assay, 31 RC samples with CNs from 2 to 9 were tested in triplicate in the same run and in singleplex assay in three independent runs for intra-assay and inter-assay variation, respectively. Concordant integer CNs were obtained among inter- and intra-assay repeats. Moreover, CVs for both intra- and inter-assay were < 0.04 (Table 2).

3.4 Validation of the ddPCR Assay in a European Population

To further validate the reliability of the ddPCR assay in a random population, DEFB CNs of all 283 samples from healthy blood donors were determined by ddPCR. To increase the reliability of the data, independent duplicates were run for each sample, and concordance between duplicates was regarded as successful determination. Eventually, concordant integer CNs between duplicates were obtained for all samples, indicating that the call rate for CNs was 100%. In addition, CNs determined by ddPCR were completely identical to those determined using MLPA (see Table 1 in the ESM). The excellent congruence of the two methods was also shown in Bland–Altman plots as the ratio was always equal to one (Fig. 5). The raw data for CNs clustered around the integer number and clear intervals between neighboring CNs were shown in a scatter plot as gaps (Fig. 6). Figure 7 shows the frequencies of the CN distribution patterns.

3.5 Quantification of DEFB CN by qPCR

Using the same primers, probes, and working conditions, we conducted qPCR with 48 samples. Inconsistent CNs between replicates were observed for 24 samples (50%). For eight samples (16.7%), the CNs were underestimated compared with ddPCR, although consistent results were obtained from replicates. Moreover, qPCR tended to underestimate CNs, especially for samples with more than four CNs (54.1%); undercounting by two copies was also observed. Only 15 samples (31.3%) with consistent CNs between replicates showed concordant results with ddPCR (see Table 2 in the ESM).
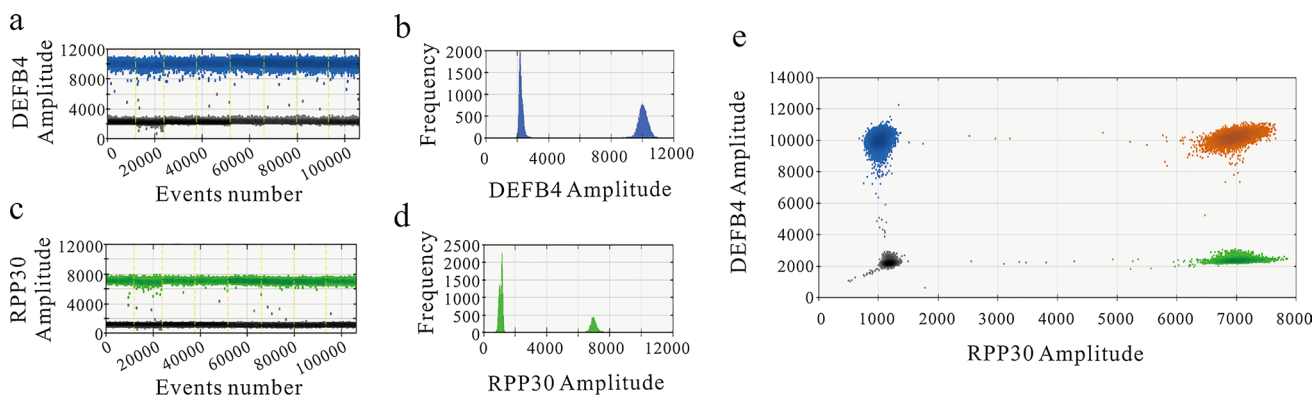
Fig. 2 Droplets plot of the digital droplet polymerase chain reaction assay. Eight samples with copy numbers from 2 to 9 previously determined using multiple ligation-dependent probe amplification were used. **a–d** One-dimensional amplitude plot. **a** *DEFB4* positive and negative droplets are presented as blue dots and gray dots, respectively; **b** the corresponding histogram. **c** *RPP30* positive and negative droplets are presented as green dots and gray dots, respectively; **d** the corresponding histogram. **e** Two-dimension amplitude plot. Gray shading indicates double negative; blue shading indicates FAM-positive; green shading indicates HEX-positive; orange shading indicates double positive

