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Statistical Analysis of Non-Uniform Volume Distributions for DropLet-based Digital PCR Assays

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Abstract

We present a method to determine the concentration of nucleic acids in a sample by partitioning it into droplets with a non-uniform volume distribution. This digital PCR method requires no special equipment for partitioning, unlike other methods that require nearly identical volumes. Droplets are generated by vortexing a sample in an immiscible oil to create an emulsion. PCR is performed and droplets in the emulsion are imaged. Droplets with one or more copies of a nucleic acid are identified and the nucleic acid concentration of the sample is determined. Numerical simulations of droplet distributions were used to estimate measurement error and dynamic range, and to examine the effects of the total volume of droplets imaged and the shape of the droplet size distribution on measurement accuracy. The ability of the method to resolve 1.5- and 3-fold differences in concentration was assessed by using simulations of statistical power. The method was validated experimentally; droplet shrinkage and fusion during amplification were also assessed experimentally and showed negligible effects on measured concentration.

Graphical Abstract

INTRODUCTION

Polymerase chain reaction (PCR) is a method for amplifying a desired segment of a genomic sequence (DNA, cDNA, or RNA). PCR is an ubiquitous tool used in medical diagnostics^{1–4} to identify genetic defects that cause diseases such as leukemia^{5,6} and Huntington's disease,^{7,8} and to identify over-expressed genes linked to cancer. Development of more accurate and sensitive PCR-based methods to quantify nucleic acids is an important ongoing area of research.

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Supporting Information. Detailed analysis procedures, LabView workflow, shrinkage and fusion figure, table for Poisson distribution-based best-fit concentrations, and other supplemental figures and equations.

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A method widely used to quantify nucleic acids is real-time PCR (qPCR).⁹ In qPCR, increasing signal from a dye that fluoresces when bound to DNA is measured at the end of each thermal cycle. The concentration of a sample is determined by comparing its fluorescence signal with that of a dilution series of a standard. A drawback is that the concentration of the standard must be determined independently, for example by measuring absorbance at 260 nm. For these absorbance measurements it is crucial to have a pure sample because contaminants such as phenol or other reagents from DNA extraction and purification contribute to absorbance at 260 nm.¹⁰

An alternative method for quantifying nucleic acids is digital PCR.^{11,12} In digital PCR, a sample is combined with PCR reagents and partitioned into discrete volumes. Nucleic acid (analyte) molecules are randomly distributed among the volumes. The number of copies of nucleic acid in each volume is assumed to follow a Poisson distribution. Thermal cycling is performed, and volumes which initially contained one or more nucleic acid molecules exhibit increased fluorescence due to DNA amplification. The concentration of nucleic acid molecules in the original sample can be inferred from the number of occupied and unoccupied volumes. An advantage of digital PCR is that it does not rely on an independent measurement of the concentration of a standard.

Compartmentalization of the sample into hundreds or thousands of well-defined volumes is a key part of digital PCR assays and has been predominantly enabled over the past two decades by micro-fabrication technology. Some approaches use micro-fluidic chips to generate and house the compartments. Examples include the SlipChip,^{13,14} Self-Digitization chips,^{15–17} and valve-controlled systems.^{18,19} One general drawback of these methods is the cost and complexity of the microfluidic devices and the custom instrumentation that are often associated with them.

Another major digital PCR approach and the most successfully commercialized is controlled emulsification of samples into droplets of well-defined and near uniform size. The number of occupied and unoccupied droplets are then detected by fluorescence. To ensure generation of uniformly sized droplets and process the droplets, these also require micro-fluidic chips and expensive instrumentation. Besides Bio-Rad's droplet digital PCR (ddPCR) system,^{20,21} other uniform droplet systems include centrifuge step emulsification²² and the Stilla crystal digital PCR system.²³ However, in these systems droplet volume uniformity is an absolute requirement for accurate analysis and is typically assumed for the commercial instruments. This may introduce some systematic errors if the volumes are only nominally uniform.

