A comparative study of ddPCR and sanger sequencing for quantitative detection of low-frequency mutation rate

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Abstract. Mutation detection has been applied to the entire diagnostic system of prevention, diagnosis, treatment, monitoring, and prognosis. However, owing to the limit of the technology, the low-frequency mutations are often identified as the false negative. Hence, this study focused on the comparison of Sanger sequencing and droplet digital PCR (ddPCR) in the quantitative detection of low-frequency mutation rate. The drug-induced deafness was chosen as the target for comparison, and the simulated samples and 15 clinical samples were detected respectively. The difference between the two methods was comprehensively analyzed in terms of repeatability, accuracy, dynamic detection range and concordance. The results showed that Sanger sequencing took advantage of acceptable cost, new mutations’ discovery, and high-throughput detection, while ddPCR performed better, especially in the detection of low-frequency mutations. In summary, this study compares in detail the merits and demerits of Sanger sequencing and ddPCR in the quantitative detection of low-frequency mutations, offering the potential to promote the further development of technology.

1. Introduction
Mutations extensively exist in the human’s genome and are mainly comprised of four forms: base substitution, frame-shift, deletion, and insertion. Some mutations contribute to the diversity of the population and thus can be used for the researches in forensic science, molecular breeding and etc. However, many reporters have shown that there are many mutations involving single base changes\textsuperscript{[1]} are harmful to the human and can drive severe cancers or syntrophus. For example, EGFR mutations\textsuperscript{[2]} are prominent drivers of non-small cell lung cancer (NSCLC), over 70% of low-grade glioma was found to possess IDH mutations\textsuperscript{[3]}. The mitochondrial mutation 12S rRNA\textsuperscript{[4]} is the leading cause of drug-induced deafness, and so on. In brief, the identification of these disease-related mutations is essential for prevention, diagnosis, treatment and prognostic prediction of cancers\textsuperscript{[5, 6]}. Besides, prenatal diagnosis\textsuperscript{[7]} can assess the risk of offspring\textsuperscript{[8]} that suffering the inherited diseases\textsuperscript{[9]}, especially in a family with defected genes.

In addition to homogeneous mutations, heterogeneous mutations are also widely existed and play an important role in disease diagnostics and forensic identifications. For example, the rise of the intrinsic mutation rate is not necessary for carcinogenesis but related to tumor growth\textsuperscript{[10]}. The mutant frequency is positively related to the proliferation rate of the tumor. Hence, the monitoring of the variation of the mutant rate during the therapy is meaningful for the evaluation of the therapeutic effect. The mutation rate can be the guidance of clinical medication and prognosis prediction.

Many available methods derived from PCR and sequencing have been developed to detect mutations. On one hand, quantitative PCR (qPCR) is a powerful tool to calculate a mutant rate of alleles depending
on a standard curve, but the current limit of detection can only reach 0.12%[11]. Droplet digital PCR (ddPCR) is considered as the third generation of PCR technology, which is feasible to achieve absolute quantification of copy number and can reduce the limit of detection of rare mutation to 0.01%[12]. In addition, Sequencing technique can simultaneously detect multiple mutations and uncover ‘new’ mutations. Theoretically, Sanger sequencing is not easily able to detect low-frequency differences below 25%[13], and thus cannot detect low-frequency mutations. Although the ability for the detection of low-frequency mutations is limited, Sanger sequencing is determined as the “gold standard”[14-16] for mutation detection due to its high accuracy and affordable cost in the clinical setting and scientific research.

All of the abovementioned three technologies have the ability to quantify the mutation rate, but their performance is various and may suitable for different applications. Wilbe M[8] had reported that SMRT sequencing could give the hereditary information which is the origin of de novo mutations between parents, while the quantitative results of ddPCR can be used to estimate the descendant of recurrence risk in the family with genetic diseases. There are many reports[11, 12, 17, 18] have shown the different performance of qPCR and ddPCR in mutation detection. Nonetheless, the comparison between ddPCR and Sanger sequencing has rarely been reported, thus it becomes the goal of this study.