Fig. 3 Quantification results of the digital droplet polymerase chain reaction (ddPCR) assay. The same eight samples with copy numbers (CNs) from 2 to 9 were used. **a** Concentrations of *DEFB4* (blue circles) and *RPP30* (green circles). The left vertical axis indicates *DEFB4* concentration. The right vertical axis indicates *RPP30* concentration. The absolute concentrations are labeled beside the circles. The error bars represent the 95% Poisson confidence interval. **b** CN determined by ddPCR. The error bars represent the 95% confidence interval of the calculated raw CN
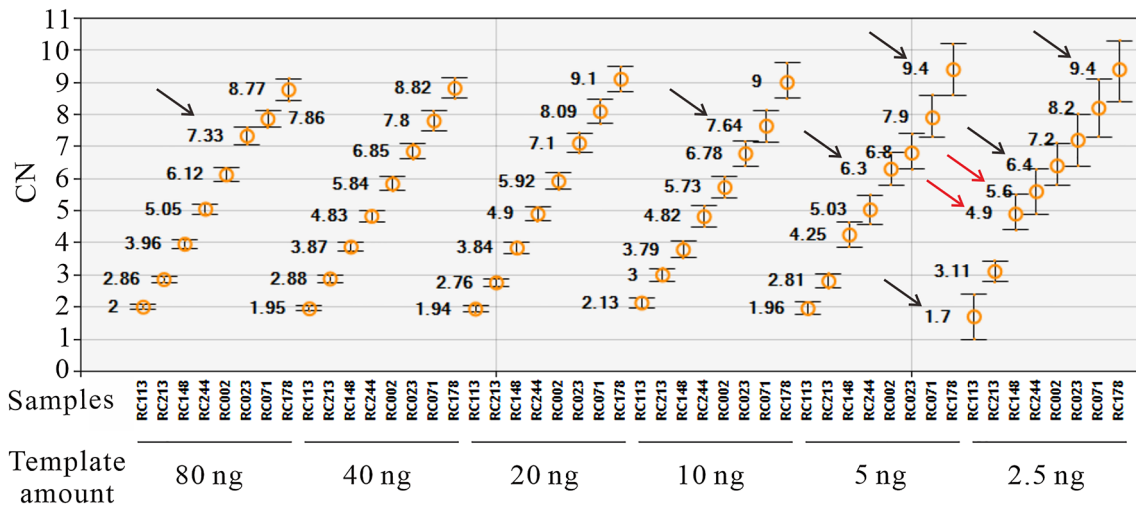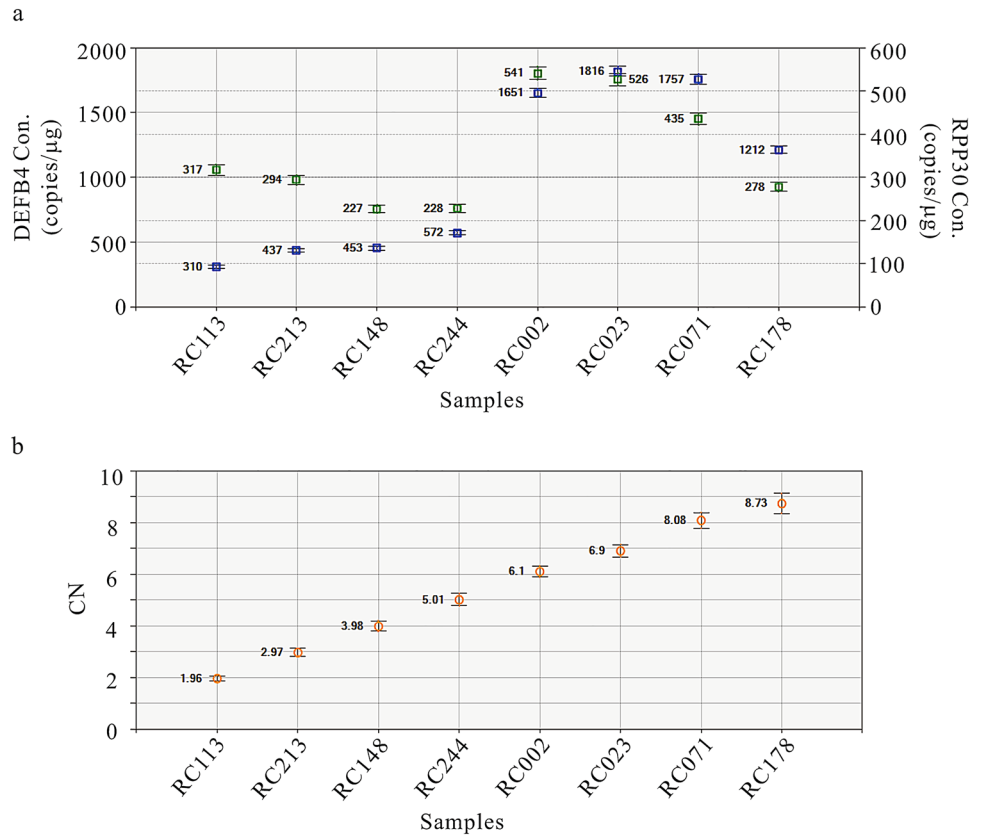
Fig. 4 Effect of template amount on β-defensin genes (DEFB) copy number (CN) determination in the digital droplet polymerase chain reaction (ddPCR) assay. The same eight samples with CNs from 2 to 9 and template amounts of 80, 40, 20, 10, 5, and 2.5ng were tested. Yellow circles represent inferred CN; bars indicate the 95% confidence interval of the raw CNs. Black arrows indicate ambiguous CN values; red arrows indicate inconsistent CN values