In the implementation here, we explore a non-uniform volume distribution digital PCR method in which a sample is rapidly partitioned into droplets by bulk emulsification (e.g. via shaking, stirring, or vortexing). This droplet emulsification method is economical, requiring no microchip fabrication and little physical space. Previously, this kind of method has been used as a means for sample containment during PCR or at very low concentrations where the sample would only be present at zero or one copy,^{24,25} but these approaches have limited quantitation ability. In cases where non-uniform droplet volume distributions were examined, the focus has typically been on error analysis of volume variability in nominally

uniform droplet populations and implementation of correction factors to address the issue.
26,27

Here, a non-uniform volume distribution of aqueous droplets are formed simply by vortexing the sample and PCR reagents in an emulsifier-oil mixture, and PCR amplification is performed directly in the resulting emulsion. The method developed here utilizes each and every unique compartment volume that was generated and analyzed to enable quantification regardless of the volume distribution pattern or occupancy fraction. The method involves directly measuring the size of each droplet after the reaction, determining its occupancy and appropriately combining the results for each unique volume using Poisson statistics. Simulations were performed to investigate the dependence of droplet occupancy on analyte concentration and droplet size distribution, and to estimate the accuracy of the measured concentration in the presence of errors in measurement of droplet volume. Simulations also provided an estimate of dynamic range for a given droplet size distribution and statistical power. The method was validated experimentally, including measurement of the effects of droplet shrinkage and fusion during the PCR reaction.

METHODS

Poisson Distribution of Analyte Molecules in Droplets with a Non-uniform Volume Distribution.

This method is used to analyze a system containing N_d droplets. Each droplet has a volume, V_i , and contains solution of nucleic acid (analyte) molecules with a concentration, C . The number of molecules per droplet is assumed to follow a Poisson distribution. The mean number of analyte molecules in a droplet with volume V_i equals CV_i . The probability that a given droplet contains exactly k analyte molecules is then²⁸

$$P(k, CV_i) = \frac{(CV_i)^k}{k!} \exp(-CV_i) \quad (1)$$

The amplification reaction causes a change in droplets containing one or more molecules, making those droplets distinguishable from empty droplets. In this method, we detect only whether a droplet is empty or occupied. The associated probabilities for empty and occupied droplets are

$$\begin{aligned} P(0, CV_i) &= \exp(-CV_i) \\ P(k > 0, CV_i) &= 1 - \exp(-CV_i) \end{aligned} \quad (2)$$

The expected number of occupied droplets (N_E) can be approximated by

$$N_E = \sum_{i=1}^{N_d} (1 - \exp(-CV_i)) \quad (3)$$

After PCR, droplet volumes are measured and the number of occupied droplets in the sample (N_s) is determined. A best-fit value of C can be obtained from Equation (3) by adjusting the value of C so that the difference $N_s - N_E$ is zero. However, it was considered better to recast the problem using the most probable number (MPN) or maximum likely estimate (MLE) formalism.^{29,30} For this case, the following equation¹³ is appropriate:

$$\sum_{i=1}^m n_i V_i = \sum_{i=1}^m \frac{(n_i - b_i) V_i}{(1 - \exp(-V_i C))} \quad (4)$$

In the approach presented here, we use Equation (4), but treat each droplet as a unique, individual volume, since none are identical. This ensures that the exact volumes in the system are utilized, eliminating sources of bias that can come from forcing non-uniform volume distributions into uniform analysis methods, or from assuming that the actual volumes match some predetermined nominal value. The left-hand term is the total volume of all droplets in the system. Since all droplets have different sizes, all n_i equal 1. Since b_i is one for an unoccupied droplet and zero for an occupied droplet, the $(n_i - b_i)$ term causes the right-hand term to be a sum over only occupied droplets. A best-fit value of C can be obtained using Equation (4) by adjusting C so that the sum on the right side of Equation (4) equals the total volume of the droplets. Further details are in the Supporting Information.

Simulation Methods.

