Accurately Detection of A-Globin Gene Copy Number Variants With Two Reactions Using Droplet Digital PCR

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Research Article

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Accurately detection of α-globin gene copy number variants with two reactions using droplet digital PCR

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Abstract

Background: α-thalassemia, with carrier rates of 11.31% and 17.55% respectively in Guangdong and Guangxi province, is a highly prevalent disease in Southern China and tropical and subtropical regions and is mainly caused by deletion in α-globin gene (*HBA1* and *HBA2*). The clinical manifestation of α-thalassemia is highly correlated with the copy number of α-globin gene. The decrease of copy number results in α-thalassemia, while duplication or triplication compounded with β-thalassemia may aggravate the clinical manifestation. However, the usual methods we used to measure the copy number variants can only detect the three common types: $--^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, which may easily miss the rare deletional type and duplication or triplication cases. Therefore, it is essential to establish a new method which allows detection of different copy number variants in α-globin genes, including deletion, duplication and triplication simultaneously and accurately.

Methods: 428 peripheral blood and fetal chorionic villus or amniotic fluid samples were recruited in this study. We used a pair of primers and two probes to perform droplet digital PCR.

Results: By performing only two reactions, we accurately detected the copy number variants in α-globin genes, including the common form α-thalassemia, triplications such as $\alpha\alpha\alpha^{anti4.2}$ and trisomy 16. The accuracy rate for detecting the copy number of α-globin genes can up to 100%.

Conclusions: In conclusion, droplet digital PCR served as an accurate and rapid method for copy number variation detection in clinical screening for α-thalassemia.

Keywords: droplet digital PCR; α-globin gene; copy number variants; deletion; triplication.

Declarations

Funding

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Conflict of interest

The authors declare no competing financial interests.

Availability of data and material

All the data in this study were available in the figures and tables in the manuscript.

Author contributions

X.B. and J.M. performed the assays, analyzed data and wrote the manuscript; D.Q., X.Z., J.W. and C.Y. collected the samples and performed the confirmed assays. L.Z. and L.D. designed the study and revised the article. All authors reviewed, edited, and approved the version to be submitted.

Ethics approval

This study was approved by the Ethics Committee of Guangdong Women and Children Hospital and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Consent to participate and for publication

The patients agreed to participate and be publicized in this study and signed the informed consent.

Background

α-thalassemia is one of the most common inherited disorders all around the world[1] and mainly occurs in tropical and subtropical regions, including southern China with a high carrier rate of 11.31% and 17.55% respectively in Guangdong and Guangxi province[2, 3]. α-thalassemia is due to the deletion or non-deletion in α-globin cluster, which located in chromosome 16. The α-globin cluster has 7 genes arranged as follow: 5'-ζ2-Ψζ1-Ψα2-Ψα1-α2-α1-θ-3'. α2 (*HBA2*) and α1 (*HBA1*) is highly homologous, except for the sequences in +55 and +119 sites in the second intron, in which the sequences are T and G in *HBA2* and G and CTCGGCCC in *HBA1* in +55 and +119 sites respectively. The clinical manifestations of α-thalassemia mainly depend on mutation in the α-globin cluster. The most common α-thalassemia mutations in southern China are caused by deletion in α-globin genes, including $--^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$. Homozygous of $--^{SEA}$ will result in Hb Bart's hydrops fetalis and lead to death during gestation or in several hours after birth whereas $--^{SEA}$ deletion heterozygous compounded with $-\alpha^{3.7}$ or $-\alpha^{4.2}$ deletion will cause Hb H disease, with which the patient may depend on irregular blood transfusion to survive. Triplication of α-globin such as $\alpha\alpha\alpha^{anti3.7}$ and $\alpha\alpha\alpha^{anti4.2}$, which compounded with β-thalassemia may aggravate the clinical manifestation, were also reported in Chinese population[4]. In addition, the carrier rate of triplication and quadruplications of α-globin is relatively not low, about 2% in southern China[5]. Therefore, it is important to detect the copy number variation (CNV) of α-globin genes while one of the parents or both are β-thalassemia carriers in prenatal diagnosis.

