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RESEARCH PAPER

Clarity™ digital PCR system: a novel platform for absolute quantification of nucleic acids

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Abstract In recent years, digital polymerase chain reaction (dPCR) has gained recognition in biomedical research as it provides a platform for precise and accurate quantification of nucleic acids without the need for a standard curve. However, this technology has not yet been widely adopted as compared to real-time quantitative PCR due to its more cumbersome workflow arising from the need to sub-divide a PCR sample into a large number of smaller partitions prior to thermal cycling to achieve zero or at least one copy of the target RNA/DNA per partition. A recently launched platform, the Clarity™ system from JN Medsys, simplifies dPCR workflow through the use of a novel chip-in-a-tube technology for sample partitioning. In this study, the performance of Clarity™ was evaluated through quantification of the single-copy human *RNase P* gene. The system demonstrated high precision and accuracy and also excellent linearity across a range of over 4 orders of magnitude for the absolute quantification of the target gene. Moreover, consistent DNA copy measurements were also attained using a panel of different probe- and dye-based master mixes, demonstrating the system's compatibility with commercial master mixes. The Clarity™ was then compared to the QX100™ droplet dPCR system from Bio-Rad using a set of DNA reference materials, and the copy number concentrations derived from both systems were found to be closely associated. Collectively, the results showed that Clarity™ is a reliable, robust and flexible platform for next-generation genetic analysis.

Keywords Tube-strip digital PCR · Absolute DNA quantification · Sample partitioning · Clarity

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Introduction

Digital polymerase chain reaction (dPCR) is the third-generation PCR technology that enables absolute quantification of target nucleic acid without using external standards. It works by dividing a PCR mixture into thousands of independent nanolitre partitions, such that each individual reaction contains zero or at least one target of interest [1]. By analysing the proportion of partitions that are positive or negative for PCR products, an absolute DNA copy number is then determined based on the Poisson statistics. Apart from obviating the use of a DNA standard, dPCR has additional advantages over real-time quantitative PCR (qPCR) such as improved precision and accuracy, lower susceptibility to PCR inhibitors and increased sensitivity for detecting rare targets amidst a high background of non-target DNA [1]. Due to its advantages, dPCR has successfully been applied to a wide array of preclinical research studies such as rare mutation detection, copy number variation studies and low-level pathogen quantification [2–6].

Commercial dPCR platforms mainly employ two different methods of sample partitioning. The QX100™/QX200™ (Bio-Rad Laboratories) and RainDrop® (RainDance™ Technologies) systems partition samples through the

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formation of water-in-oil droplets while the BioMark™ HD (Fluidigm) and QuantStudio® 3D (Thermo Fisher Scientific) systems employ chip-based technology to generate partitions. Droplet-based platforms typically have wider dynamic ranges of detection as they generate a higher number of partitions per sample. Their use of conventional multi-well PCR plates also reduces the complexity of performing dPCR. Droplets, however, may coalesce during thermal cycling [7, 8], and this results in sample loss which is a major drawback for analysis of samples with low abundance DNA.

On the other hand, chip-based partitions are more stable and since the partitions are detected concurrently, shorter time is required for data analysis. Nonetheless, the multiple steps required for handling the chips often result in a more cumbersome workflow. Chips might also require dedicated thermal cyclers which, in turn, limit the number of reactions that can be performed in a single experiment. More recently, a new dPCR system known as Clarity™ was launched by JN Medsys which combines the advantages of both droplet- and chip-based systems using a novel chip-in-a-tube approach for sample partitioning. Each reaction mix is sub-divided into 10,000 partitions on a high-density chip within a PCR tube strip. Consequently, PCR can be performed using most conventional thermal cyclers. This format provides stable chip-based partitions and simplifies the cumbersome workflow typically associated with dPCR (Fig. 1).

Here, the accuracy and precision of the Clarity™ dPCR system in absolute quantification of nucleic acid target was investigated through quantification of the single-copy human *RNase P* gene. The performance of Clarity™ was also evaluated with different probe- and dye-based master mixes to assess the robustness of the system with these different mixes. Lastly, DNA quantification by Clarity™ was compared to the QX100™ droplet dPCR system from Bio-Rad Laboratories using a set of reference materials previously certified by the Fluidigm BioMark™ system.