For each simulation, a number of droplets, N_d , and a simulation concentration of analyte molecule, C_s , were chosen. A random number generator was used to choose diameters for N_d droplets within a chosen size range and distribution. Equation (2) and a random number generator were used to determine if a particular simulated droplet was occupied or empty. The number of occupied droplets was used with Equation (3) or (4) to obtain a best-fit concentration, C , which was compared with C_s . Unless otherwise stated, the reported concentrations were for best-fit results using Equation (4). Differences between C and C_s reflect the variability in the number of occupied droplets due to randomness in the distribution of analyte molecules.

Our method uses microscopy to determine droplet diameters. The accuracy of this measurement depends on the numerical aperture (NA) of the microscope objective and other aspects of the imaging system. For imaging a large number of stationary droplets, the NA would likely be <1 . In our experience, errors in diameter measurements are typically $<0.5 \mu\text{m}$, independent of the size of the droplet. In order to estimate the effect of measurement error on best-fit concentrations, we simulated a set of droplets and determined whether each droplet was occupied using Equation (2) and the droplet's simulated volume. To estimate the effect of errors in measurement of droplet diameter on the best-fit results, a Gaussian-distributed measurement error was added to the diameter of each droplet, from which a simulated measured volume was calculated. For simulations which incorporate measurement error, the occupancy of each droplet was determined using its simulated volume (without error), while the best-fit concentration was obtained using the simulated measured volume (with error). With measurement error, equations (3) and (4) become

$$N_E = \sum_{i=1}^{N_d} (1 - \exp(-\hat{C}\hat{V}_i)) \quad (5)$$

$$\sum_{i=1}^m n_i \hat{V}_i = \sum_{i=1}^m \frac{(n_i - b_i) \hat{V}_i}{(1 - \exp(-\hat{V}_i \hat{C}))} \quad (6)$$

where $\hat{V}_i$ is the simulated measured volume of each droplet and $\hat{C}$ is the best-fit concentration obtained using those measured volumes. In Equation (6), the left-hand term is the total measured volume of the droplets. The process of fitting data using Equation (5) was more robust with respect to choosing an initial guess of $\hat{C}$ than when using Equation (6), so Equation (5) was used to obtain an initial guess of $\hat{C}$ for Equation (6). Comparisons of best-fit concentrations using Equation (6) with simulated concentrations using Equation (4) illustrate the effects of the Poisson distribution and errors in measurement of droplet volume. We simulated two magnitudes of measurement error, E_1 and E_2 , which are worst-case scenarios chosen to test the robustness of the method. For E_1 , the standard deviation of the Gaussian-distributed error added to each droplet diameter was the larger of 1 μm or 8% of the droplet's diameter. Relative error was included so that the simulation included a non-trivial measurement error for the largest droplets. For E_2 , the standard deviation of the Gaussian-distributed error added to droplet diameter was the larger of 2 μm or 15% of the droplet's diameter. E_1 simulates the worse measurement error that our experiments might produce, and E_2 is roughly twice as large as E_1 . Since the E_2 simulated error is so large the exact shape of the distribution of errors is unlikely to be important so long as it is unbiased.

In practice, it is likely that the imaging system will have upper and lower limits in terms of droplet size. Any droplet whose diameter falls outside these limits will be ignored. Accordingly, we simulated a droplet size distribution with a range that exceeded the desired limits by two standard deviations of the simulated measurement error. For analysis, only droplets with a simulated diameter or simulated measured diameter within the limits were used, and Equation (2) was used with simulated droplet size to determine droplet occupancy.

Since this method involves measuring the diameters of the droplets, there is no requirement that the droplet sizes conform to any particular distribution. To illustrate this, we have simulations using two different distributions of droplet sizes, an even distribution and a clipped lognormal distribution (both described later).

Limits of Quantitation.

Ismagilov and coworkers¹³ developed a body of theory to describe their SlipChip device, which is composed of multiple chambers with predetermined volumes. Some of that theory is applicable to this work. They defined the lower detection limit (LDL) as the concentration at which there is a 95% probability that one or more chambers are occupied, and the upper

limit of quantification (ULQ) as the concentration at which there is a 95% probability that one or more chambers are unoccupied. From Poisson statistics, these limits correspond to concentrations at which, on average, three chambers are expected to be occupied and three unoccupied, respectively. For each distribution simulated, a random number generator was used to choose diameters for N_d droplets within a chosen size range and distribution. Data were then fit twice using Equation (3), once with $N_E = 3$, and once with $N_E = N_d - 3$. This fit was repeated 500 times for each N_d and distribution, and the results were averaged to obtain LDL and ULQ.