At present, the main methods used for the detection of copy number variation in α-globin genes include multiplex gap-PCR[6], multiplex ligation-dependent probe amplification (MLPA) [7] and real-time quantitative PCR (qPCR) [8, 9] and so on. However, these methods have certain

limitations. Although the multiplex gap-PCR is relatively low cost and does not require high equipment, it can only detect the known common deletional mutations. The variation types of α-globin gene copy number are complex and diverse, and multiple gap-PCR will inevitably lead to missed diagnosis. MLPA is mainly used for the detection and genotype confirmation of CNVs of unknown types in individual cases because of its complicated operation, long time consuming and high cost. QPCR is needed to establish standard curves and CT values for quantitative copy number analysis, rather than direct quantitative analysis.

Droplet digital PCR (ddPCR), with high throughput and easily operation, can accurately and directly detect CNV without standard curves and CT values. It stands out in CNV detection due to the using of microfluidic technology to separate each reaction mix into thousands of micro spherical droplets[10]. Detection of $--^{SEA}$ deletion by using ddPCR was established in 2013 by Watcharee Prasing from Thailand[11]. This was the first time for using ddPCR in α-thalassemia diagnosis, however, it can only detect $--^{SEA}$ deletion. In 2016, Elizabeth George from Malaysia used ddPCR for triplications and deletions detection of α-globin gene [12], in which four CNV assays were performed in duplications for each sample which includes HS-40, HBA2, HBA3.7 and HBA1 genes. Although they can accurately detect triplications and deletions of α-thalassemia, it was highly cost and reduced throughput. In this study, we established a new method by using ddPCR with only two PCR reactions to detect the CNVs of α-globin genes, which included the detection of deletions and triplications.

Methods

DNA samples

A total of 428 DNA samples with characterized genotype were recruited in this study, including

385 heterozygous and homozygous or compounded heterozygous of common deletional α-thalassemia, 25 normal samples, a rare deletional α-thalassemia --^{THAI} compounded with -$\alpha^{3.7}$, 2 $\alpha\alpha\alpha^{anti4.2}$ heterozygous and 14 trisomy 16. One sample with --^{SEA} deletion compounded with HKαα was also enrolled in this study. Informed consent was obtained from all the participants prior to the study following presentation of the nature of the procedures. The study was conducted in accordance with the Declaration of Helsinki.

Droplet digital PCR (ddPCR)

Two PCR reactions with a pair of primers and two probes specific to *HBA1* and *HBA2* respectively was performed for CNV of α-globin gene detection. The location of the primers and probes were showed in Figure 1. ddPCR was conducted as the protocol recommended by the manufacturer (ddPCR supermix for probes, 1863024, Bio-rad, USA). Each reaction contained 1× ddPCR supermix for probes (no dUTP), 250 nM primers and probes as follow: Primer F: 5'-GGTTGCGGGAGGTGTAGC-3', primer R: 5'- GTGCTCACAGAAGCCAGGAAC-3'; HBA1 probe (specific to *HBA1* gene detection assay): 5'-FAM-CCCTCGGCCCCACTGACCCTCTT-BHQ1-3'; HBA2 probe (specific to *HBA2* gene detection assay): 5'- FAM-CCTGGGCCGCACTGACCCTCTTC-BHQ1-3'. *RPP30* was served as reference gene and the specific primers and probe (RPP30 F: 5'- GATTTGGACCTGCGAGC-3'; RPP30 R: 5'- GGTTGGCCAGGCGCGAAG-3'. Probe: 5'-VIC-CTGACCTGAAGGCTCT-MGB-3'), were also added into each reaction. 5-10 ng DNA samples were added as template. Ultrapure water was added up to 20 μl for each reaction. A total of 20 μl reaction mix was loaded into a sample well of a DG8-cartridge for QX200 Droplet Generator (Bio rad), followed by 70 μl of droplet generator oil into the oil wells. After the droplet

were generated, carefully transfer droplets into a clean 96-well plate. Seal the plate using the PX1 PCR Plate Sealer at 180℃ for 5 second. Then proceeding to thermal cycling as follow: 25℃ hold for 3 minutes, 95 ℃ for 10 minutes; then at 40 cycles at 94 ℃ for 30 second, 61 ℃ for 1 minute. After 40 cycles, incubate in 98 ℃ for 10 minutes and hold at 4℃. After thermal cycling, transfer the plate in the QX200 Droplet Reader for data acquisition.

