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ORIGINAL ARTICLE

Digital polymerase chain reaction quantification of hematopoietic chimerism by insertion/deletion: A personalized selection for different chimerism status

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Abstract

Background: Clinical decision-making after allogeneic stem cell transplantation (HSCT) is partially based on hematopoietic chimerism analysis. Short tandem repeat (STR), the current gold standard for quantitative chimerism analysis, has limited sensitivity. Digital polymerase chain reaction (dPCR) offers precise quantification and high reproducibility with excellent sensitivity (usually ≤0.1%) across a wide measurement range. However, the reported dPCR-based chimerism detection methods were developed in non-Chinese cohorts and may not be directly applicable to the Chinese population.

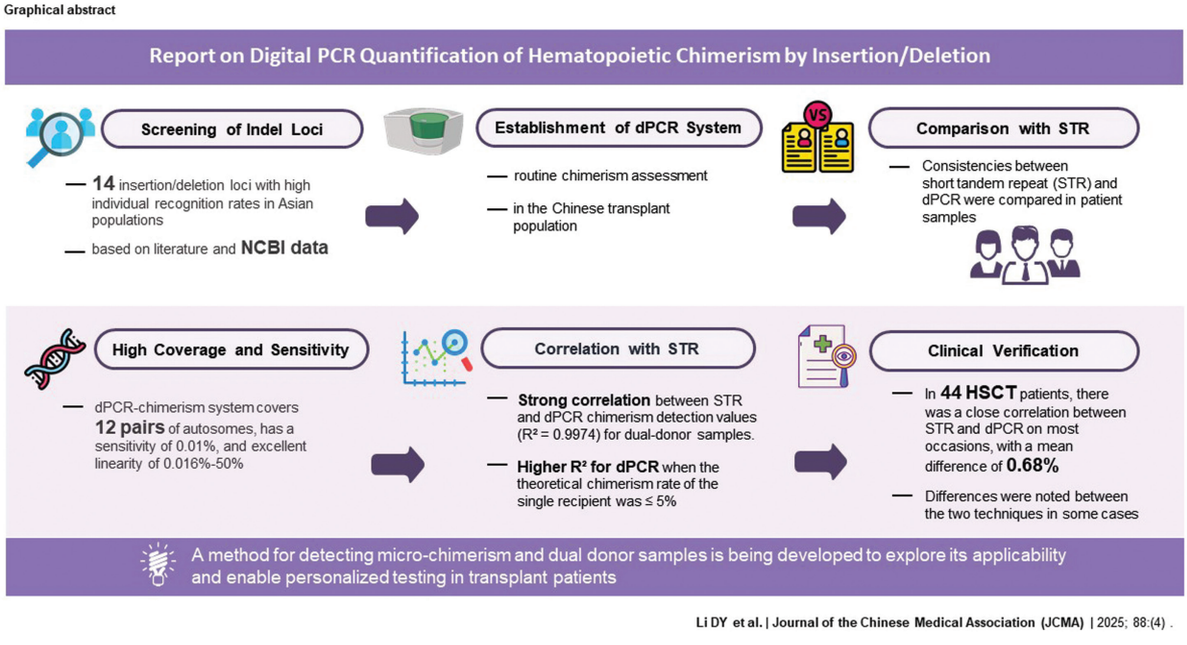
Methods: To improve sensitivity and accuracy, we first screened out 14 insertions/deletions (indel) loci with high individual recognition rates in Asian populations based on literature and NCBI data. Then, we established a dPCR detection system for routine chimerism assessment ("dPCR-chimerism system") in Chinese transplant recipients. We compared the concordance between STR and dPCR in patient samples.

Results: The newly developed dPCR-chimerism system covers all 12 pairs of autosomes, achieves a sensitivity of 0.01%, and demonstrates excellent linearity from 0.016% to 50%. For dual-donor samples, there was a strong correlation between STR and dPCR-chimerism detection values (R^2 = 0.9974). The R^2 of the dPCR results was higher than STR when the theoretical chimerism rate of the single recipient was ≤5%. Clinical validation in 44 HSCT patients showed strong overall concordance between STR and dPCR (mean difference: 0.68%), although discrepancies were noted in some cases.

Conclusion: Our newly developed system demonstrates excellent repeatability and sensitivity, particularly in detecting. It is expected to show good applicability in Chinese transplant patients. Selecting between dPCR and STR testing based on individual chimerism status can facilitate sensitive and accurate analysis, enable timely therapeutic intervention, and support effective relapse monitoring in clinical practice.