Materials and methods

Quantification of the human *RNase P* gene using the Clarity™ dPCR system

The accuracy and precision of the Clarity™ dPCR system were assessed by quantifying the single-copy *RNase P* gene from eight serial dilutions of human genomic DNA with expected target concentrations ranging from 2240 to 0.38 copies/μL reaction mixture. These dilutions were yielded from a 202 ng/μL stock (equivalent to approximately 60,000 target copies/μL) purchased from Promega. The probe and primers used were custom designed in JN Medsys and synthesized by Integrated DNA Technologies. Each reaction mix consisted of 0.25 μM forward and reverse primers, 0.25 μM probe, 1× FastStart Essential DNA Probes Master (Roche), 1× Clarity™ JN solution, 3 μL DNA sample and PCR grade water top up to 15 μL. The JN solution is a

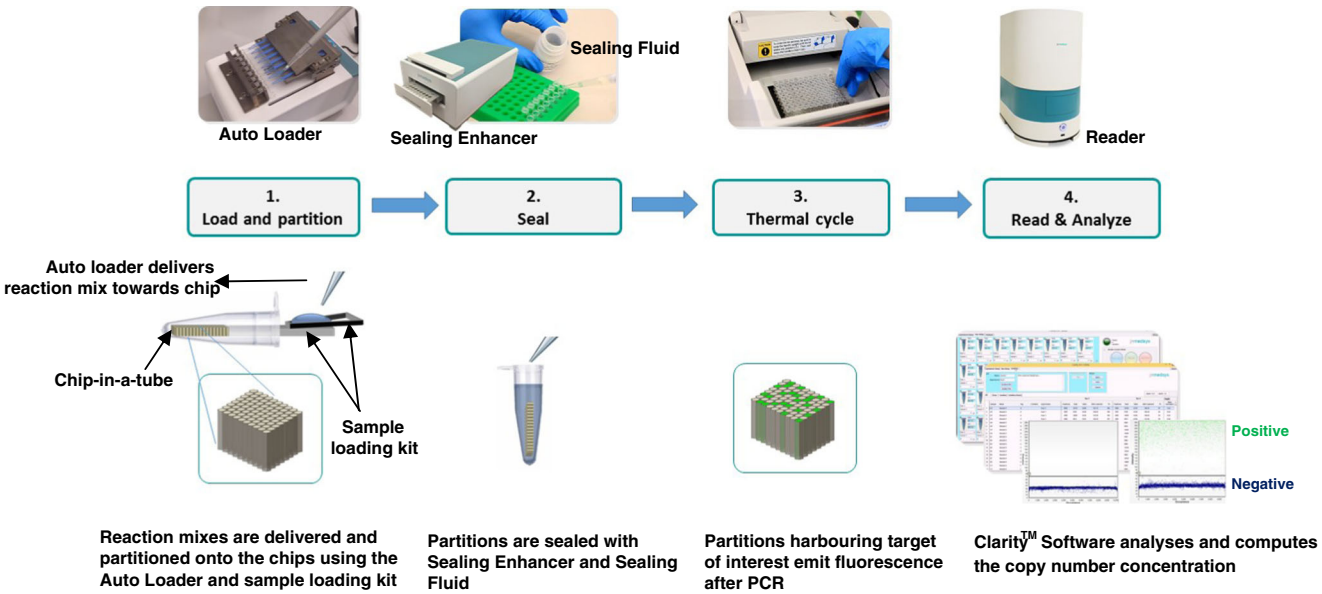

Fig. 1 Experimental workflow of Clarity™ digital PCR. The Clarity™ system adopts a unique chip-in-a-tube format which allows digital PCR to be performed with ease and speed. Loading and partitioning of eight reaction mixes can be performed simultaneously using the auto loader (*1*). The partitions are then sealed with the sealing enhancer following by addition of sealing fluid (*2*). Following which, reactions are subjected to thermal cycling using most conventional thermal cyclers (*3*). After PCR amplification, the tube strips are transferred to the reader where fluorescent signals from every partition of a reaction are detected concurrently (*4*). The number of positive and total partitions is determined using the Clarity™ software, and based on this information, the copy number concentration of each sample is calculated using the Poisson statistics