CNV analysis

To calculate the copy number, we used *RPP30* as reference gene, which has two copy numbers. Copy number were calculated as follows: CNV = (X/Y) N_{ref}. X = the concentration of target genes. Y = the concentration of *RPP30*. N_{ref} = the copy number of *RPP30* (usually 2). The error bars were 95% confidential interval of Poisson distribution.

Statistical analysis

Data are shown as mean ± SD.

Results

To detect the CNV of α-globin genes in deletional α-thalassemia, we used a pair of primers and two probes specific to *HBA1* and *HBA2* respectively to perform ddPCR. We found that ddPCR can accurately detect the CNVs in α-globin genes (Figure 2A and Table 1). 25 normal individuals with all intact α-globin genes have a copy number ratio of close or equal to two in both *HBA1* (1.95 ± 0.19) and *HBA2* (1.78 ± 0.24). 160 heterozygous for $--^{SEA}$ deletion have only one copy number for both *HBA1* and *HBA2* while 125 heterozygous for $-\alpha^{4.2}$ or $-\alpha^{3.7}$ deletion have two copy number for *HBA1* and one copy number for *HBA2*. There were one copy number for *HBA1* and 0 for *HBA2* in 50 samples with $--^{SEA}$ deletion compounded with $-\alpha^{3.7}$ or $-\alpha^{4.2}$. The homozygous of $--^{SEA}$ deletion has 0 copy number both in *HBA1* and *HBA2* while $-\alpha^{3.7}$ or $-\alpha^{4.2}$ homozygous have 2 copy number

for *HBA1* respectively and 0 copy number in both for *HBA2*. Two samples with compounded heterozygous of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ have 2 copy number for *HBA1* and 0 copy number for *HBA2*. We also found that $--^{THAI}$ compounded with $-\alpha^{3.7}$ deletion has 1 copy number for *HBA1* and 0 copy number for *HBA2* (Figure 2B). The genotypes of these samples were previously characterized by using gap-PCR or MLPA. These data showed that ddPCR can accurately detect the CNVs in deletional α-thalassemia.

Furthermore, to estimate the accuracy and sensitivity of ddPCR in triplications or multicopy genes detection, we performed ddPCR by recruiting two individuals with heterozygous for $\alpha\alpha\alpha^{anti4.2}$ and 14 individuals with trisomy 16. We observed that the $\alpha\alpha\alpha^{anti4.2}$ heterozygous had 2 copies for *HBA1* and 3 copies for *HBA2* (Figure 2C). In addition, the trisomy 16 had 3 copies for both *HBA1* (2.91 ± 0.21) and *HBA2* (2.75 ± 0.45) (Figure 3). These data indicated that ddPCR was able to detect the triplications and multicopy genes accurately and sensitively.

Discussion

α-thalassemia, mainly caused by deletion in α-globin cluster, is one of the most common inherited disease in southern China and tropical or subtropical regions. In southern China, the most common α-thalassemia deletions included $--^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$. Therefore, the routine PCR-based method to detect the CNVs mainly focus only on these three genotypes, but not for triplications such as $\alpha\alpha\alpha^{anti3.7}$ and $\alpha\alpha\alpha^{anti4.2}$, which might lead to misdiagnosis[13]. For example, individuals with $HK\alpha\alpha/--^{SEA}$ will be diagnosed as $-\alpha^{3.7}/--^{SEA}$, but they will have α-thalassemia trait hematological data (Hb > 100 g/L) instead of Hb H disease hematological data (Hb < 100 g/L). Considering the carrier rate of HKαα (0.07%) and anti-HKαα (0.02%) in Guangxi province[13], the potential of misdiagnosis cannot be ignored. In addition, even though the individual with triplications has no

clinical manifestation, it will aggravate the symptom when compounded with β-thalassemia. Therefore, it is very important and meaningful to detect triplications and quadruplications, which cannot be detected by using routine PCR-based method.

