DNA copy number concentration measured by digital and droplet
digital quantitative PCR using certified reference materials

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Abstract The value assignment for properties of six certified
reference materials (ERM-AD623a–f), each containing a plasmid DNA solution ranging from 1 million to 10 copies per µL,
by using digital PCR (dPCR) with the BioMark™ HD System
(Fluidigm) has been verified by applying droplet digital PCR
(ddPCR) using the QX100 system (Bio-Rad). One of the critical
factors in the measurement of copy number concentrations by
digital PCR is the partition volume. Therefore, we determined
the average droplet volume by optical microscopy,
revealing an average droplet volume that is 8% smaller than
the droplet volume used as the defined parameter in the
QuantaSoft software version 1.3.2.0 (Bio-Rad) to calculate
the copy number concentration. This observation explains
why copy number concentrations estimated with ddPCR and
using an average droplet volume predefined in the QuantaSoft
software were systematically lower than those measured by
dPCR, creating a significant bias between the values obtained
by these two techniques. The difference was not significant
anymore when the measured droplet volume of 0.834 nL was
used to estimate copy number concentrations. A new version
of QuantaSoft software (version 1.6.6.0320), which has since
been released with Bio-Rad’s new QX200 systems and
QX100 upgrades, uses a droplet volume of 0.85 nL as a defined
parameter to calculate copy number concentration.

Keywords Digital PCR · Droplet digital PCR · Reference
materials · Optical microscopy · Areal equivalent diameter

Introduction

Quantification of nucleic acid molecules by quantitative PCR
(qPCR) has been developed during the last 25 years in many
applications of green and red biotechnologies and has progressive-
ly reached a level of maturity [1]. qPCR has been applied
to gene expression quantification [2, 3], forensic DNA quantifi-
cation [4, 5] and clinical [6, 7] and veterinary [8] virology
diagnostics, all reviewed recently.

Quantification of nucleic acids by qPCR is commonly
achieved by using a calibration curve, constructed by measur-
ing the number of cycles required for the fluorescent signal to
reach a threshold level for known amounts of target DNA in a
parallel set of reactions. The threshold fluorescent level is
calculated from the initial cycles, and this cycle number (Cq
value) is proportional to the number of copies of template in
the sample. The use of calibration curves has basically two
consequences.

First, it requires that the exact quantity value carried by a
calibration material (measurement standard) is determined by
independent means using, e.g. spectrophotometry or an inter-
calating dye. Spectrophotometric methods rely on the molar
absorptivity (or the molar absorption coefficient) of pure
nucleic acid solutions used in the equation based on the
Lambert-Beer law [9]. The molar absorptivity depends upon
the chemical nature (deoxyribonucleic or ribonucleic) and
conformation (single or double stranded) of the nucleic acid
molecule as well as its degree of purity. The fraction of the
absorbed light will depend on how many molecules the light
interacts with, being the molecule of interest but also
contaminants that have been co-extracted. Despite those limitations, spectrophotometric methods are widely used to quantify nucleic acids in solutions. Fluorometric methods using intercalating dyes have as a major advantage their higher sensitivity compared to spectrophotometric methods, allowing a detection down to 25 pg/mL dsDNA [10]. However, these measurements need to be calibrated using the quantity value of a material having ideally the same chemical nature and molecular weight as the analyte of interest. This circular problem cannot be solved without the use of a reference material that is certified for its nucleic acid concentration, a reason why several metrology institutes are developing such materials.

The use of an external measurement standard being an amplicon [11], cut or uncut plasmid DNA [12, 13], cosmid [14] or complementary DNA [15] to calibrate qPCR has a second drawback. A number of strategies have been used to prepare and apply a standard with similar properties to the target DNA. These include treating genomic DNA with a cocktail of restriction enzymes, DNA ultrasonication [16], shredding methods [17] or using a Bayesian approach that takes into account several sources of uncertainty [18]. A few studies have shown that those standards could behave in a similar way as the nucleic acid molecules that were targeted [19, 20]. However, factors such as DNA stability, base composition, secondary structure and presence of complex mixtures of non-target DNA can significantly alter the PCR amplification performance, making the DNA quantification dependent and only traceable to the calibrants that have been used [21].