Keywords: Chimerism; Digital PCR; Hematopoietic stem cell transplantation; Indel

Graphical abstract



1. INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an effective treatment for various hematologic diseases. A total of 13 415 HSCTs were performed in China in 2020, of which approximately 75% were allogeneic HSCT (allo-HSCT). The majority (6187) of allo-HSCT cases were haploidentical.[1] Allo-HSCT outcomes can be evaluated by chimerism analysis, which monitors the proportion of viable donor cells and residual recipient cells in peripheral blood or bone marrow samples.[2] This tool is used for routine clinical monitoring of allo-HSCT status and as a reference for surrogate biomarkers of tumor-specific measurable residual disease (MRD).[3] MRD monitoring is particularly useful in cases of malignant hematologic diseases.[4,5] Additionally, chimerism testing may help predict potential relapse following allo-HSCT[3] if the MRD assay is unavailable (lack of identified disease-specific somatic mutations) or in cases of clonal heterogeneity.[6]

Chimerism could be classified into two major categories: complete chimerism (more than 95% of hematopoietic cells post-transplant are of donor origin) and mixed chimerism (MC, 5%-95% cells in hematopoietic tissues are of donor origin).[7] In MC cases, whether the amount of patient cells is 50% or 100% has therapeutic significance.[6] Long-standing evidence indicates that early MC detection and appropriate treatment can improve disease outcomes.[8,9] With technological innovation, various approaches can help determine chimerism and MRD levels. Short tandem repeat (STR) is a feasible, quantitative chimerism analysis recommended by the EuroChimerism consortium for relapse prediction.[10,11] However, its limited sensitivity of 1% to 5%, allelic imbalance, and high CV at a low MC percentage hinder early detection and relapse prediction.[12]

Micro-chimerism, first proposed by Starzl et al,[13–15] is a subgroup of chimerism in which the proportion of foreign cells from the peripheral blood does not exceed 1%.[3,16] Micro-chimerism is detectable at an early stage and can help to predict relapse. Its correlation with the risk of hematological relapse emphasizes the importance of quantitative detection of micro-chimerism.[13,17] Several studies have proposed real-time quantitative polymerase chain reaction (qPCR) to detect short insertions/deletions (indel) or single nucleotide polymorphisms for chimerism quantification.[14,18,19]

A novel qPCR approach based on more robust indel polymorphisms was then published. This method is highly sensitive, and capable of detecting up to 0.01% of recipient cells (one patient cell in >10 000 donor cells). This approach has an increased leukemia relapse early detection rate of 88%, compared to fragment analysis.[10] indel-qPCR was expanded to include an initial map of the human indel variant containing over 400 000 unique indel polymorphisms.[20] The indel-qPCR method applies to almost 99% of the population with a 0.001% detection rate and 0.1% sensitivity. This made micro-chimerism detectable by indel-qPCR. However, qPCR is less accurate than STR in measuring MC and is extremely hindered by the efficiency of every amplification reaction and calibration curve. Also, replicate assays are required due to restricted technical precision.[14] This highlights the importance of method selection based on a patient's chimerism status.[6,12]

By contrast, digital PCR (dPCR), an end-point assay that determines the positive droplet fraction and Poisson statistics to calculate the absolute number of starting copies, is a promising routine chimerism surveillance tool.[6] This method has easy operability, a low DNA requirement (65 ng corresponds to a sensitivity of approximately 0.03%),[6] does not require calibration curves or duplicate analysis,[9] and has a limit of detection of 0.008% (LOD, allowing the reliable determination of micro-chimerism.[14,15,21]) Despite the current lack of routine dPCR platforms in clinics, many studies on dPCR confirm its hematopoietic chimerism detection abilities. A dPCR-based detection system is highly repeatable (deviation <5%), accurate, and sensitive, particularly in the "difficult" range of MC.

For clinicians, decisions regarding post-transplant interventions depend on the chimerism rate or dynamic analysis of MC status, which indicates the persistence of recipient hematopoietic cells. Due to the potential relapse or transplant failure in different disease states, timely intervention is often required. High-performance testing for different chimerism levels is important to evaluate implantation status and guide immunosuppressants and donor lymphocyte infusion. Unfortunately, few studies on the dPCR-based chimerism detection system have used a Chinese population.[6,9,14,20,22] Given that racial difference is a principal factor for the individual recognition rate of indel loci, the reported indel loci panels may be inapplicable to the Chinese population. Thus, it is crucial to develop a dPCR-chimerism system with high coverage and great detection for the vast Chinese transplant population. The development of a dPCR platform could help to manage costs and optimize reporting progress optimization.

2. METHODS

2.1. Loci screening and primer/probe design

We selected indel loci from 12 pairs of autosomes with unique insertions or deletions (easy-to-design primers and probes) from the literature[23] and the NCBI database with the Alt Allele screening criteria close to 0.5 (Supplementary Table 1, http://links.lww.com/JCMA/A316). The chimerism detection system was developed on a dPCR platform ("dPCR-chimerism system"). The self-designed primers and probes (including 28 primers) were synthesized and purified through HPLC by Sangon Biotech Co., Ltd (Shanghai, China). We dissolved and diluted primers and the TaqMan probe were dissolved by RNase-Free Distilled Water and then stored them in a –20°C refrigerator in dark.