Estimation of Confidence and Power.

We also characterized the method by determining the range of concentrations over which it can resolve specified differences in concentration. The ability of a method to distinguish a difference in concentration can be described in terms of confidence and power.^{31,32} If measurements on two samples yield two different best-fit concentrations ($\hat{C}_1$ and $\hat{C}_2$), it is useful to know how confident one would be in asserting that the concentrations were different. The risk of a false-positive result (Type I error) is controlled by requiring that the results have a specified minimum confidence. The risk of a false-negative result (Type II error) is controlled by requiring that the method have a specified minimum power.

A 3-fold change (0.5 log) in HIV viral load at a confidence level of 95% and a power level of 95% is considered clinically significant.^{33,34} A proposal for a similar standard has been made for quantitation of cytomegalovirus.³⁵ Therefore, we determined the range of concentrations for which our method could resolve a 3-fold change in analyte concentration at confidence and power levels of at least 95%. To illustrate the method's capabilities in more demanding situations, we also determined the range of concentrations for which our method could resolve a 1.5-fold change in analyte concentration.

For comparison of best-fit concentrations from two samples, the null hypothesis would be that the two samples have the same (unknown) concentration. If α is the probability that two samples with the same concentration result in best-fit concentrations that differ in magnitude by more than $|\hat{C}_1 - \hat{C}_2|$, then $(1 - \alpha)$ is the confidence associated with rejecting the null hypothesis. The acceptable minimum value of $(1 - \alpha)$ is chosen by the researcher or clinician to limit false-positive results.

One method used to estimate confidence levels is the Z-test:

$$Z = \frac{\hat{C}_1 - \hat{C}_2}{\sqrt{\sigma_1^2 + \sigma_2^2}} \quad (7)$$

A confidence level of 95% is a common choice and requires $Z > 1.96$. $\hat{C}_n$ are the best-fit results of Equation (6) and σ_n^2 is the variance associated with measurement of $\hat{C}_n$. Kreutz et al.¹³ derived an expression for σ_n^2 used in their Z-test. A tractable analytical expression for σ_n^2

does not appear to exist for our method, so it was necessary to estimate σ_n^2 by simulation. Furthermore, as noted by Kreutz et al., one limitation of the Z-test is that it assumes that the measurement results are normally distributed. Since this is not necessarily the case, a second simulation to estimate the confidence was performed and compared with the Z-test results. The method of determining the confidence levels from a simulation using the Z-test will be called the Z-method. The alternate method, which does not assume that the measurements are normally distributed, will be called the P-method. These two methods are described in detail in the Supporting Information. The results in the Supporting Information show that these two methods yield very similar results for the confidence levels and so either may be used. This agreement is important because, in practice, an experimenter will only have the information used in the Z-method. This provides reassurance that confidence can be estimated without detailed knowledge of either the droplet size distribution or the measured diameters if all measurement error could be eliminated. While the analytical expression of Kreutz *et al.* is an appealing feature of their work, the Z-method appears to be reasonably accurate. While we used the P-method to calculate statistical power, in practice the Z-method is a reasonable alternative and computationally less expensive.

Simulations were performed to estimate the power at a given concentration (C_{s1}) and resolution. 200 simulations were performed using the P-method with C_{s1} and a second concentration (C_{s2}) which was either 1.5-fold or 3-fold larger. For each set of 200 simulations, the power was defined as the fraction of simulations for which the confidence ($1 - \alpha$) exceeded 0.95. This was the probability that two samples with concentrations C_{s1} and C_{s2} would be resolved as different if a confidence of 95% was required. This process was repeated over a range of concentrations to obtain plots of the statistical power. The results yielded an estimate of the power for 1.5- and 3-fold resolutions for the simulated droplet size distribution.

PCR Reagents and DNA.