Digital PCR (dPCR) technology [22] is an end-point measurement that overcomes the dependency on a DNA calibrant, and it has recently been used as an alternative method to assign copy number concentrations of defined nucleic acids in solution [23–26]. Some prerequisites of the technology have already been discussed [27], and recommendations were published on how to properly report digital PCR results [28]. The nucleic acid targets must be randomly distributed among the partitions, and the conditions of the PCR assays must be optimised to ensure that a single copy of the target is indeed amplified during the PCR. Since the calculation of the copy number concentration includes dividing the copy number estimate by the assay volume, knowing the correct partition or droplet volume is a key factor when measuring DNA concentrations using digital PCR [29]. The partition or droplet volumes as provided by the manufacturer are utilised by the majority of laboratories without further independent verification. The potential error on that volume is not important if a ratio between two concentrations of DNA targets is reported, but it can generate a significant bias when an absolute copy number concentration is measured.

In this study, we used a set of reference materials that has been certified by using dPCR for the absolute copy number concentration of a BCR-ABL target [24, 30] applying the BioMark™ HD System (Fluidigm). The certified concentrations were verified using results from the QX100 droplet digital PCR (ddPCR) system (Bio-Rad). In parallel, the average droplet volume was measured and the copy number concentration reported by the ddPCR QuantaSoft software (Bio-Rad) was corrected by taking into account the real average droplet volume.

Materials and methods

Test material

ERM-AD623a, ERM-AD623b, ERM-AD623c, ERM-AD623d, ERM-AD623e and ERM-AD623f (Joint Research Centre-Institute for Reference Materials and Measurements (JRC-IRMM)) were used as certified reference materials containing (1.08×10^6±0.13×10^6), (1.08×10^5±0.11×10^5), (1.03×10^5±0.10×10^4), (1.02×10^3±0.09×10^3), (1.04×10^2±0.10×10^2) and (10.0±1.5) copies (cp)/μL, respectively, of a double-stranded linearised plasmid [24]. The plasmid is in a 1-mmol/L Tris, 0.01-mmol/L EDTA pH 8.0 buffer (T_{1E0.01}) supplemented with 50 mg/L of transfer RNA from Escherichia coli and used undiluted in the ddPCR experiment unless otherwise mentioned. The copy number concentrations and related uncertainties were established using the measurement data of three (National) Metrology Institutes each using their own dPCR apparatus from Fluidigm (i.e. the BioMark™ HD System) and two validated assays as described earlier [24].

Digital PCR

Eppendorf DNA LoBind tubes (VWR International, cat no. 0030108051, Leuven, Belgium) as well as calibrated Rainin Pipet-Lite XLS+ single channel micropipettes (Mettler-Toledo S.A., Zaventem, Belgium) were used throughout this study. For the measurement of copy number concentrations of the six plasmid solutions by dPCR, 10 μL of ERM-AD623a, ERM-AD623b, ERM-AD623c and ERM-AD623d was gravimetrically diluted in T_{1E0.01} buffer to obtain DNA samples at a nominal concentration of 500 cp/μL. ERM-AD623e and ERM-AD623f were used undiluted in the dPCR assay. A volume of 19.7 μL of the DNA sample was further mixed with 30.3 μL of pre-sample mix solution, and 9 μL of this mixture was loaded on five panels of the 12.765 digital Array™ IFCs from Fluidigm (BIOKE, Leiden, Netherlands). The pre-sample mix solution contained the primers and probes for the BCR-ABL b3a2 transcript and for the ABL transcript (duplex PCR conditions) at final concentrations mentioned in Table 1 together with 20× GE sample loading reagent (Fluidigm) and TaqMan® Universal PCR MasterMix (Applied Biosystems, Ghent, Belgium) as recommended by the
Table 2  Thermal cycle protocol used for both dPCR and ddPCR protocols

| Name            | Phase      | Time [s] | Temperature [°C] | Repeats |
|-----------------|------------|----------|------------------|---------|
| UNG and hot start | UNG        | 120      | 50               | 1       |
|                 | Hot start  | 600      | 95               |         |
| PCR cycles      | Denaturation | 15      | 95               | 50      |
|                 | Annealing  | 60       | 60               |         |