2.2. Detection system construction

We randomly selected peripheral blood samples from unrelated donors to simulate chimeric samples of different proportions. The total genomic DNA was purified using a Blood DNA Extraction Kit (GenMagBio, Changzhou, China) following the manufacturer's instructions. We screened the homozygous samples of 14 loci by qPCR (verified by Sanger sequencing). Each individual was paired with another individual with a different homozygous genotype to simulate diverse chimerism levels, which were diluted by the Elution Buffer into seven theoretical mosaic rates (50%, 10%, 2%, 0.4%, 0.08%, 0.016%, and 0.0032%) to simulate the post-transplantation samples for the subsequent construction of the dPCR-chimerism detection system. The study workflow is shown in Supplementary Fig. 1, http://links.lww.com/JCMA/A312.

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2.3. Gene primers and probe amplification efficiency detection

We adopted primers and probes corresponding to the 14 loci to prepare the qPCR system for the gradient diluted samples (5 μL TaqMan Fast Advance Master Mix, 2 μL Primer Mix, and 4 μL sample DNA). We then amplified them on an Applied Biosystems (ABI) 7500 real-time PCR system cycled under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 amplification cycles of 95°C for 15 seconds and 60°C for 30 seconds. We calculated the amplification efficiency of each locus using the slope of the standard curve.

2.4. Optimization of the amplification system

We prepared the dPCR reaction system by mixing 10 μL 2 × dPCR Mix, 2 μL Primer Mix, and 8 μL template DNA. We then amplified the system on the Quantstudio 3D dPCR (Life Technologies) according to the following procedure: 95°C for 10 minutes, followed by 39 amplification cycles of 95°C for 30 seconds and 55°C for 1 minute, one cycle of 98°C for 10 minutes, and one final cycle of 20°C for 2 minutes. The appropriate annealing temperature was adjusted and selected based on the experimental data.

2.5. Technical efficacy test

We randomly selected DNA samples from each locus to detect the limit of blank (LOB). We found only two to three homozygous samples on the N1-3 (+), N7-2 (–), N21-1 (–), and N21-1 (+) loci. Thus, we tested the corresponding samples three times. We obtained samples with theoretical mosaic ratios of 0.02%, 0.01%, and 0.005% by pair dilution of homozygous samples corresponding to each locus. LOD was preliminarily set according to the LOB.[24]

For linearity, we selected homozygous samples (verified by Sanger sequencing) to prepare six samples with different degrees of theoretical chimerism (50%, 10%, 2%, 0.4%, 0.08%, and 0.016%). We tested linearity under each different theoretical chimerism of the reaction system using R^2 three times. We verified the indel status of the 14 homozygous loci by Sanger sequencing (ABI 3730XL sequencer) and compared them with the PubMed database. If there was an insertion sequence, it was inserted, otherwise, it was deleted. For stability (repeatability), we obtained samples with a theoretical chimerism ratio of 0.1% or 0.05% by pair-to-pair dilution of homozygous indel samples at each locus.

2.6. Dual-donor type sample verification

First, we selected dual-donor post-transplant samples (each corresponding to a recipient and two donors) for STR testing (as previously described[25]) and a newly established dPCR-chimerism system. We obtained different groups of data to confirm whether the dPCR system could detect the mosaic rate of dual-donor type samples. We calculated the R^2 between the two measured mosaic rates to reflect the correlation between STR and dPCR. Chimerism testing looks at the differences between the recipient and donor genomes. The specific equations are as follows:

$$\%\ \text{Chimerism of the recipient} = \text{Rate}_{(\text{recipient} + \text{donor } 1)} - \text{Rate}_{(\text{donor } 1)}$$
$$\%\ \text{Chimerism of the donor 1} = \text{Rate}_{(\text{donor } 1)}$$
$$\%\ \text{Chimerism of the donor 2} = 100\ \% - \text{Rate}_{(\text{recipient} + \text{donor } 1)}$$

We then used the ABL dPCR system[20] to accurately quantify the genomic DNA copy number in donors' peripheral blood to make artificial mixtures of dual-donor post-transplant samples with six theoretical mosaic rates. We used this to evaluate the accuracy of the new dPCR-chimerism system and STR. We verified and compared the sensitivities of both systems using artificial mixtures of dual-donor post-transplant samples with eight theoretical chimerism degrees (20.00%, 10.00%, 5.00%, 2.50%, 1.00%, 0.50%, 0.10%, and 0.05%).

2.7. Clinical validation

To verify the accuracy of the dPCR-chimerism system in clinical samples, we collected peripheral blood samples from patients with malignant hematological diseases who were hospitalized in Ruijin Hospital (Shanghai, China) between August 2018 and March 2021 who had been tested for STR in our laboratory and were hospitalized at Ruijin Hospital (Shanghai, China) between August 2018 and March 2021. A total of 44 patients were included, all of whom provided informed consent.

2.8. Statistical analysis

We performed statistical analyses and generated graphs using Prism 8 for Windows (Version 8.4.2, GraphPad Software, LLC, San Diego, CA). We used Pearson's test for correlation analysis, and drew the Bland-Altman plot to reflect the consistency of the two methods.