In this work, Sanger sequencing and ddPCR were used to detect mutant rates of plasmids and clinical samples, and the workflow, sensitivity, repeatability, and concordance between the two methods are compared. The mutation we chose is m.1555A>G, which is located in the MTRNR1 gene and related with hearing loss. The significance of this study is to clarify the strengths and weaknesses of Sanger sequencing and ddPCR in the detection of mutation, especially low-frequency mutation, aiming to guide the clinical application of mutation rates in cancer and heredopathia.

2. Materials and methods

2.1 Primers and probes
A 200-bp DNA sequence of MTRNR1 gene was download from GenBank (http://www.ncbi.nlm.nih.gov/genbank) to design the corresponding primers and probes by the Oligo 7 and Primer Express 3.0 software, respectively. The forward primer (5'-GCACATTCCAGTACACTTACCAT-3') and the reverse primer (5'-CGTCACCTCCTCAAGTATRACT-3') were synthesized by TSINGKE Biological Technology Corporation (Beijing, China). The wild-type probe (5'-FAM- TTACGACTTGCTTCCT-MGB-3') and mutant probe (5'-VIC- TTACGACTTGCCCTC-MGB-3') were synthesized by Invitrogen (Shanghai, China).

2.2 Plasmid construction
The wild and mutant plasmids containing a mutant base were constructed by the following procedure: First, the genomic DNA was extracted from DBSs by TIANamp Blood Spots DNA Kit (Tiangen, Beijing, China). Then, a DNA fragment containing the MTRNR1 gene was amplified from the genomic DNA of DBSs and ligated to pGM-T vector using pGM-T Ligation Kit (Tiangen, Beijing, China). Afterwards, the recombinant plasmids were transferred into TOP10 competent cells, and a blue-white spotting test was performed to select positive clones containing wild-type and mutant sequences. After culturing the positive clones for about 6 h, the genotypes of clones with wild-type and mutant alleles were verified by Sanger sequencing. Finally, the plasmid DNA was extracted by TIANprep Rapid Mini Plasmid Kit (Tiangen, Beijing, China) and linearized by Scal-HF (New England Biolabs, MA) for the followed detection.

2.3 Sample processing
Fifteen clinical samples we tested in this study were dry blood spots stored at room temperature, providing by Beijing CapitalBio Medical Laboratory (Beijing, China). Table 1 shows the detailed
information of the samples. Genomic DNA was extracted from DBSs by TIANamp Blood Spots DNA Kit (Tiangen) and quantified by Qubit 3.0 Fluorometer (Thermo Fisher Scientific, MA).

2.4 ddPCR
PCR reactions were performed in a 30-μL reaction containing 1× Probe ddPCR MasterMix (no UNG; Targeting One), 200 nM each probe, 400 nM each primer, 2 μL containing <2 ng gDNA and sterile water. Droplets were generated and analyzed using the Xinyi TD-1 Digital PCR Platform (Targeting One, Beijing, China). The PCR program is as follows: 95℃ for 10 min, 40 cycles at 94℃ for 30 sec and 56℃ for 60 sec, followed by 1 cycle at 98℃ for 10 min. The sample was incubated at 4℃ until further processing. The quantification results of the samples with wild-type alleles and mutant alleles were estimated by modeling as a Poisson distribution using ChipReader R1 software (Targeting One).

2.5 Sanger sequencing
A 102-bp DNA fragment with 1555 A>G alleles was amplified by conventional PCR following the standard manufactures. A 50-μL PCR reaction mixture contained 2 μL template DNA, 2 μL forward primer (10 μM), 2 μL reverse primer (10 μM), 1×Hieff™ PCR Master Mix (With Dye; YeasenBiotech) and 19 μL sterile water. The PCR program is as follows: Pre-denaturation at 94 ℃ for 5 min; 35 cycles with 94 ℃ for 30 sec, 56 ℃ for 30 sec and 72 ℃ for 30 sec; 72 ℃ for 10 min. Then the amplicon was purified and sequenced by TSINGKE Corporation. The sequencing results were analyzed by Chromas software.