Table 1  Primers and probes used to amplify the BCR-ABL b3a2 transcript by dPCR and ddPCR

| PCR assay  | Primers and probe | Sequence                        | Final concentration [µM] | Amplicon size [bp] |
|------------|-------------------|---------------------------------|--------------------------|--------------------|
| BCR-ABL b3a2 | F-primer         | 5'-TCCGCTGACCATCAAYAAGGA-3'    | 0.3                      | 149                |
|             | R-primer         | 5'-CACCAGGCCCTAGGGCTCAA-3'     | 0.3                      |                    |
|             | Probe            | 5'-6VIC)CCCTTCACGGCCGAGTGATCTGA-(MGB)-3' | 0.2                     |                    |
| ABL        | F-primer         | 5'-TGGAGATAACACTCTTAAGTAAAGGT-3' | 0.3                     | 122                |
|             | R-primer         | 5'-GATGTAAGTGGCTGGGACCCA-3'    | 0.3                      |                    |
|             | Probe            | 5'-6FAM)CCATTTTTGTTTTGGCTACACCAT-(TAMRA)-3' | 0.2                     |                    |
Droplet volume sizing by optical microscopy

Average droplet volumes were determined for three DG8-186-3008 cartridges, each prepared on a different day. Droplet generation and acquisition of the optical microscopy images was performed on the same day. Image analysis was performed afterwards, off-line. For each of the three cartridges, a volume of 10 μL containing the droplets generated in the DG8-186-3008 cartridges was carefully pipetted into the chamber of an Ibidi plate (Ibidi μ-slide VI-flat non-coated, Proxylab sprl, Beloeil, Belgium). The Ibidi plates were held at an angle to allow the formation of a uniform monolayer of droplets for better imaging. Four wells were randomly selected from each of the three different droplet generator cartridges for this analysis. Between 140 and 160 droplets were measured in each selected well. Each cartridge was analysed on a different day providing measurement of 1794 droplets in total.

An optical microscope (Leica DM 4000M, Diegem, Belgium) with a digital CCD camera (Leica DFC 290 HD) was used to image droplets in four wells, randomly selected from each of the three different droplet generator cartridges. All images were recorded under uniform illumination in a bright field imaging mode, using sufficient magnification (×200). The accuracy of the scale of the images was verified with a field imaging mode, using sufficient magnification (×200). The length scale of the microscope used at IRMM was calibrated with a calibration grid, leading to an estimated calibration uncertainty of 0.15 %. The National Measurement Institute of Australia (NMIA) produced a more detailed type B uncertainty evaluation of the individual droplet volume measurement via the area equivalent diameter and estimated the relative expanded uncertainty of this measurement as 2.0 %. This uncertainty includes factors such as operator bias in the imaging process, calibration, off-focus image and a component for the (minor) non-sphericity of the droplets.

Results

Concentration measurements using ddPCR

In the first experiment, the comparability of results obtained by two different ddPCR platforms (chip vs. droplet based) was investigated by measuring the six plasmid solutions present in the ERM-AD623 set of certified reference materials. A volume of 8.5 μL of each of the six concentrations ranging from 1.08×10^6 to 10 cp/μL was tested in eight replicates in ddPCR. In Fig. 1, the highest concentration was diluted 10× in a 1-mmol/L Tris, 0.01-mmol/L EDTA pH 8.0 buffer to avoid an overloading of positive droplets. The conditions of the PCR assay were optimal as the population of positive droplets could clearly be discriminated from the negative droplets using a threshold value of 4002.

The certified values as determined by ddPCR were plotted against the average copy number concentration determined by ddPCR using the QuantaSoft software version 1.3.2.0 (Fig. 2). A linear regression fitted the data with a coefficient of determination of 0.9994 in a log-log plot suggesting good agreement between the two digital PCR methodologies based on chip partitions and droplets.