Table 1 Oligonucleotides used for *BCR-ABL b3a2* amplification

Oligonucleotide	Sequence (5′–3′)	Source
BCR-ABL b3a2 forward	TCCGCTGACCATCAAYAAGGA [10]	Integrated DNA technologies
BCR-ABL b3a2 reverse	CAC TCA GAC CCT GAG GCT CAA [10]	
BCR-ABL b3a2 probe	(HEX) CCC TTC AGC (ZEN) GGC CAG TAG CAT CTG A (3IABkFQ) [10]	

proprietary formulation optimized for robust performance on Clarity™ high-density chips. Using the Clarity™ auto loader, the resultant mix was then delivered onto the chip where it was sub-divided into 10,000 partitions. The partitions were then sealed with the Clarity™ Sealing Enhancer and 230 μL Clarity™ Sealing Fluid, followed by thermal cycling using the following parameters: initial cycle of 95 °C for 10 min and 40 cycles of 95 °C for 50 s and of 58 °C for 90 s (ramp rate = 1 °C/s). After PCR amplification, the tube strips were transferred to the Clarity™ Reader which detects fluorescent signals from each partition concurrently. The data were analysed with the Clarity™ software (version 1.0), and a proprietary algorithm is used for setting each threshold based on fluorescent intensities to determine the proportion of positive partitions out of the total. Based on this information, the software determines the DNA copies per microlitre of dPCR mix using the Poisson statistics. The mean partition volume of 1.336 nL was used for copy number calculation.

DNA quantification by Clarity™ using different probe- and dye-based master mixes

A panel of ready-to-use master mixes were evaluated for their compatibility with the Clarity™ system. Four probe-based master mixes tested were the FastStart Essential DNA Probes Master (Roche), GoTaq® Probe qPCR Master Mix (Promega), Kapa Probe Force qPCR Master Mix Universal (Kapa Biosystems) and SensiFAST™ Probe No-ROX Kit (Bioline). For dye-based assays, the following master mixes were used: FastStart Essential DNA Green Master (Roche), GoTaq® qPCR Master Mix (Promega), Power SYBR® Green PCR Master Mix (Applied Biosystems) and SensiFAST™ SYBR® No-ROX Kit (Bioline). Probe-based master mixes were assessed based on the BCR-ABL b3a2 assay with each reaction mix consisted of the respective test master mix, 0.1 μM forward and reverse primers, 0.1 μM probe, 1× Clarity™ JN solution, 3 μL ERM-AD623 plasmid DNA (consisted of 9000, 900 or 90 target copies/μL) and PCR grade water top up to 15 μL. The templates were serially diluted from the ERM-AD623c solution (see the "Quantification of ERM-AD623 by Clarity™ and QX100™" section for details on its quantification). The sequence of the primers and probe is listed in Table 1. For evaluation of the dye-based master mixes, a dPCR assay for *Escherichia coli GapA* detection was performed. Each reaction mix consisted of the respective dye-based master mix, 0.15 μM custom-designed forward and reverse primers, 1× Clarity™ JN solution, 3 μL *E. coli* genomic DNA (consisted of 9000, 1500 or 250 target copies/μL) and PCR grade water top up to 15 μL. The templates were serially diluted from a stock solution consisted of approximately 20,000 target copies/μL (the copy number was derived from the DNA concentration measured by UV

Table 2 Accurate and precise quantification by the Clarity™ digital PCR system. The accuracy and precision of Clarity™ were assessed by quantifying the single-copy *RNase P* gene from eight serial dilutions of human genomic DNA with the following expected concentrations

Expected concentration (copies/μL)	Mean of measured concentration (copies/μL)	Relative standard deviation (%)	Difference[a] (%)
2240	2275	0.450	2
560	583	2.39	4
140	137	0.749	2
35	35	4.82	1
8.75	8.31	6.46	5
2.19	2.39	11.9	9
0.73	0.67	10.4	8
0.38	0.37	15.2	3

The results represent the mean of the measured concentration of five replicates and are expressed as copies per microlitre of dPCR mix. The relative standard deviation is standard deviation divided by the mean of each quintuplicate expressed as a percentage

[a] Difference (%) is calculated by the formula (|Mean measured concentration − Expected concentration|/Expected concentration) × 100