3. Results
3.1. Process comparison
As shown in Figure 1, both the Sanger sequencing and ddPCR can calculate the mutation ratio of genes, but the principles they followed are quite different. The percentage of mutation was calculated by the following formula, in which M is the copy number of mutant alleles, W is the copy number of wild-type alleles.

\[
\text{mutation rate(%) = } \frac{M}{W+M} \times 100%
\]  

Sanger sequencing distinguishes different bases by capillary electrophoresis. Generally, the height or area of the peak represents the numbers of DNA fragments, so the ratio of the height or area of the corresponding loci roughly is approximately equal to the mutation rate of the sample. In this study, the peak height is used to calculate the mutation rate. Sanger sequencing requires a high enough sample concentration, but the DNA content of direct clinical samples often cannot meet the needs of the method. The standard solution is to amplify the target fragment exponentially by traditional PCR, but it will generate amplification preference, which is also an essential factor affecting the interpretation of the results.
Figure 1. The flowchart of two methods for mutation detection. For Sanger sequencing, the ratio of the mutated and wild-type DNA fragments is finally calculated by the peak height of different bases at the same location. For ddPCR, the ratio is finally obtained by calculating the number of positive droplets in two different fluorescence channels.

On the contrary, for the ddPCR method, a pair of probes with different fluorescence dyes are used to state the mutated and wild-type DNA fragments. First, the mixture was divided into each enclosed droplet equably except template DNA. According to Poisson distribution, each droplet contained only 0 or 1 template DNA molecule. After amplification, droplets were classified by the intensity of the fluorescence signal. The mutation rate of samples could be calculated by counting the number of different fluorescent droplets. The method does not have amplification preference since each droplet is an independent reaction unit. In short, ddPCR is a digital and accurate detection method which is not needed for a standard curve.

Besides, compared with Sanger sequencing, ddPCR is fewer sample requirements, less time-consuming, and lower cost of the machines. However, the throughput of ddPCR is lower (8 v. s. 96), of which consumables are more expensive. Moreover, it can only detect known mutations, and the reaction system is limited by using probes. In short, ddPCR is superior to Sanger sequencing in performance, but its cost is higher.

3.2 Stability analysis
To compare the performance of these two methods, a pair of plasmids containing a different base was prepared as the simulation samples for testing. The plasmids were named as 1555A and 1555G, respectively. The property of systems was evaluated in two aspects: stability and dynamic detection range. First, the steadiness of the system was determined by the intra-group repeatability of the test results. After the quantification and gradient dilution, plasmids were mixed proportionally to produce simulated samples with a mutation rate of 30% and 70%. The final concentration of the samples was 2 ng/μL. Two methods were used to detect the above samples successively.
Figure 2. The comparison of the repeatability of Sanger sequencing and ddPCR. In the graph, every point represents one detection, the horizontal line in the middle represents the mean, and the error bar represents the standard deviation. The mutation rates are 30% and 70% for the results with dark and grey dots, respectively.

Figure 2 displays the results of repetitive experiments, and the error bar refers to the error value, which is equal to the maximum absolute value of the difference between each data and the average. The repeatability of two methods within-run was available within an acceptable error range. It was obviously observed that the error bar of ddPCR group was smaller than that of Sanger sequencing group, which manifested that the stability of ddPCR was better than Sanger sequencing. In the meantime, the relative error caused by measurement can reflect the accuracy of the results. For example, in the experimental group with the theoretical mutation rate of 30%, the average value of ddPCR detection results was 29.85%, and the relative error was -0.5%; while that of Sanger sequencing detection results was 17.22%, and the relative error was -42.6%. In brief, the repeatability and accuracy of Sanger sequencing measurement were inferior to ddPCR. Therefore, as a detection tool for mutation rate quantification, ddPCR was more reliable than Sanger sequencing.

3.3 Analysis of dynamic detection range and concordance

To further compare the dynamic detection range and concordance of the two methods, the plasmids were mixed to prepare several standards with different mutation rates. The 1555A and 1555G plasmids were quantified and diluted to 10,000 copy number per microliter by the methods mentioned above, and then the two standard solutions were mixed in the proportion of 0%, 5%, 10%, 30%, 50%, 70%, 90%, 95%, and 100%. After samples preparation, two methods were used to detect the above samples successively.
Figure 3. The linear fitting curves of the two methods. The gradient of the theoretical mutation rate is 0%, 5%, 10%, 30%, 50%, 70%, 90%, 95% and 100%. Linear fitting curve based on standard dilution coefficient by Sanger sequencing and ddPCR are shown in the (A) and (B), respectively. (C) illustrated the concordance between the two methods to detect mutation rate expect 5% and 10%.