A PrimeTime® qPCR Assay (HS.PT.47.3482985.g) was purchased from Integrated DNA Technologies (Coralville, IA) and was used to extract ERBB2 dsDNA (235 base pairs) from human male DNA (Applied Biosystems, Foster City, CA). The ERBB2 product was purified using a Min- Elute PCR Purification Kit (Qiagen, Valencia, CA) and verified by gel electrophoresis. The assay and purification procedure was repeated on the purified product to remove off-target sequences. The forward and reverse primer sequences were 5'-TCACACCATAACTCCACACATC-3' and 5'-CGAGACAGAGTACCATGCAG-3', respectively, and the double-quenched probe (internal ZEN and 3'-Iowa Black FQ) contained a 5' 6-carboxyfluorescein (6-FAM) fluorophore.

The PCR stock reagents used consisted of 10 mM dNTP (Life Technologies, Carlsbad, CA), 20 mg/mL bovine serum albumin (BSA; Roche Diagnostics, Indianapolis, IN), 50x carboxy-X-rhodamine (ROX) passive reference dye (Bio-Rad, Hercules, CA), and Bio-Rad's iTaq kit, which contained 10x iTaq buffer (200 mM Tris-HCl, pH 8.3, and 500 mM KCl), 50 mM MgCl₂, and 5,000 U/μL iTaq DNA polymerase. The general reaction (50 μL) was prepared following the manufacturer's protocol: 31.5 μL nuclease-free H₂O, 5 μL iTaq buffer, 1.5 μL

MgCl₂, 1.5 μL BSA, 1 μL ROX, 1 μL dNTP, 2.5 μL 20x primers and probe mix (Integrated DNA Technologies), 5 μL DNA template, and 1 μL iTaq polymerase.

Generating Water-in-Oil Droplets.

An emulsifier-oil mixture was prepared using 7% (w/w) Abil WE09 (Evonik Industries, Essen, Germany), 20% (w/w) light mineral oil (M8410; Sigma-Aldrich, St. Louis, MO), and 73% (w/w) Tegosoft DEC (Evonik Industries). Abil WE09 serves as the emulsifier, and Diehl *et al.* has previously demonstrated that water-in-oil emulsions generated with this mixture are stable under PCR conditions²⁴. The oil mixture was vortexed for 10 s at 3000 rpm and incubated at room temperature for 30 min before use. A 300 μL aliquot of emulsifier-oil and 50 μL of PCR solution were combined in a microcentrifuge tube and vortexed at 3000 rpm for 30 s at 10 s intervals. The resulting water-in-oil emulsion of non-uniform volume distribution was aliquoted into two or more PCR tubes (50 μL each) and heated in a C1000 Thermal Cycler (Bio-Rad) for 3 min at 95°C followed by 50 cycles of 30 s each at 95°C, 54°C, and 72°C. A schematic of the procedure is shown in Figure 1.

Droplet Imaging.

Emulsion droplets were imaged using a Zeiss LSM 510 confocal microscope in multi-track mode and a PLAN APO 20X, 0.75 NA objective. Laser source excitation wavelengths of 543 nm (LP 610) and 488 nm (BP 500–530) were used to collect fluorescence signals from the ROX and FAM dyes. For each field of view, a series of optical sections (z-stack) was imaged at varying z-axis depths at 6.4 μs per pixel and a scan speed of 15.73 s to build a 3D profile of droplet dimensions. A single image was created from the mean of four scans. Using the instrument's native operating program, a minimum of four random field of views were imaged for each sample without overlap. For most of the experiments discussed herein, optical sections were imaged at 3 μm apart and 24–30 μm along the z-axis.

A custom program (LabView 2010; National Instruments, Austin, TX) used a background threshold and blob analysis on the ROX signal to identify droplets in a single image (Figure S2, Supporting Information). Droplets large enough to appear in more than one image of the z-stack were identified by position tracking through successive images. Droplets imaged multiple times often had a different apparent diameter in each image; the largest measured diameter was used. The program also determined ROX and FAM signals of each droplet to determine droplet occupancy.

RESULTS AND DISCUSSION

Sensitivity of Best-fit Concentrations to Errors in Measured Droplet Diameter.