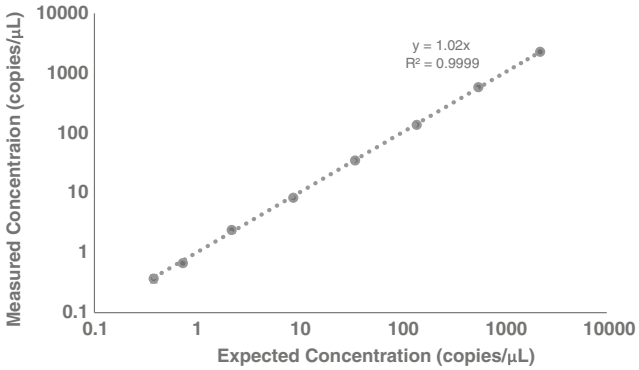

Fig. 2 Measured concentrations of *RNase P* using Clarity™ against its expected values. The graph is represented in a log-log format with each *data point* representing the mean ± standard deviation of five replicates

spectrophotometry). The prepared master mixes were then subjected to Clarity™ dPCR analysis using experimental procedures as described in the "Quantification of the human *RNase P* gene using the Clarity™ dPCR system" section.

Quantification of ERM-AD623 by Clarity™ and QX100™

The ERM-AD623 reference materials provided by the Joint Research Centre-Institute for Reference Materials and Measurements (JRC-IRMM) were used as DNA standards for comparing the absolute quantification of DNA by Clarity™ and QX100™. The reference materials consist of six solutions (ERM-AD623a–f) of double-stranded plasmid DNA whose concentrations have been established previously using the BioMark™ HD system by Fluidigm (Table 4) [9]. Quantification of the standards by both dPCR systems was performed through amplification of the *BCR-ABL b3a2* target. ERM-AD623a, ERM-AD623b and ERM-AD623c were serially diluted 1000×, 100× and 10×, respectively, prior to use, while ERM-AD623d, ERM-AD623e and ERM-AD623f were analysed in undiluted form.

Each dPCR assay mix prepared for Clarity™ consisted of 0.1 μM forward and reverse primers, 0.1 μM probe, 1× FastStart Essential DNA Probes Master (Roche), 1× Clarity™ JN solution, 3 μL template DNA and PCR grade water top up to 15 μL. The experimental procedures were carried out similar to that discussed in the "Quantification of the human *RNase P* gene using the Clarity™ dPCR system" section.

Each reaction mix prepared for QX100™ analysis consisted of 0.1 μM forward and reverse primers, 0.1 μM probe, 1× Bio-Rad ddPCR™ Super Mix for Probes, 4 μL template DNA and PCR grade water top up to 20 μL. dPCR was performed according to protocols as described in [10] with similar thermal cycling profile: initial cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and of 60 °C for 60 s (ramp rate = 2.5 °C/s). Results were analysed using the QuantaSoft software (version 1.3.2.0), and the partition volume of 0.85 nL [10] was used for copy number calculation.

Table 3 Compatibility of Clarity™ with different probe- and dye-based master mixes. The compatibility of probe-based master mixes was evaluated using the BCR-ABL b3a2 assay (A) while that of dye-based master mixes was assessed through amplification of the *E. coli GapA* gene (B). Serial dilutions of ERM-AD623 plasmid and *E. coli* genomic DNA were used as DNA targets for the probe- and dye-based assays, respectively

Expected concentration (copies/μL)	Mean of measured concentration (copies/μL)	Relative standard deviation (%)	Mean of measured concentration (copies/μL)	Relative standard deviation (%)	Mean of measured concentration (copies/μL)	Relative standard deviation (%)	Mean of measured concentration (copies/μL)	Relative standard deviation (%)
A								
	FastStart Essential DNA Probes Master Mix (Roche)		GoTaq® Probe qPCR Master Mix (Promega)		Kapa Probe Force qPCR Master Mix (Kapa Biosystems)		SensiFAST™ Probe No-ROX Master Mix (Bioline)	
9000	9052	0.373	8965	0.233	8813	1.81	8904	1.55
900	909	0.504	895	1.19	873	2.47	885	1.07
90	91	4.27	90	3.13	87	4.67	88	1.23
B								
	FastStart Essential DNA Green Master Mix (Roche)		GoTaq® qPCR Master Mix (Promega)		Power SYBR® Green PCR Master Mix (Applied Biosystems)		SensiFAST™ SYBR® No-ROX Kit (Bioline)	
9000	8852	0.983	8601	0.872	8696	2.19	8847	1.66
1500	1617	3.59	1501	2.37	1586	3.87	1641	0.459
250	260	3.24	250	4.87	240	2.65	267	1.45

The results represent the mean of the measured concentration of three replicates and are expressed as copies per microlitre of stock DNA. The relative standard deviation is standard deviation divided by the mean of each triplicate expressed as a percentage