To date, many methods such as MLPA[14, 15] and next generation sequencing (NGS) [5, 16] have been developed for CNVs analysis. These methods can detect the large deletions or unknown deletions, but also have some limitations in clinical screening. MLPA is complicated operation, long time consuming and high cost. NGS needs high-end equipment and is high cost, which limits its application in clinical screening of α-thalassemia. DdPCR, with high sensitivity and time saving, has showed more advantages compared with other methods. DdPCR requires more less time. We can detect the CNVs within 4 hours from DNA extraction to data analysis by using the method showed in this study. In addition, in this new method, only two reactions are sufficient to cover the detection of deletions and triplications or quadruplications of α-globin gens, which is more suitable for clinical screening compared with the method reported by Elizabeth George from Malaysia in 2016, in which four CNV assays were performed in duplications for each sample. Furthermore, our purpose is to establish a new method by using ddPCR to cover the detection of most of the CNVs of α-globin genes to avoid or reduce the misdiagnosis or missed diagnosis in clinical screening and prenatal diagnosis. This new method can accurately and sensitively detect the CNVs of α-globin genes, by which even only 1 ng DNA can be detected.

However, there are some limitations by using ddPCR to detect CNVs. Firstly, given that there are only two channels (FAM and HEX or VIC) for the QX200 Droplet Reader, it requires two reactions in which contains probe specific to *HBA1* or *HBA2* and *RPP30* to detect the CNVs. Secondly, by using this method we can detect the CNVs of α-globin genes but cannot distinguish

the genotypes. For example, it is not possible using this method to be sure about the rare $--^{THAI}$ and $--^{FIL}$ deletions, which will appear as the same as $--^{SEA}$ deletion. However, even though this method cannot characterize the exact genotypes, it can make out primary diagnosis. One individual with Hb H disease will be detected with one copy for *HBA1* and 0 copy for *HBA2*. And the homozygous or compounded heterozygous of $-\alpha^{3.7}$ or $-\alpha^{4.2}$ will be detected with 2 copies for *HBA1* and 0 copy for *HBA2* while the heterozygous of $-\alpha^{3.7}$ or $-\alpha^{4.2}$ will be detected with 2 copies for *HBA1* and 1 copy for *HBA2*. In addition, by using this new method we can also accurately detect the copy number even with 5 or 6. Therefore, it is sufficient to use ddPCR to detect CNVs as clinical screening purpose but other methods such as gap-PCR and MLPA should be used to make confirmation test.

In summary, we have established a new method to detect the CNVs in α-globin genes by using a pair of primers and two probes to performed ddPCR in two reactions. This new method was highly sensitive, with an accuracy up to 100%.

Acknowledgement

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Figure legends

Figure 1. Schematic of *HBA* genes. E1-E3, exon 1-3. The black boxes showed the probes specific to *HBA1* and *HBA2* respectively.

Figure 2. The ratio of copy number of *HBA1* and *HBA2* in different genotypes. (a) the copy number for *HBA1* and *HBA2* in different deletional α-thalassemia. (b, c) individuals with $--^{THAI}$ compounded with $-\alpha^{3.7}$ deletion (b) and $\alpha\alpha\alpha^{anti4.2}$ heterozygous (c), respectively. Blue droplets indicated positive *HBA1* or *HBA2*, while green droplets represented *RPP30*.

Figure 3. DdPCR assay for quantification of trisomy 16. (a, b) plots showed the distribution of positive and negative droplets. Blue droplets indicated positive *HBA1* or *HBA2*, while green droplets represented *RPP30*. (c) the copy number of 14 trisomy 16. Data were showed as mean ± SD.