3. RESULTS

3.1. Amplification efficiency detection of gene primers and probes

Supplementary Fig. 2, http://links.lww.com/JCMA/A313, shows the variation in amplification efficiency (represented by the threshold cycle [Ct] value) of the primers and probes designed for 14 indel loci at different DNA concentrations. The amplification efficiency of both primers and probes at each locus was within the range of 90% to 110%. Only the amplification efficiency of the N7-2 and N21-1 loci was slightly higher (111% and 112%, respectively). This indicates that the above primers and probes had demonstrated good amplification performance and specificity under the designed conditions.

3.2. Construction and optimization of dPCR amplification system

Utilizing N13-1 as a randomly selected example, we initially set the annealing temperatures at 52°C, 55°C, 57°C, and 60°C (with a temperature gradient of 2°C-3°C) and tested them separately, as seen in Supplementary Fig. 3, http://links.lww.com/JCMA/A314. According to the quadrant diagram, HEX fluorochrome's clustering effect became more obvious with increasing temperature. FAM fluorochrome's clustering effect became more obvious with a decrease in temperature. Both probes could well distinguish negative from positive when the annealing temperature was at 55°C. Additionally, when the other 13 loci were amplified at an annealing temperature of 55°C, FAM and HEX fluorochromes both expressed a great clustering effect for each locus. Therefore, we used 55°C as an optimized annealing temperature for the dPCR system.

3.3. Performance verification

We placed the homozygous samples of 14 loci screened by qPCR in the dPCR-chimerism system for LOB detection in primers and probes. The detection results were all below the LOD of 1.0E-04 (0.01%) (Fig. 1). Therefore, the LOB of the dPCR-chimerism system was 0.01%. According to Fig. 2, when we tested samples with theoretical mosaic rates of 0.005%, 0.01%, and 0.02% via the dPCR-chimerism system, all loci could be detected at 0.01% and 0.02%, while several loci were undetected at the rate of 0.005%. Therefore, we set 0.01% as

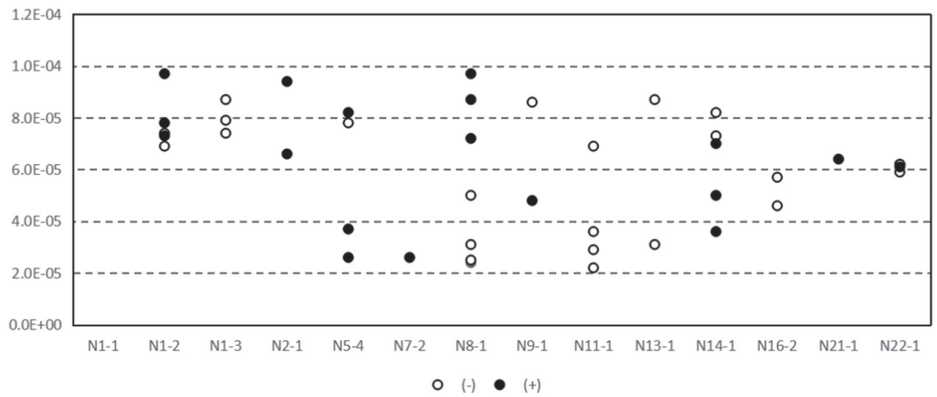

Fig. 1 LOB of 14 indel loci in the dPCR-chimerism system. The open and filled circles represent the negative and positive of each locus, respectively. dPCR = digital polymerase chain reaction; LOB = limit of blank.

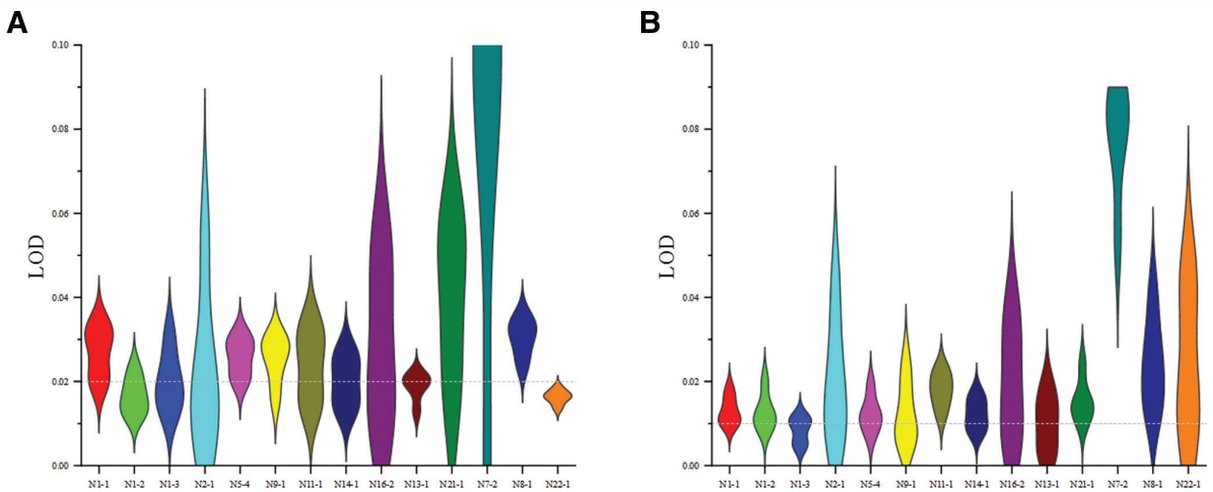

Fig. 2 Determination of the LOD of the dPCR-chimerism system at theoretical mosaic rate of (A) 0.02% and (B) 0.01%. dPCR = digital polymerase chain reaction; LOB = limit of blank.