Figure 3A and 3B describe the detection results of Sanger sequencing and ddPCR, respectively. In Figure 3A, the detection results of 5% and 10% of experimental groups were wild type by Sanger sequencing measurement, not in line with expectations. The possible reason for Sanger sequencing to get false negative was these mutation rates are beyond the detection capability of this method. Besides, the accuracy of the results of other dilution factors was inadequate. It was the absence of false negative and the lack of accuracy that the determinant coefficient $R^2$ equal to 0.9735. However, as shown in Figure 3B, the relative error of each set of data obtained by ddPCR measurement was minimal, and the determination coefficient $R^2$ was equal to 0.9996. In short, the linearity of results detected by ddPCR was better than that of Sanger sequencing.
Figure 3C represents the concordance analysis of the results detected by the two methods in every standards dilution factor, excluding the two groups of 5% and 10% with the false negative by Sanger sequencing method. As is shown in 3C, the abscissa represents the results of Sanger sequencing, and the ordinate represents that of ddPCR. In the range of 30% to 100%, the two methods were consistent in genotyping and different in quantification analysis, and other gaps could be the excess of the sensitivity of Sanger sequencing.

Many researches[13] have indicated that the limit of the detection (LOD) by Sanger sequencing is 25%, which was consistent with our results. Beyond that, for the identification of homozygote phenotype, the results obtained from the two methods were accordant. In other words, within the available sensitivity of the two methods, there was no difference between the two test results, and there was no comparability out of the detection range. However, ddPCR had a higher dynamic detection range than Sanger sequencing, and its accuracy in mutation rate quantification was more superior.

3.4 Real samples detection

The performance of the two detection methods was compared in detail using the simulated samples prepared by the plasmids in the previous section. Next, 15 dried blood spot specimens from newborns were used to explore the differences in practical applications (details in Table 1). DNA was extracted and quantified to control the quality of samples. Five of the fifteen samples were below 1 ng/μL, which did not affect the detection of the two methods. For ddPCR, the input of the sample was controlled to below 2 ng/μL to prevent the copy number of DNA from exceeding the number of droplets, avoiding the accuracy of the quantitative results. For Sanger sequencing, the input of the sample was not controlled because all samples were amplified by PCR to meet the requirement of sequencing.

The results of the two methods were consistent between the eight wild-type samples, while the mutant samples were different. Among the two samples with low-frequency mutations, the results of ddPCR were 0.08% and 13.58% separately, while Sanger sequencing showed no variation. For the specimen 5 with the high-frequency mutation, the result of ddPCR was 98.63%, while Sanger sequencing showed the homozygotic mutation. The four remaining samples with high-frequency mutations were consistent in genotyping, but Sanger sequencing gave lower quantitative results than ddPCR.

In general, compared to the Sanger sequencing method, ddPCR has the superiority of more extensive detection range and more accurate quantitative results in the application of gene mutation rate detection.

| Sample | Age   | Type | Concentration (ng/μL) | ddPCR(%)       | Sanger sequencing(%) |
|--------|-------|------|-----------------------|----------------|----------------------|
| 1      | Newborn | DBS  | 0.286                 | m.1555A>G(51.31) | m.1555A>G(26.96)    |
| 2      | Newborn | DBS  | 0.240                 | m.1555A>G(13.58) | Normal(hom)         |
| 3      | Newborn | DBS  | 1.26                  | m.1555A>G(98.38) | m.1555A>G(83.26)    |
| 4      | Newborn | DBS  | 0.878                 | m.1555A>G(98.68) | m.1555A>G(78.60)    |
| 5      | Newborn | DBS  | 0.170                 | m.1555A>G(98.63) | Abnormal(hom)       |
| 6      | Newborn | DBS  | 0.152                 | m.1555A>G(98.66) | m.1555A>G(88.35)    |
| 7      | Newborn | DBS  | 2.26                  | m.1555A>G(0.08)  | Normal(hom)         |
| 8      | Newborn | DBS  | 2.2                   | Normal(hom)     | Normal(hom)         |
| 9      | Newborn | DBS  | 4.44                  | Normal(hom)     | Normal(hom)         |
| 10     | Newborn | DBS  | 2.12                  | Normal(hom)     | Normal(hom)         |
| 11     | Newborn | DBS  | 2.08                  | Normal(hom)     | Normal(hom)         |
| 12     | Newborn | DBS  | 2.42                  | Normal(hom)     | Normal(hom)         |
| 13     | Newborn | DBS  | 2.82                  | Normal(hom)     | Normal(hom)         |
4. Conclusions