4 Discussion

In the present study, we developed a new assay for DEFB CN determination using ddPCR. We also found that, with a call rate of 100%, ddPCR could accurately and reproducibly determine DEFB CNs. Validation of ddPCR in 283 samples of healthy blood donors showed integer CNs consistent with those obtained with MLPA.

The reliability and high reproducibility of our ddPCR assay for DEFB CN determination could be attributed

Table 2 Intra- and inter-assay variation

| CN	Intra-assay variation			Inter-assay variation		
	Mean	SD	CV	Mean	SD	CV
2	1.99	0.05	0.03	1.97	0.06	0.03
3	2.99	0.07	0.03	3.01	0.06	0.02
4	3.93	0.08	0.02	3.94	0.08	0.02
5	4.94	0.08	0.02	5.01	0.09	0.02
6	5.94	0.15	0.02	5.93	0.13	0.02
7	6.90	0.15	0.02	6.97	0.15	0.02
8	7.87	0.10	0.01	7.93	0.11	0.01
9	9.00	0.14	0.02	9.13	0.20	0.02

CN copy number, *CV* coefficient of variation, *SD* standard deviation
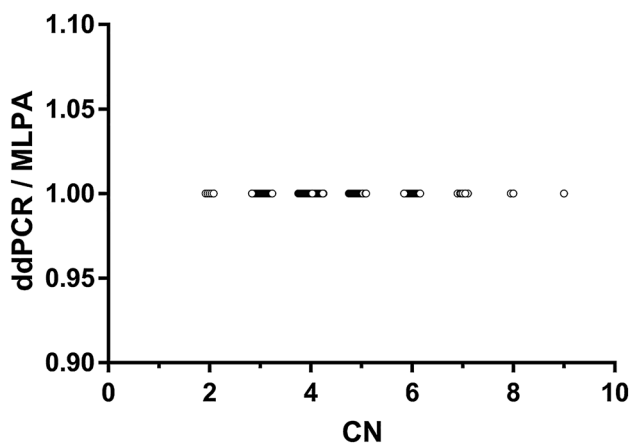
Fig. 5 Bland–Altman plot for comparison between digital droplet polymerase chain reaction (ddPCR) and multiple ligation-dependent probe amplification (MLPA). The plots show the ratio of the two methods against their mean. The dots were offset to allow individual measurements to be distinguished
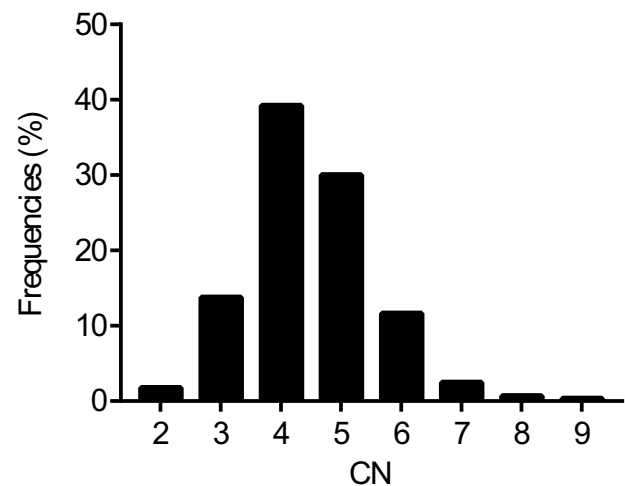
Fig. 7 β-Defensin genes (DEFB) copy number (CN) distribution in 283 individuals of European ancestry. DEFB CNs were determined by digital droplet polymerase chain reaction
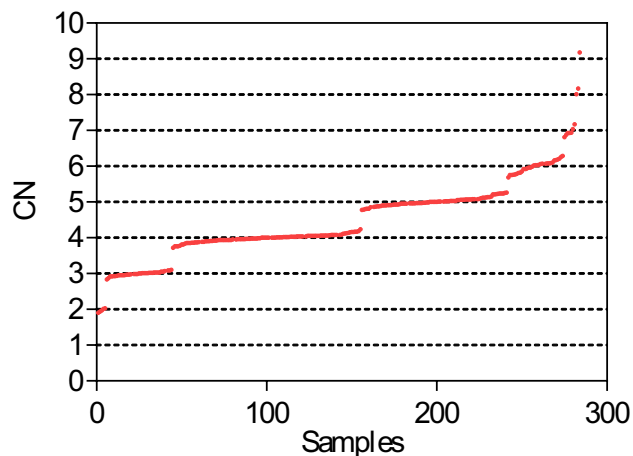
Fig. 6 Scatter plot of raw copy number (CN) values of 283 samples determined by digital droplet polymerase chain reaction. Raw CN values were plotted in ascending order

to the following. First and most important, ddPCR is an endpoint but not real-time quantification method. After PCR amplification, droplets are assigned as either positive or negative in a binomial way, and nucleotide acid concentration is further calculated using Poisson statistics. For positive droplets, defined as fluorescent signals above the threshold, no perfect amplification efficiency is needed [19, 20, 36]. This feature makes ddPCR much more tolerant to inhibitory factors, which can decrease the amplification efficiency [37–39]. Second, the primers and probes were designed to target the second exon of the *DEFB4* gene, which is a sequence conservative region. It has been reported that perfect binding of the primer and probes to template is essential to reduce the intermediate droplets between the positive and the negative (also called rain) [40, 41]. As shown in our results, a clear separation between positive and negative clusters (little or even