the minimum LOD of this system, which was consistent with the literature.[9]

For the linear range test, the standard curves were in line with the average of the result and the theoretical chimerism value of the 14 loci in the above samples (Supplementary Fig. 4, http://links.lww.com/JCMA/A315), with the R^2 exceeding 0.99. This indicates that the dPCR was strongly correlated with the theoretical value within the range of 0.016% to 50%. Our dPCR-chimerism system provided linear results within this range. Fig. 3 shows that the average results at low chimerism levels (0.05% and 0.1%) were close to the theoretical values. The inter-batch CV of the loci was below 20%, except for N2-1, N13-1, and N21-1 (23.26%, 21.01%, and 21.96%, respectively), indicating the dPCR-chimerism system was stable.

3.4. Dual-donor type sample verification between dPCR and STR

Fig. 4 suggests a strong correlation between STR and dPCR results for dual-donor type samples (R^2 = 0.9974 > 0.99). This indicates that the dPCR-chimerism system was feasible for the analysis of dual-donor type samples and that the dPCR system can be used when the target is outside the LOD of STR. Then, we compared the theoretical and actual (STR and dPCR) values of simulated chimeric samples. We obtained R^2 values by comparing the theoretical value and the actual result according to the recipient, donor 1, and donor 2. All R^2 values were >0.99. This indicates that the dPCR and STR could accurately detect the rate of chimerism (Fig. 5, Table 1). All of the R^2 between the theoretical value and the dPCR results exceeded the R^2 between the theoretical value and the STR.

To compare the accuracy of the two methods, we compared the theoretical value of each sample with the R^2. As shown in Table 1, when the theoretical chimerism rate of the recipient was ≤5%, the R^2 of the dPCR results was almost always higher than that of STR. The dPCR result of group A in number 3 was significantly closer to the theoretical value compared to the STR, although the R^2 of the STR was slightly larger than the dPCR (0.9998 vs 0.9984, respectively). In addition, out of 12 samples, the R^2 of the four samples between STR and the theoretical value was lower than 0.94: group B in number 4, group A in number 5, and groups A and B in number 6 (0.9314, 0.7806, 0.1006, and 0.0002, respectively). The R^2 of their corresponding dPCR results remained above 0.9604, suggesting the dPCR-chimerism system is more accurate than STR.

In terms of sensitivity, the detection rate of the STR method was 100% when the theoretical mosaic rate was ≥1%, but it failed to reach 100% at a rate theoretical mosaic rate of <1%. In

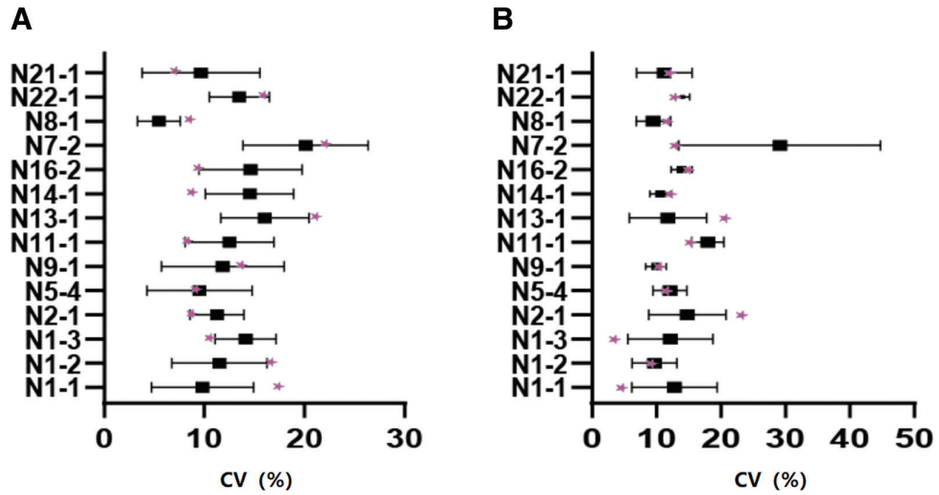

Fig. 3 Stability test of the dPCR-chimerism system at theoretical mosaic rate of (A) 0.05% and (B) 0.1%. *Inter-batch. dPCR = digital polymerase chain reaction.