The apparently good agreement between the certified values and the measured values was verified by a structured and quantitative approach that allows making a statement on the evidence of any bias. The approach takes into account the certified value and its uncertainty, the measurement result and their respective uncertainties. These uncertainties were subsequently combined, and the expanded uncertainty was compared to the difference between the measured values and the certified values as explained in [32] and detailed in Table 3.

The copy number concentration of the plasmid measured by ddPCR appeared to be slightly underestimated when compared to the certified values. A statistically significant bias (at 95 % confidence level) varying between 14 and 17 % was observed for ERM-AD623a, ERM-AD623b, ERM-AD623c, ERM-AD623d and ERM-AD623e corresponding to the copy number concentration level ranging from 1,080,000 to 104 cp/μL. No significant bias was observed for the lowest concentration certified at 10 cp/μL.
Underestimation of copy number concentration can have several origins, related either to the PCR assay itself, to the density value used for the gravimetric dilution, to the volume of the assay or to a combination of those three factors. The PCR assays used in this study have been optimised to reduce to a minimum the rain effect, so that the setting of a threshold value to segregate positive droplets from negative droplets was not an issue. Indeed both populations of droplets were clearly segregated (Fig. 1). The plasmid solutions used as test materials were small linearized plasmid to reduce the false negative droplets that may occur with larger unrestricted DNA molecules. Special care was also taken to always dilute the DNA in buffered solutions to reduce potential degradation of the targets prior amplification, reducing as such the probability to miss some droplets containing the target of interest.

The PCR platform used was a C1000 Touch Thermo Cycler recommended by Bio-Rad for having uniform, slow ramping temperatures through the plate reducing border plate effects and particularly adapted for this digital PCR application. Special care was also taken to carefully correct the gravimetric dilutions using the density of the pre-mix. Having taken all those precautions, one obvious parameter which is the final volume of the assay had to be investigated.

Therefore, the hypothesis that the observed bias could be the result of an incorrect average droplet volume used in the QuantaSoft software to convert positive droplets into a copy number concentration was tested in subsequent experiments.

Droplet volume measurements using optical microscopy

To test the above hypothesis, ERM-AD623c, certified to contain 10,300±1000 cp/μL was measured by ddPCR under repeatability and intermediate repeatability conditions by analysing samples from three vials in three independent experiments (N=3) in eight replicates (n=8). The droplets generated in the DG8-138-3008 cartridges were examined under an optical microscope and simultaneously analysed in the droplet reader.

In order to avoid that the examination under the microscope would alter the size of the droplets, the photography of the generated droplets was done in less than 30 min. (The manufacturer recommends starting thermal cycling within 30 min of sealing the PCR plate.)

Over the 3 days, a total of 1794 droplets were analysed and imaged by optical microscopy to calculate an average droplet volume. A typical image of a droplet layer is provided in Fig. 3A together with the image obtained after the image analysis in Fig. 3B. For reasons of surface energy minimisation, the liquid droplets in the liquid (oil) matrix have a strong tendency to take a perfectly spherical shape. This shape could be distorted under the action of mechanical stress, for example due to gravity, but during the microscopy investigation, the droplets were submersed in oil, and therefore, the spherical shape is not strongly challenged in the vertical direction. Also, as shown in Fig. 3, there is no indication that the interaction between neighbouring particles results in lateral deformation.
In the 2D image analysis step, two approaches were followed to access the 3D droplet volume (see “Materials and methods” section). The average Feret diameter of the droplets was 3.4 % larger than the diameter calculated using the area equivalent diameter. This observation was explained by noting that the measured Feret diameters are affected by small artificial deviations or tiny protrusions on the droplet perimeter caused by the digital processing of the droplet images. This is the reason why the Feret diameter is consistently larger than the major axis of the best fitting ellipse, and why the area equivalent diameter was chosen to estimate the diameter of the droplets.

The average volume of the droplets generated by the droplet generator in the DG8-186-3008 cartridge (Table 4) was measured as 0.834 nL which is much smaller than the value of 0.91 nL used in the QuantaSoft software version 1.3.2.0 to calculate the copy number concentration.