Results and discussion

Clarity™ dPCR system quantifies DNA with high accuracy and precision

As Clarity™ is a newly launched dPCR system, the current study was initiated to evaluate its accuracy and precision in the absolute quantification of target DNA. To achieve this, eight serial dilutions of the purified human genomic DNA were prepared and used as templates for dPCR amplification of *RNase P*. In this experiment, the mean number of partitions detected was 9782 with a standard deviation of 142 partitions (see Electronic Supplementary Material (ESM) Table S1). The mean effective reaction size was 13.1 μL based on the partition volume of 1.336 nL (ESM Table S1). As summarized in Table 2, the measured concentrations of *RNase P* were found to be closely associated (<10% difference) with their expected concentrations. In addition, the relative standard deviations observed for most samples were ≤10% except for the three lowest dilutions (2.19, 0.73 and 0.38 copies/μL) where the relative standard deviations obtained were expectedly higher than the more concentrated samples. When the measured concentrations were plotted against their expected values, linear regression analysis revealed an R^2 value of above 0.999 across a dynamic range of over 4 orders of magnitude (Fig. 2). Together with the capacity to detect and quantify down to <1 copy of DNA per microlitre of reaction, the data obtained demonstrate that Clarity™ is a useful platform for genetic analysis that requires a high level of sensitivity, precision and accuracy.

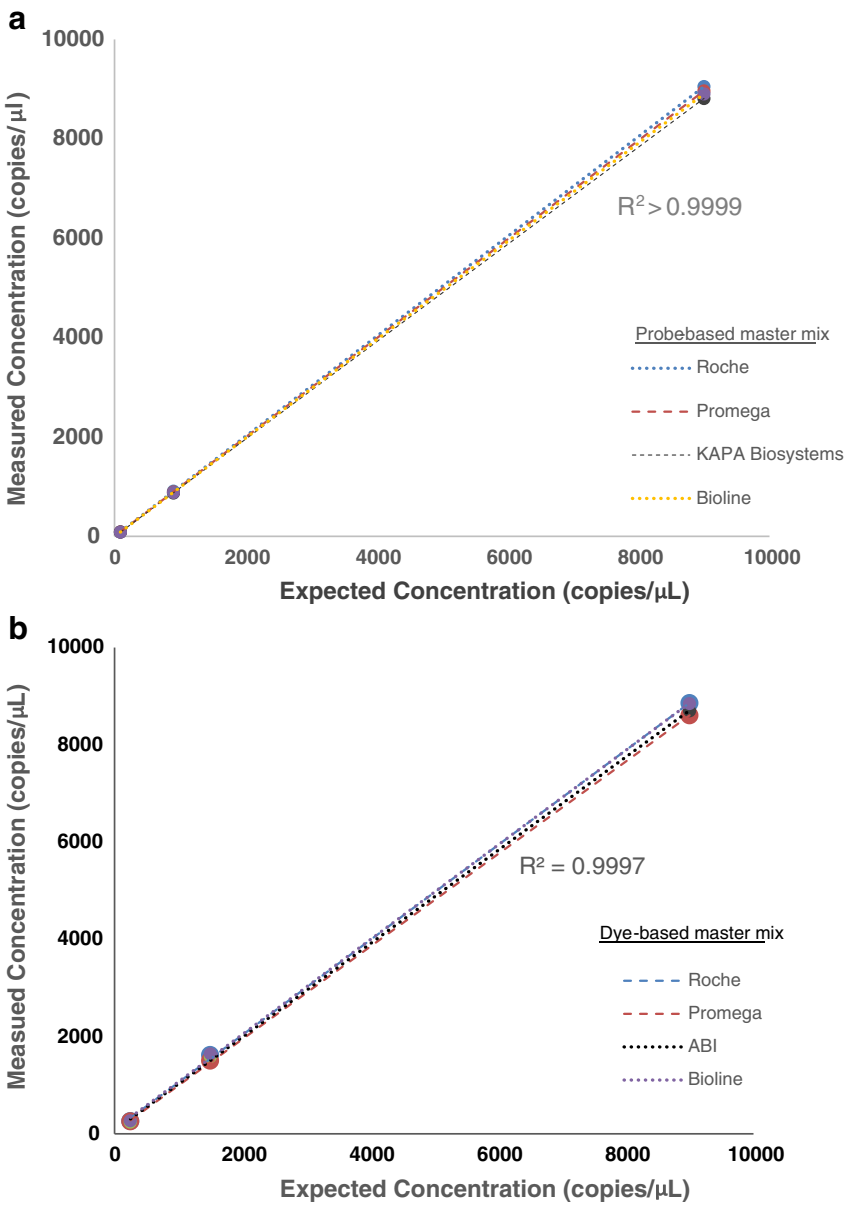

Fig. 3 DNA concentrations measured by Clarity™ using different probe- (**A**) and dye-based (**B**) master mixes