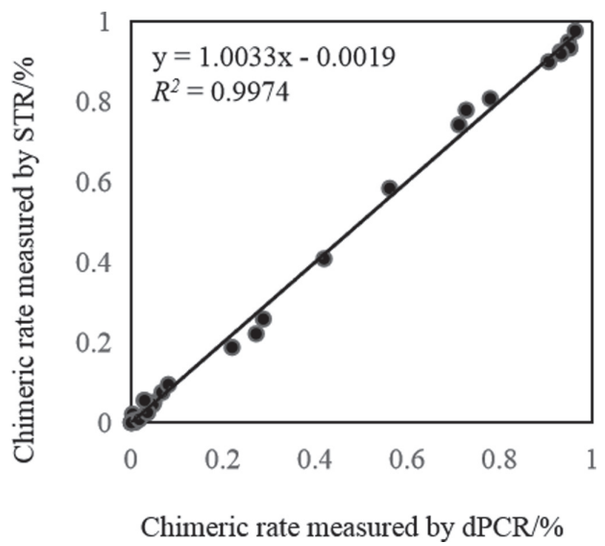

Fig. 4 Correlation of chimerism detection values between STR and dPCR in dual-donor type samples. dPCR = digital polymerase chain reaction; STR = short tandem repeat.

contrast, the dPCR result was preferred for full detection within the theoretical chimerism rate range of 0.05% to 20% (Fig. 6). When compared with the SDs of both methods, the dPCR-chimerism system was superior to STR in sensitivity and stability when the theoretical mosaic rate was <20%.

3.5. Evaluation of dPCR and STR technical characteristics

A comparison of dPCR and STR (Fig. 7, refer to Navarro-Bailón et al[25]) reveals that dPCR is slightly superior to STR in quantification capacity, reproducibility, accuracy, and time cost. dPCR provides superior information if a sample contains more than one donor or when a sample requires better sensitivity at a low mosaic rate (<5%). STR specialties depend on multiplex feasibilities, the total amount of DNA required, the ease of results interpretation, the validation of the sample identity, hands-on time, price, and sorting cells. In general, dPCR or STR can be appropriate depending on the individual theoretical chimerism level.

3.6. Clinical validation

To visualize the agreement between STR and dPCR data in clinical samples (n = 44), we performed a Bland-Altman analysis. As seen in Fig. 8, the observed mean value of the difference between STR and dPCR (0.68%) and the agreement range (95% limit of

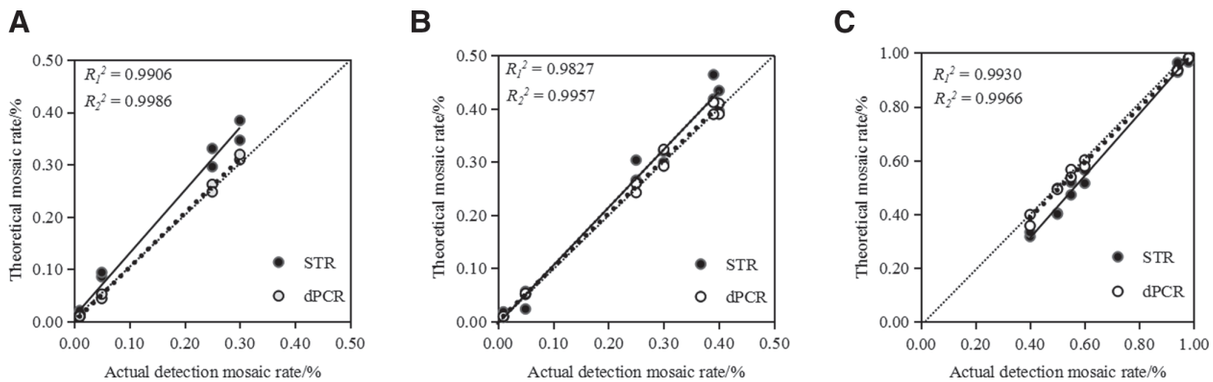

Fig. 5 Comparison of theoretical and actual detection values of simulated mosaic samples for recipient (A), donors 1 (B) and 2 (C). R^2 of STR and dPCR are represented by R_1^2 and R_2^2, respectively. dPCR = digital polymerase chain reaction; STR = short tandem repeat.

Table 1

Comparison between the theoretical mosaic rate of each sample and the measured mosaic rate obtained by dPCR and STR methods

		R^2 for group A		R^2 for group B	
Number	Theoretical mosaic rate/%[a]	STR/%	dPCR/%	STR/%	dPCR/%
1	1:1:98	1	1	1	1
2	1:5:94	0.9992	1	1	1
3	5:40:55	0.9997	0.9983	0.9584	0.9984
4	1:39:60	0.9904	1	0.9315	0.9950
5	25:25:50	0.7805	0.9953	0.9954	0.9980
6	30:30:40	0.1006	0.9788	0.0002	0.9882

dPCR = digital polymerase chain reaction; STR = short tandem repeat.
[a]For recipient: donor 1: donor 2.