Simulations were performed with 10,000 droplets and seven concentrations: 1.0×10^7 , 1.0×10^6 , 1.0×10^5 , 1.0×10^4 , 1.0×10^3 , 1.0×10^2 , and 1.0×10^1 molecules/μL. Droplet sizes were drawn from an even distribution of diameters between 4 and 190 μm. The total volume of the droplets was 9.5 μL. The smallest droplets (4 μm) were not substantially larger than typical errors in measuring droplet diameters by light microscopy (1 μm standard deviation). The resulting errors in droplet volume could be significant and might be expected to degrade the accuracy of this method. To test this, simulations obtained the best-fit

concentration of analyte using both simulated volumes and simulated measured volumes (calculated from diameters with Gaussian-distributed error of magnitude E_1 or E_2). Table 1 compares the average and standard deviations for the best-fit concentrations from fitting Equations (4) and (6) to a series of simulations. Sets of simulations were performed using E_1 and E_2 . For each concentration, 1000 simulations were performed, each with 10,000 droplets. Simulation results using Equation (4) represent best-fit concentrations obtained when droplet volumes were known with perfect accuracy; results using Equation (6) include the effect of measurement error.

Differences between best-fit concentrations using Equations (4) and (6) were small except at the largest concentrations. Even when the droplet diameter measurement error was $\pm 2 \mu\text{m}$ or 15% (E_2), the error in the concentration measurement reached 20% only for the largest and smallest concentrations. Furthermore, for each concentration, the standard deviations of the best-fit concentrations using Equations (4) and (6) were nearly the same (except for the largest concentration), without any apparent systematic difference. This suggests that for most C_s values in Table 1, the largest source of variability in best-fit concentration was the variation in droplet occupancy number from one simulation to the next. Any unbiased variability in best-fit results due to droplet size measurement error had a small effect on best-fit concentration. This was despite the fact that, for the smallest droplets ($\sim 4 \mu\text{m}$) and simulations using a $2.0 \mu\text{m}$ error (E_2), one standard deviation of the error was close to half the diameter. This is important since it means that this method does not require unreasonably high accuracy in the measurement of droplet diameter. Instead it requires that any measurement error be mostly unbiased. This unexpected result can be explained with the help of Figure 2 and (Equation 3).

The cumulative number of occupied droplets for a single simulation of 10,000 droplets and a concentration of 2.0×10^{-6} molecules/fL is shown in Figure 2a. Droplets diameters were evenly distributed between 4 and $190 \mu\text{m}$. The droplets were sorted from smallest to largest and the probability that a droplet was occupied was calculated for each droplet. The ordinate of the n th point is the sum of these probabilities for all droplets with diameters less than or equal to that of the n th droplet. It equals the expected cumulative number of occupied droplets among the n smallest droplets in the distribution. The curve is divided into three regions: Low, Transition, and High. In the Low region, the probability that a droplet is occupied is essentially zero. In the High region, the probability that a droplet is occupied is close to one, and the curve rises to a near unit slope. In the Transition region, the probability that a droplet is occupied increases from zero to one. Measurement errors for droplets within the Low or High regions had little or no effect on the total number of occupied droplets. A measurement error on a droplet in the Low region simply replaced a term in the sum in (Equation 3) which was nearly zero with a different term which was also nearly zero. A similar effect occurred in the High region, where measurement errors replaced terms in Equation (3) which were nearly 1.0 with other terms also nearly 1.0. Neither of these measurement errors is expected to make a significant change in the best-fit concentration. For droplets in the Transition region, some measurement errors resulted in terms in Equation (3) being replaced with larger or smaller values. If the errors were unbiased, as in the simulations in Table 1, there appeared to be sufficient cancellation to result in modest error for most simulated concentrations. Since the best-fit concentrations using Equations (3) and

(5) were nearly identical to those using Equations (4) and (6), cancellation of errors must have occurred in the latter case as well.