Figures

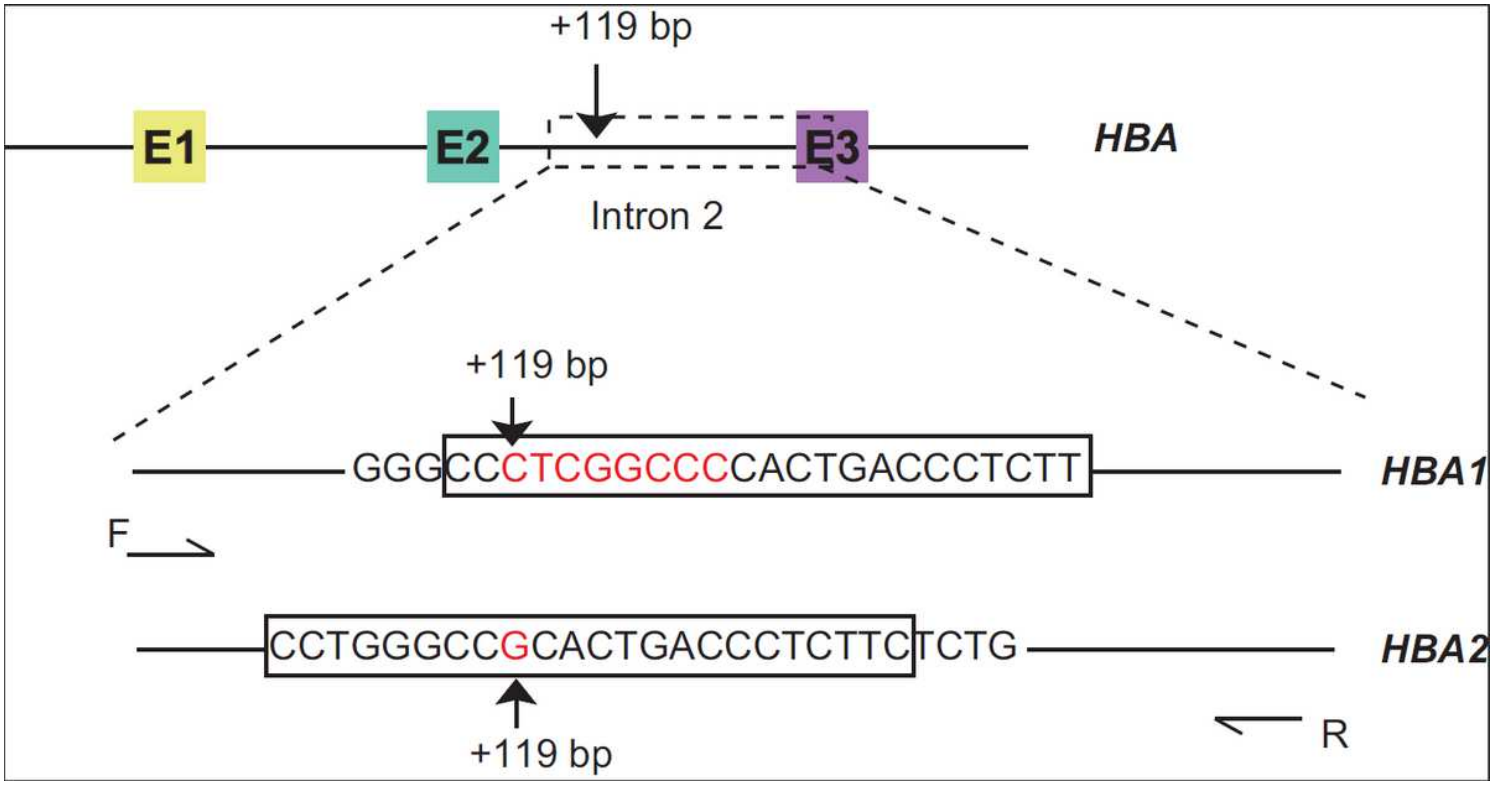

Figure 1

Schematic of HBA genes. E1-E3, exon 1-3. The black boxes showed the probes specific to HBA1 and HBA2 respectively.