Table 4 Quantification of the ERM-AD623 reference materials using the Clarity™ and QX100™ digital PCR systems

BioMark™		Clarity™			QX100™		
ERM-AD623	Certified concentration (copies/μL)	Mean of measured concentration (copies/μL)	Relative standard deviation (%)	Difference[a] (%)	Mean of measured concentration[b] (copies/μL)	Relative standard deviation (%)	Difference[a] (%)
a	1,080,000	1,100,334	2.00	2	923,494	3.67	14
b	108,000	104,847	4.72	3	81,636	4.30	24
c	10,300	10,601	3.82	3	8595	3.72	17
d	1020	1007	2.68	1	815	4.35	20
e	104	110	5.53	6	79	5.50	24
f	10	11	8.44	10	13	16.4	30

The results shown are the mean of the measured concentrations derived from three independent experiments performed in triplicates and are expressed as copies per microlitre of stock DNA. The relative standard deviation is standard deviation divided by the mean of each experiment

[a] Difference (%) is calculated by the formula (|Measured concentration − Certified concentration|/Certified concentration) × 100

[b] The copy number concentrations measured by QX100™ were recalculated based on a more accurate droplet volume (0.85 nL) as reported in [10]

Clarity™ is compatible with different probe- and dye-based master mixes

Some dPCR systems require the use of specific master mixes in order to achieve optimized results. For instance, QX100™/QX200™ is only compatible with the Bio-Rad ddPCR™ supermixes for droplet stabilization. Likewise, assays analysed using QuantStudio® 3D require the use of the QuantStudio® 3D Digital PCR Master Mix. However, Clarity™ is designed as an open platform which supports the use of different commercial qPCR master mixes. Therefore, it is desirable to validate the robustness and precision of the system with various mixes. For this investigation, dPCR was conducted using a panel of four probe- and four dye-based qPCR master mixes. The use of different master mixes resulted in DNA copy measurements that are similar (<5% difference) to the expected concentration (Table 3, ESM Tables S2 and S3). In addition, the relative standard deviations obtained for all master mixes were below 5%. When a linear graph was plotted using the data, the linearity of R^2 >0.999 was observed for all master mixes (Fig. 3). Taken together, these data highlight that the Clarity™ dPCR system is compatible with different commercially available master mixes to generate reproducible and precise DNA copy number measurements.

Copy number concentrations measured by Clarity™ correlate well with measurements determined by the BioMark™ HD and QX100™ dPCR systems

Following evaluation of the performance of Clarity™, subsequent investigations were performed to gain insights into the

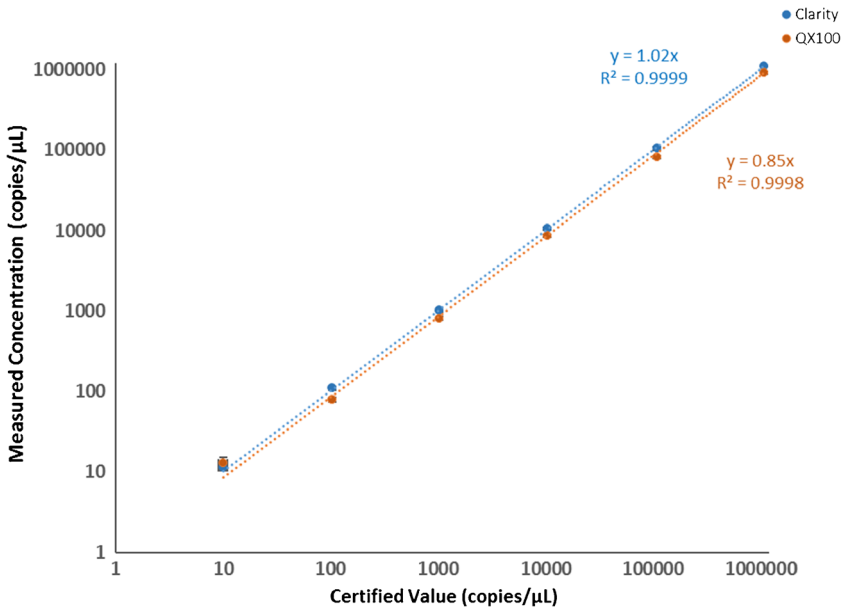

Fig. 4 Measured concentrations of ERM-AD623 standards using Clarity™ and QX100™ against values certified by BioMark™ HD. The graph is represented in a log-log format with each *data point* representing the mean ± standard deviation of three independent experiments