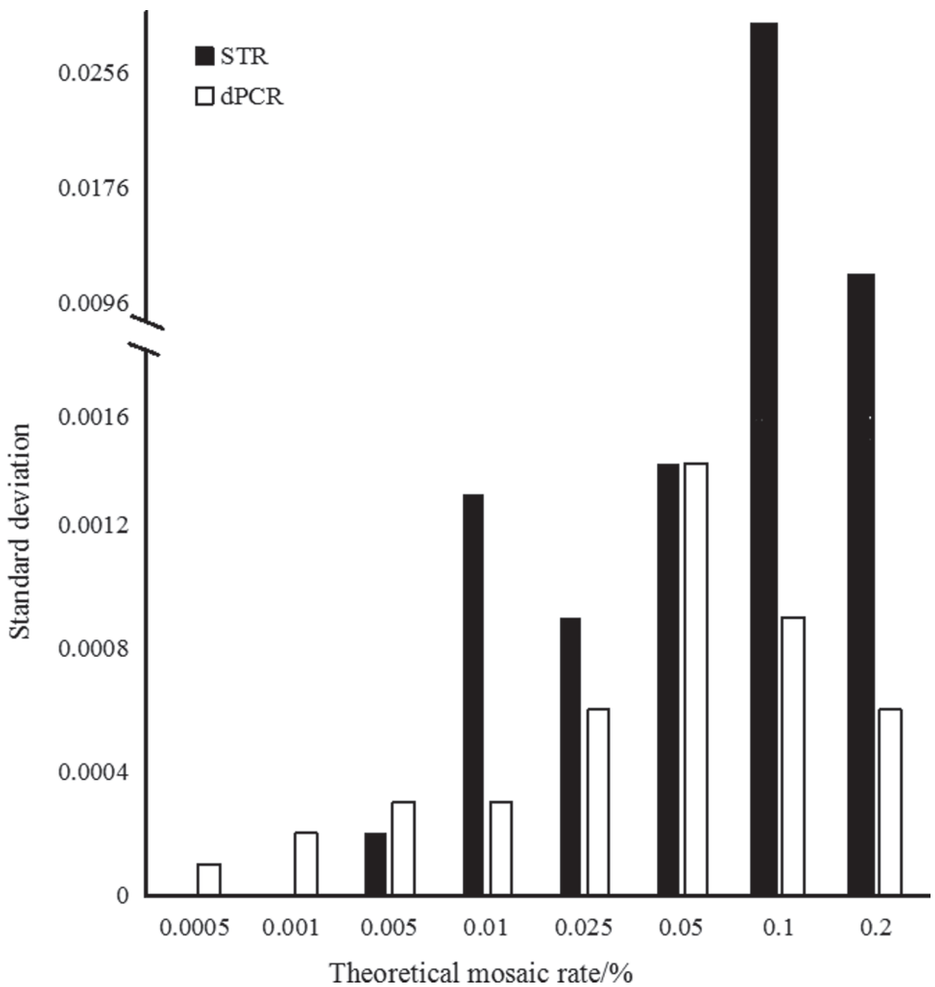

Fig. 6 Sensitivity comparison between STR and dPCR under different theoretical mosaic ratios. The black and white columns represent the SD values of STR and dPCR, respectively. dPCR = digital polymerase chain reaction; STR = short tandem repeat.

agreement) from –2.48% to 3.83% underline the close correlation of the data obtained with the two techniques. Several points were distributed outside of the agreement range, confirming that there can be differences between STR and dPCR.

4. DISCUSSION

In this study, we successfully established and clinically verified a new dPCR-chimerism system. This system features a simple, single-locus detection approach with automated result interpretation. It demonstrates good repeatability and high sensitivity (detection range: 0.01%–99.99%) and is expected to be highly applicable to Chinese transplant patients due to its inclusion of indel loci with high individual recognition rates in Asian populations. Its good performance in detecting both micro-chimerism and dual-donor samples will accelerate the evaluation of the implantation status and eventual disease recurrence. This will lay a solid foundation for effective early treatment intervention.

Fig. 7 Performance characteristics evaluation of dPCR and STR. Colors in graph state for qualitative evaluation of each characteristic: very good (dark green), good (light green), bad (orange). Length of bars state for quantitative evaluation of each characteristic (high, medium, low, none). dPCR = digital polymerase chain reaction; STR = short tandem repeat.

One major strength of this research is the selection of indel loci. Indels are valuable in population genetic analyses, including inferring biogeographic ancestry and determining the population substructure.[26] The considerable potency of a multi-indel panel was verified in an ancestry inference of subpopulations in China.[27] Specific indel markers show significant allele-frequency differences between Han Chinese and ethnic minorities.[28] The commercially available Investigator DIPplex kit, which contains 30 autosomal indel loci and an amelogenin gene, has been validated in a large population to evaluate its efficiency for forensic applications.[29] Here, for the first time, we screened 14 unique indel loci with high individual recognition rates for Asian populations based on the literature[23] and NCBI. Then, we designed specific primers and probe combinations for each locus. Based on theoretical considerations and empirical data, this system may be suitable for nearly 99% of HSCT patients (unpublished data). This guarantees the applicability of the newly established dPCR system in a Chinese transplant population. For individuals with different degrees of chimerism, the new dPCR-chimerism system can be applied for dynamic relapse surveillance.