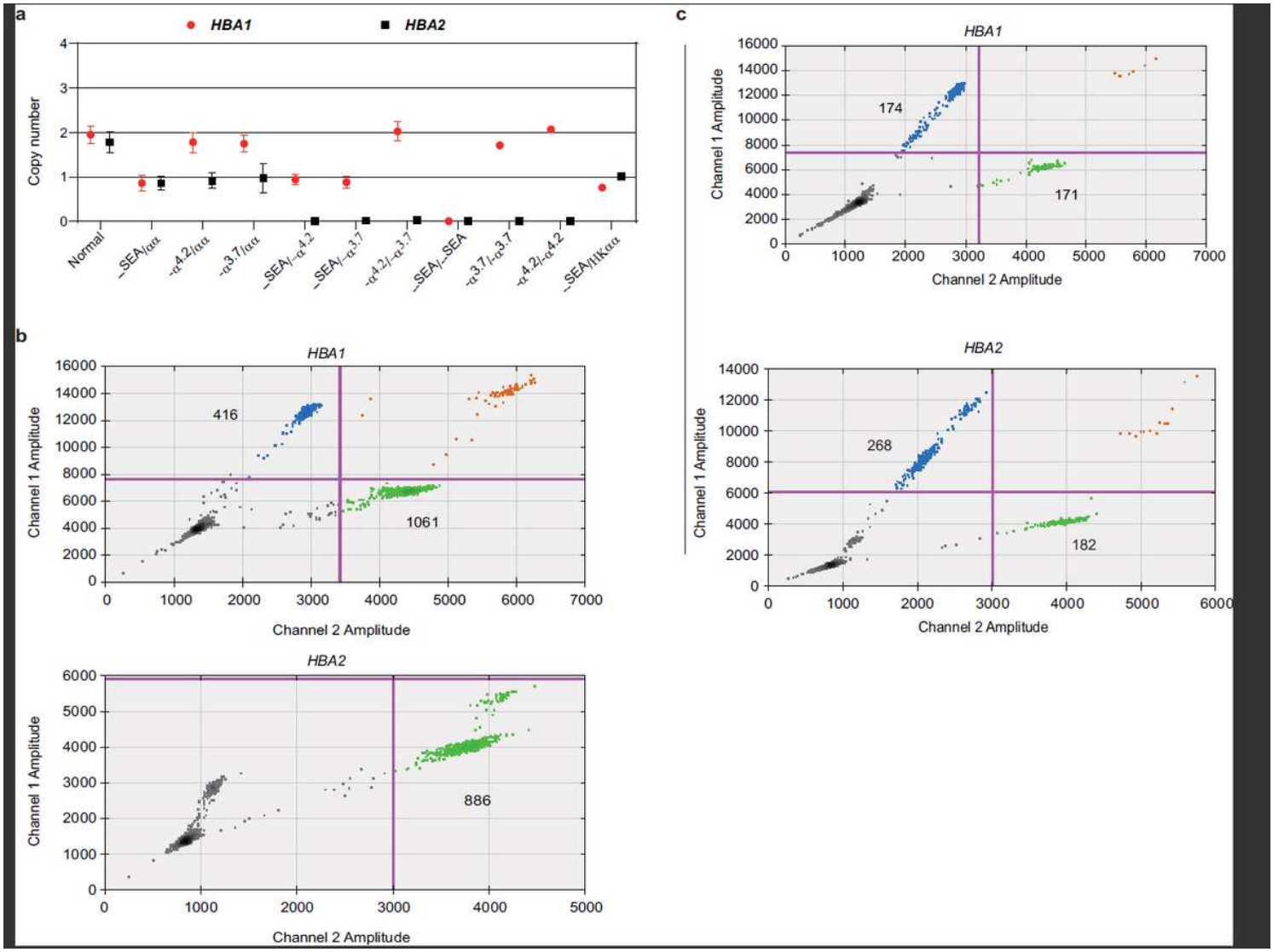

Figure 2

The ratio of copy number of HBA1 and HBA2 in different genotypes. (a) the copy number for HBA1 and HBA2 in different deletional α-thalassemia. (b, c) individuals with --THAI compounded with -α3.7 deletion (b) and ααανti4.2 heterozygous (c), respectively. Blue droplets indicated positive HBA1 or HBA2, while green droplets represented RPP30.