Similar experiments have been performed at the NMIA in Sydney reporting volumes of 0.833 and 0.830 nL using

**Table 3** Comparison between the certified copy number concentrations and concentration estimated by ddPCR in the ERM-AD623 series. A droplet volume of 0.91 nL has been used to calculate the copy number concentration

| ERM-AD623 | Certified value (cp/μL) | U_{CRM} | Average measured values (cp/μL), n=8 | std dev | Δ_{m} (cp/μL) | u_{m} (cp/μL) | U_{Δ} (cp/μL) | Significant bias | % difference |
|-----------|------------------------|--------|-----------------------------------|---------|----------------|--------------|---------------|-----------------|--------------|
| f         | 10                     | 1.5    | 9                                 | 2       | 1              | 1            | 2             | No              | 12           |
| e         | 104                    | 10     | 87                                | 10      | 17             | 7            | 14            | Yes             | 16           |
| d         | 1020                   | 90     | 846                               | 80      | 174            | 60           | 121           | Yes             | 17           |
| c         | 10,300                 | 1000   | 8851                              | 669     | 1449           | 602          | 1203          | Yes             | 14           |
| b         | 108,000                | 11,000 | 90,951                            | 8105    | 17,049         | 6832         | 13,663        | Yes             | 16           |
| a         | 1,080,000              | 130,000| 908,543                           | 58,766  | 171,457        | 71,333       | 142,665       | Yes             | 16           |

The calculated bias is significant with a confidence level of 95 %

U_{CRM} expanded uncertainty of the certified value; Δ_{m} absolute difference between mean measured value and certified value; U_{Δ} combined uncertainty of result and certified value; u_{m} uncertainty of the measurement result, calculated as the standard deviation divided by the square root of the number of replicates.
16 sub-samples from a single well was 2.4 % \((k=2)\) (Table 5), indicating little variability and good within-well precision. The similarity in values reported from two different laboratories demonstrates good agreement and reproducibility between laboratories applying the same methodology, but using different lots of cartridges and different master mixes.

The relative expanded uncertainty associated to the average droplet volume estimated by IRMM and NMIA for the ddPCR™ Supermix for Probes was 3.6 % \((k=2)\) and 4.2 % \((k=2)\), respectively, indicating an appreciable small variation in the volume of the droplets. For the QX200™ ddPCR™ EvaGreen® Supermix, a very similar relative expanded uncertainty associated to the average droplet volume \([3.3 \% (k=2)]\) was estimated indicating also only a small variation of the volume of the droplets with those two different master mixes. On some images, noticeable smaller droplets were observed (results not shown), but according to the manufacturer, those very small droplets are eliminated by the reader and are not used by the software as countable droplets.

**Discussion**

The droplet volume measured using the DG8 cartridge is slightly smaller than the volume generated using the beta-version of the droplet generator \([29]\) demonstrating the importance of re-measuring the droplet volume when new generators or cartridges are released. Equation (1) illustrates that the copy number concentration is inversely related to the average droplet volume \(V_d\). Hence, if the number of positive droplets \(H\) and total number of droplets \(C\) are constant, the use of a smaller droplet volume \(V_d\) in the formula will increase the estimated copy number concentration. This is illustrated in Fig. 4, showing how the estimated copy number concentration per microliter of the plasmid in ERM-AD623c varies depending on the droplet volume \(V_d\) used in the equation.

The copy number concentration measured by ddPCR for the ERM-AD623c was 9454 cp/μL with an expanded uncertainty of 200 cp/μL taking into consideration the measured droplet volume of 0.834 nL in our experimental setup.
Table 4. The uncertainty associated to the average copy number concentration was determined taking into account the variance of the repeatability estimated by the residual of the ANOVA and the variance of the between-day repeatability or the intermediate precision. The certified concentration of 10,300 cp/μL remains slightly higher than the value measured by ddPCR. However, the remaining bias of 8% is statistically no longer significant as it is smaller than the combined uncertainty of the certified value and the measured value.