no rain) ensured accurate CN inference. It is reasonable that single nucleotide variations (SNVs) in primer- or/and probe-binding sites of the target gene may lead to unsuccessful amplification or/and indication of some droplets. Consequently, they can cause underestimation of nucleotide acid concentration for the target gene and eventually undercount the gene CNs. For this reason, primer- and probe-binding sites must be checked using tools such as NCBI Variation Viewer to avoid SNVs before starting the assay. Third, we used the optimal DNA template amount in the ddPCR assay. The theoretical and calculation fundament of ddPCR also has limitations when the percentage of positive or negative droplets are at their extremes [18]. As tested in our ddPCR assay, 80 ng DNA input showed an extremely high percentage of positive droplets for the *DEFB4* gene and caused ambiguous gene CN determination, especially for high CN samples (nucleic acid concentration of target gene increases fold-wise after enzyme digestion) (Fig. 4; Fig. 1a in the ESM). Meanwhile, Härmälä et al. [42] also found that a high DNA template amount could lead to ambiguous CN determinations using ddPCR for the haptoglobin gene. We think that too high nucleic acid concentrations of the target gene could lead to nearly saturated positive target droplets and consequently affect CN calculation. Conversely, low template amounts (10, 5, and 2.5 ng) led to a too low proportion of positive droplets for *DEFB4* and/or *RPP30* gene(s) and also affected the correct interpretation of target and/or reference gene(s) concentrations (Fig. 1b in the ESM). Accordingly, the CNs cannot be calculated correctly (Fig. 4). A wide range of template amounts, from 7 to 80 ng was reported for determining different gene CNs [29, 31, 41, 42]. Considering our results (20 ng), we speculate that the optimal DNA template amount is gene and assay specific. Therefore, we suggest that the template amount be tested before applying a new ddPCR assay for CN determination.