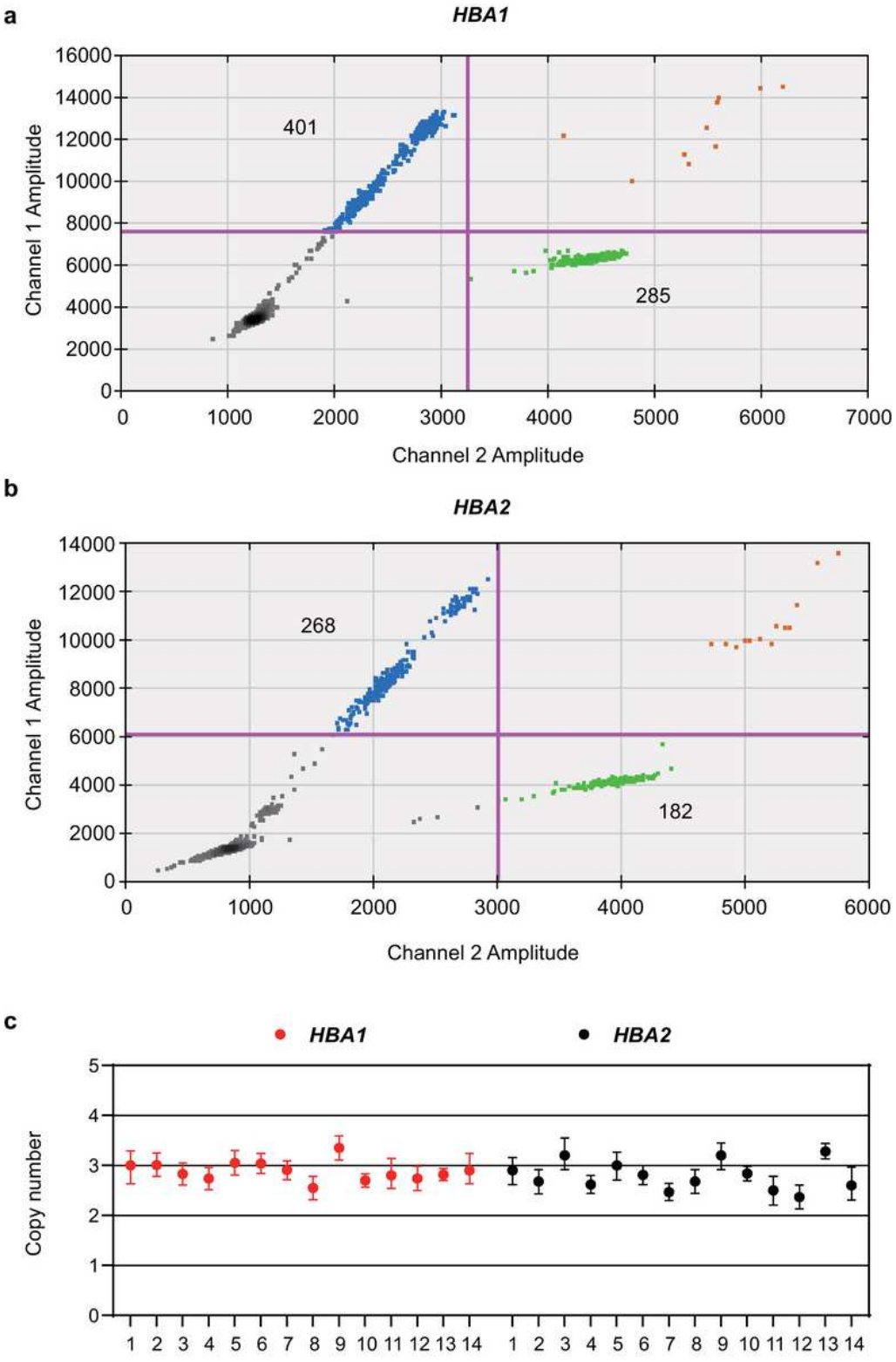

Figure 3

DdPCR assay for quantification of trisomy 16. (a, b) plots showed the distribution of positive and negative droplets. Blue droplets indicated positive HBA1 or HBA2, while green droplets represented RPP30. (c) the copy number of 14 trisomy 16. Data were showed as mean ± SD.