The bias observed in the first set of experiments using the manufacture-specified droplet volume of 0.91 nL (Fig. 4(a)) disappears when taking into account the real droplet volume as determined in this study (Fig. 4(c)). Indeed, the measured copy number concentration taking into account the real droplet volume increased by 8% bringing it closer to the certified values assigned by dPCR. When applying the measured volume of 0.834 nL to recalculate the copy number concentrations reported in Table 3, the bias observed for the values on the ERM-AD623 series is not statistically significant anymore. The copy number concentrations determined by ddPCR remain nevertheless slightly lower (between 4 and 9%) than those reported by dPCR. This could be attributed to a variability in the partition volume in the Fluidigm 12.765 digital array, since the relative standard uncertainty of the average partition volume for the 12.765 digital array has previously been estimated at 5% [27]. More extensive studies to verify the accuracy of the partition volume in dPCR would be required to exclude this possibility.

The finding of two independent laboratories concerning the droplet volume generated by the DG8-186-3008 has been

### Table 5

| Cartridge | Mastermix                     | Droplet volume (nL) | Total number of droplets |
|-----------|-------------------------------|--------------------|--------------------------|
| DG8       | ddPCR™ Supermix for Probes    | 0.833±0.035        | 622                      |
| DG8 (lot nos. C000021590, C000024241 and C000031616) | QX200™ ddPCR™ EvaGreen® Supermix | 0.830±0.027        | 805                      |
| DG8 (production year 2013) | ddPCR™ Supermix for Probes | 0.860±0.021        | 463                      |

a) Sixteen 2 μL samples were collected sequentially from droplets generated from a single well of a DG8 cartridge and the average droplet volume measured
communicated to the instrument producer Bio-Rad, who has since launched a newer version of the QuantaSoft software (version 1.6.6) using an average droplet volume of 0.85 nL. This newer software version coincides with the placing on the market of a newer droplet generator cartridge (DG8-186-4008) that is supposed to replace the earlier cartridges [Bio-Rad, personal communication]. The value of 0.85 nL used in the software version 1.6.6 is not accompanied with an uncertainty but is lowered enough to avoid the significant bias observed with the earlier software version. No further details on the method used to determine this volume could be obtained from Bio-Rad.

The variability in the volume of droplets generated from different cartridge wells and across different channels correlated with changes in the average fluorescence amplitude reported by the QuantaSoft software (Fig. 5A, B). A slightly stronger fluorescence signal for both positive and negative droplets was observed in larger droplets. This is not unexpected since larger droplets will contain more fluorescence molecules than smaller droplets. It means that a small variation of the fluorescence amplitude among replicates of the same assay (around 10 % in this study) is an indication that droplets with different average volumes were produced in each channel of the droplet generator. In an optimised assay, it is theoretically possible to recalculate the copy number concentration with a correction factor that takes into account the variability of the droplet volume among replicates. This feature may be exploited in the future to improve the precision of ddPCR measurements.

Conclusion

In this study, a reference material certified for the copy number concentration of a plasmid solution over a concentration range from 1 million to 10 cp/μL was used to compare the certified values determined by dPCR with results from another digital PCR method based on droplet emulsion. Copy number concentrations measured by those two digital PCR methods were in good agreement, if the real average volume of the droplets was used to recalculate the assay volume. The volume of 0.91 nL used in the QuantaSoft software version 1.3.2.0 was found to be inaccurate and was the source of the bias observed between the results using the two digital PCR platforms. This study illustrates on the benefit of using a certified reference material to validate methods and procedures involving digital PCR and the risk of relying on data generated by a software which makes assumptions on the value of one or more experimental variables.

For many clinical applications, where copy numbers are expressed as ratios, the uncertainty on the volume—provided the volume stays constant—is not so critical. However, when digital PCR is used to assign ‘absolute’ copy number concentrations, the volume of the assay needs ideally to be confirmed. The droplet volume of 0.85 nL used in the latest software version from Bio-Rad is a value that is very close to the average droplet volume measured in this paper. Assuming that other parameters that affect the number of positive droplets, the total number of droplets and the dilution process are under control, an ‘absolute’ quantification of copy number using that pre-set volume can be reliable. However, digital PCR-based enumeration techniques to quantify bacteria, viruses or whole cells that are encapsulated in the droplets from which the volume is unknown or fluctuating with the applied experimental conditions will require the real volume of the droplets to be taken into consideration [33, 34].

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