comparability of Clarity™ with the Bio-Rad QX100™. For this purpose, dPCR was performed on six ERM-AD623 plasmid solutions whose concentrations were previously certified by the BioMark™ HD system [9]. As shown in Table 4 and ESM Table S4, the results obtained by Clarity™ are well correlated (≤10% difference) with the certified concentrations. On the other hand, the copy number concentrations measured by QX100™ are consistently lower than the certified values by 14–30% (Table 4, ESM Table S5). This trend is similar to an earlier report where measurements by QX100™ were shown to vary from those by BioMark™ by 12–17% [10]. Nonetheless, when the data were plotted against the certified concentrations (Fig. 4), both Clarity™ and QX100™ measurements showed strong linear correlation with the BioMark™ measurements (R^2 values of 0.9999 and 0.9998, respectively) and variability of less than 20% (regression coefficient of Clarity™ of 1.02 vs that of QX100™ of 0.85). This minor variation could be due to the inherent difference between the chip- and droplet-based platforms and also the different master mixes used in the respective dPCR assays. Collectively, these data showed that DNA copy number measurements using Clarity™ are comparable with the established QX100™ and BioMark™ systems.

Conclusion

This study marks the first investigation to validate the performance of the Clarity™ dPCR system and its comparability with the more prevalent QX100™ and BioMark™ HD systems. Clarity™ demonstrated excellent linearity across its dynamic range and is capable of quantifying different complexities of DNA samples (i.e. human genomic DNA and bacterial DNA) with high precision and accuracy without the need for any pre-dPCR processing (e.g. DNA fragmentation). Coupled with the flexibility to use different qPCR master mixes, as well as the fast and user-friendly workflow provided by the tube-strip partitioning format, Clarity™ is well suited to be incorporated into high-throughput genetic analysis that requires precise and accurate DNA quantification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

This article does not contain any research with human participants or animals.

References

1. Huggett J, Cowen S, Foy C. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem. 2014;61(1):79–88.
2. Hall Sedlak R, Jerome K. The potential advantages of digital PCR for clinical virology diagnostics. Expert Rev Mol Diagn. 2014;14(4):501–7.
3. Lamy P, Castan F, Lozano N, et al. Next-generation genotyping by digital PCR to detect and quantify the BRAF V600E mutation in melanoma biopsies. J Mol Diagn. 2015;17(4):366–73.
4. Belgrader P, Tanner S, Regan J, Koehler R, Hindson B, Brown A. Droplet digital PCR measurement of HER2 copy number alteration in formalin-fixed paraffin-embedded breast carcinoma tissue. Clin Chem. 2013;59(6):991–4.
5. Gutiérrez-Aguirre I, Rački N, Dreo T, Ravnikar M. Droplet digital PCR for absolute quantification of pathogens. Plant Pathol. 2015;1302:331–47.
6. Pavšič J, Žel J, Milavec M. Digital PCR for direct quantification of viruses without DNA extraction. Anal Bioanal Chem. 2015;408(1):67–75.
7. Pompano R, Liu W, Du W, Ismagilov R. Microfluidics using spatially defined arrays of droplets in one, two, and three dimensions. Annu Rev Anal Chem. 2011;4(1):59–81.
8. Zhong Q, Bhattacharya S, Kotsopoulos S, et al. Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR. Lab Chip. 2011;11(13):2167.
9. White H, Deprez L, Corbisier P, et al. A certified plasmid reference material for the standardisation of BCR–ABL1 mRNA quantification by real-time quantitative PCR. Leukemia. 2014;29(2):369–76.
10. Corbisier P, Pinheiro L, Mazoua S, et al. DNA copy number concentration measured by digital and droplet digital quantitative PCR using certified reference materials. Anal Bioanal Chem. 2015;407(7):1831–40.