STR's relatively low sensitivity (1%-5%) makes it weaker in relapse monitoring,[12] whereas the above-mentioned characteristics of dPCR contribute to better relapse prediction. The dPCR-chimerism system is a promising alternative to traditional STR analysis, which provides a flexible, individualized strategy for HSCT patients. When the theoretical mosaic rate is ≤5%, the dPCR method guarantees high stability, with 100% detected. When the theoretical mosaic rate is 5% to 20%, both STR and dPCR could achieve complete detection, but the latter is preferred in terms of stability. In other cases, STR is recommended due to testing costs and template quantity. Patients could select dPCR or STR according to their chimerism status.

Moreover, indel loci have been used to quantify the micro-chimerism status of DNA from the peripheral blood of patients who underwent bone marrow transplantation.[30] Our stability verification results showed that at low chimerism (theoretical value, 0.05% and 0.1%), the dPCR detection value was close to the theoretical value, with the CV below 20%. This was stable and acceptable for micro-chimerism, which prefers a CV of 16% at 1:999 dilution.[31] This suggests good performance in micro-chimerism detection. Examples of real dPCR graphs demonstrating the detection of a low concentration of the target indel indicate high sensitivity. In a comparison of several

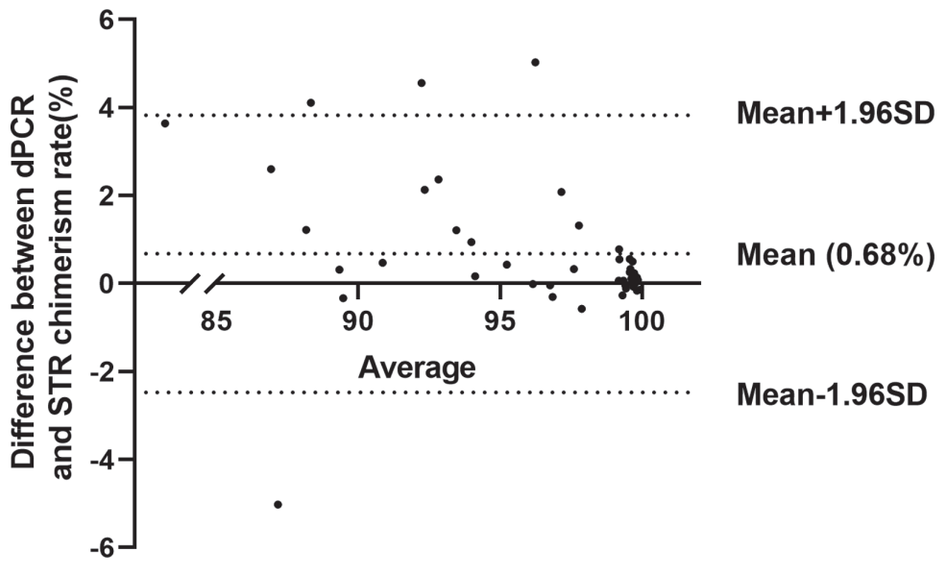

Fig. 8 Bland-Altmann analysis of dPCR as compared to STR data. The difference between STR and dPCR quantification is plotted against their mean. Ninety-five percent limits of agreement have been computed by average difference ±1.96 × SD of difference. dPCR = digital polymerase chain reaction; STR = short tandem repeat.

different methods, STR sensitivity is insufficient in cases of low chimerism, and qPCR fails to achieve accurate quantification due to its impact on amplification efficiency.[2] Therefore, by collecting clinical samples that had been tested for STR, we confirmed the dPCR-chimerism system's accuracy at low chimerism. In addition, the dPCR-chimerism system is applicable for dual-donor type samples. Indeed, the main advantage of STR lies in its multiallelic (up to 16 distinct variants) nature and consequential high informativity rate. It is applicable in all donor-recipient pairs or transplants with multiple donors.[3]

However, this study has several limitations: First, it is difficult to completely exclude the influence of large amounts of genomic DNA and potential sample contaminants (e.g., proteins, salts) in the analysis of two individuals during dPCR-based chimerism detection.[24] This may explain why the N21-1 locus cannot achieve stable detection under the theoretical mosaic rates of 0.05% and 0.1% (with the CV often exceeding 20%). Second, because this study focused primarily on methodological development, we lacked a sufficient number of clinical samples for broader validation. We will continue to collect samples from HSCT patients with different disease types in multiple centers to conduct a stratified evaluation while verifying this new system. This will enable more individualized chimerism analysis and relapse prediction strategies for patients of various ages and disease categories.

In conclusion, our newly established dPCR-chimerism system, characterized by high coverage and excellent sensitivity and linearity, will help patients select dPCR or STR detection according to their chimerism status. Our system is feasible for clinical evaluation after allo-HSCT, organ transplantation, and micro-transplantation. Further verification of within-system accuracy is warranted as more clinical samples are collected.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://links.lww.com/JCMA/A312, http://links.lww.com/JCMA/A313, http://links.lww.com/JCMA/A314, http://links.lww.com/JCMA/A315, and http://links.lww.com/JCMA/A316.

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