ddPCR has advantages over MLPA for DEFB CN determination. As reported in our previous work, compared with the paralog ratio test and qPCR, MLPA is the most reliable and accurate method for DEFB CN determination [16, 17]. Here, we showed that results obtained with ddPCR are as accurate and reproducible as those with MLPA. The intra- and inter- assay variations of ddPCR were low, as indicated by small CVs (< 0.04), which is comparable to that with MLPA in our previous study [17]. However, ddPCR is easier to establish and less time- and labor-intensive because fewer experimental procedures and calculation steps are needed. For this reason, it may be widely applied for large population screening. In addition, a lower template amount is needed for ddPCR. This feature is important in clinical settings with limited experimental material. Moreover, ddPCR is more tolerant to low-quality (and even degraded) DNA templates

Table 3 Comparison between digital droplet polymerase chain reaction and multiple ligation-dependent probe amplification

Attribute	ddPCR	MLPA
Accurate	√	√
Reproducible	√	√
Easy to establish	√	
Easy to infer CN	√	
Time saving	√	
Labor saving	√	
Small DNA template	√	
Tolerant of DNA quality	√	
Cheap	√	

CN copy number, *ddPCR* digital droplet polymerase chain reaction, *MLPA* multiple ligation-dependent probe amplification

because the amplicons are short and the PCR efficiency is not as important. In contrast, MLPA is quite sensitive to the quality of the DNA template, which could affect hybridization and subsequent amplification. Lastly, ddPCR is less expensive to use than MLPA (at ~ $US1 vs. ~ $US20 per assay, respectively). Table 3 summarizes a comparison of ddPCR and MLPA.

ddPCR is even more advantageous over qPCR for DEFB CN determination. In the present study, we showed that qPCR could underestimate DEFB CNs and that CN results were unreproducible even when we used the same reagents and reaction conditions as in ddPCR. The major difference in experimental procedures is the generation of microdroplets in ddPCR, through which PCR reaction is divided into thousands of parts and PCR is performed in independent compartments. In this manner, ddPCR may suffer from less competition for materials, including templates, primers, probes, and polymerase. In contrast, this competition cannot be avoided in qPCR so could cause dropout of some copies, especially in high CN samples. The other major difference is the calculation algorithm for CN in which PCR efficiency is less dependent for ddPCR. However, qPCR depends on PCR efficiency and cycle threshold values to infer template concentrations. Thus, qPCR is more sensitive to PCR inhibitors.

5 Conclusions

This study showed that ddPCR is an accurate, reproducible, easy-to-use, cheap, high-throughput method for DEFB CN determination. ddPCR could be applied to DEFB CN quantification in large-scale case–control studies.

Declarations

Conflict of interest Tingting Wen, Xianghong Zhang, Christoph Lippuner, Marcel Schiff, and Frank Stuber have no conflicts of interest that are directly relevant to the content of this article.

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of the Canton of Bern (No. KEK 041/09).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication Participants signed informed consent regarding publishing their data.

Availability of data and material All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability Not applicable

Author contributions FS initiated and coordinated the study. XZ designed the study. WT, MS, CL and XZ performed the experiments. WT and XZ analyzed the data and drafted the manuscript. CL and FS revised the manuscript. All authors read and approved the final manuscript.

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