Gene mutations are ubiquitous in healthy humans and patients. However, most genetic mutations are unfavorable and could lead to the formation of tumors or the occurrence of hereditary diseases. Therefore, it is of considerable significance and full application for the qualitative and quantitative analysis of mutant genes in basic medical research and clinical practice.

In the case of tumors, lung cancer is the top of morbidity and mortality worldwide\(^2\). Many studies\(^5,6\) have shown that NSCLC patients cancer cells are often observed with EGFR dysfunction. It is the driving gene mutation, and the over-activation of EGFR activity this brings, which explains promoting the lung cancer course. Clinical trials\(^1,6\) have demonstrated new anti-tumor drugs such as gefitinib and erlotinib are highly responsive to patients with sensitive EGFR-mutant NSCLC, compared with standard chemotherapy. Hence, the precise surveillance of EGFR mutations is a crucial role in the EGFR mutation-positive NSCLC patients. In the case of monogenic disorder, drug-induced deafness ranks third in the Chinese deaf population, with an incidence of 3.8% of 1555A>G. Only after aminoglycoside-sensitive families used drugs such as aminoglycosides can they suffer from disabling irreversible hearing loss. And the degree of hearing impairment is positively correlated with the defected gene mutation rate. As a consequence, the precise detection of mitochondrial gene mutations is significant on neonatal screening and progeny phenotype prediction in genopathy.

For the quantitative detection of low-frequency mutations, Sanger sequencing has been active in the laboratory and hospital, while ddPCR attracts more attention in the absolute quantitative capability. This study focused on the property comparison of the two methods (details in Table 2). The simulated samples prepared by a pair of plasmids containing one point mutation site and 15 real clinical DBSs were used to support the study, the results indicated that ddPCR had better performance than Sanger sequencing.

More specifically, ddPCR has the following advantages than Sanger sequencing in the detection of low-frequency mutations. (1) Lower requirements for samples: several copy numbers is enough for ddPCR to gain the absolute quantification results, which can meet the detection of rare specimens. (2) Better performance: ddPCR took advantage of accurate quantification of low-frequency mutation samples (<25%), while Sanger sequencing results were false negatives. Briefly, ddPCR had more extensive dynamic detection range, better reproducibility, and accuracy. (3) Fool-style operation: it only takes a quarter of the consuming time of Sanger sequencing to finish the process, and the platform is more comfortable to train operators. (4) Free interference: ddPCR has tens of thousands of independent reaction chambers to avoid endogenous signal crosstalk ideally. However, as the limitations of low-

|   | ddPCR | Sanger sequencing |
|---|---|---|
| Sample capacity | Several copy number | >0.5 μg |
| Detection range | >1% | >25% |
| Reproducibility | Stable | Unstable |
| Period | ~6 h | ~24 h |
| Platform | Easy | Complexed |
| Amplification preference | No | Yes |
| Cost for per sample | >5 $ | ~2 $ |
| Detection throughput | 8 | 96 |
| Mutation site | Known | Known and unknown |
throughput, high-cost and mutation sites, Sanger sequencing is a better choice in precise genotyping and new mutations discovery for the samples with a mutation rate over 25%.

In summary, this study comprehensively compares the advantages and disadvantages of Sanger sequencing and ddPCR in the detection of low-frequency mutations from four aspects: accuracy, repeatability, dynamic detection range, and consistency. The results showed that ddPCR has better stability and dynamics than Sanger sequencing. In practical applications, when genotyping and exploring unknown mutation sites, Sanger sequencing should be preferred. Although the cost of ddPCR is unaffordable, it still plays an essential role when precise quantification, too little samples, and low-frequency mutation rate. This comparison provided a reference in the application of Sanger sequencing and ddPCR, expecting to promote the further development of precise detection technologies.

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