The effect of the Gaussian-distributed measurement errors (Table 1) on the distribution of diameters was noticeable if the Transition region occurred at one end of the distribution. For example, the smallest droplets in these simulations had diameters $\sim 4 \mu\text{m}$. When measurement error was included, some simulated droplets with diameters $< 4 \mu\text{m}$ had simulated measured diameters $> 4 \mu\text{m}$ and were included in the fit. The probability that those droplets were occupied would be less than suggested by their simulated measured diameters. Unlike the case where the Transition region was in the middle of the size distribution, there was no cancellation, since droplets with diameters $> 4 \mu\text{m}$ but simulated measured diameters $< 4 \mu\text{m}$ were simply not included in the fit. For large concentrations, this resulted in fewer occupied droplets than expected, and the best-fit concentration was too small, as seen in Table 1. The opposite effect occurred at low concentrations, where the Transition region was at the upper end of the size distribution, resulting in best-fit concentrations which were too large. It should be noted that this affect was appreciable only for the unrealistically large E_2 measurement error.

The range of droplet diameters used in these simulations was chosen so that any reasonably sized droplet volume measurement error might be expected to have a noticeable effect on attempts to estimate the concentration from the total number of occupied droplets. However, this did not appear to occur even though the range of concentrations simulated (10^6) was larger than the range of droplet volumes simulated (10^5).

Any real process used to generate varying sizes of droplets is more likely to produce a peaked rather than an even distribution. So in a further test, a clipped lognormal distribution was used to generate droplet diameters. An example of a droplet diameter distribution from one simulation is shown in Figure 2b, where only droplets with diameters between 5 and 61 μm are included. The distribution qualitatively resembles some of the droplet size distributions obtained in our emulsions. This distribution was used in the same type of simulations performed for Table 1 and the results shown are in Table 2. These simulations had 10,000 droplets with a total volume of 0.06 μL .

For simulations with an analyte concentration of 1.00×10^2 molecules/ μL , a small fraction ($< 3\%$) of the simulated droplet samples had no occupied droplets (not shown). For the purposes of Table 2, the lowest concentration included was 5.00×10^2 molecules/ μL . As in Table 1, for most concentrations there was little difference between concentrations obtained with and without inclusion of measurement error. The largest effect occurred for the largest concentration.

The results shown in Tables 1 and 2 suggest that it should be possible to obtain an accurate estimate of analyte concentration over a large concentration range. Noticeable errors in concentration were observed only if an unreasonably large error was assumed for measurement of droplet size. Thus, this method should be robust with respect to unbiased error in measurement of droplet size. The experimental results described below also show

that measurement error is not a significant problem for the emulsion we used, even with the possibility of biased error.

Limits of Quantification.

LDL and ULQ were determined for four different distributions (Table 3). Simulations of 10,000 droplets from either the even or lognormal distribution were performed, and the resulting distributions were fit using Equation (5) with N_E set first to 3 and then to 9,997. The measurement error in droplet sizes was E_1 . The simulations were performed 500 times and the resulting best-fit concentrations were averaged to obtain LDL and ULQ for the two distributions. For comparison, LLD and ULQ were obtained for two distributions of uniform sized droplets with diameters of 122 and 22.6 μm , with total volumes of 9.5 and 0.06 μL , respectively, corresponding to the average total volumes of the even and lognormal distributions. No measurement error was included in these simulations. In Table 3, LDL and ULQ of the even and lognormal distributions are compared with those of the uniform size distributions with the same number of droplets and total volume. LDL values were similar, in all cases being the concentration which, on average, resulted in three copies of analyte molecule in the total volume. However, ULQ values were significantly larger for the even and lognormal distributions than for the corresponding uniform droplet size distributions. For the uniform distributions, ULQ corresponded to the concentration at which, on average, only three of 10,000 droplets were unoccupied. The even and lognormal distributions contained droplets that were both larger and smaller than the uniform distributions, for the same total volume. As a result, the concentration necessary to reduce the number of unoccupied droplets to an average of only three of 10,000 droplets was larger than for the corresponding uniform distribution. This resulted in a larger ULQ and a larger range of quantitation for the even and lognormal distributions than for uniform distributions. These results are similar to those obtained by Ismagilov and coworkers using SlipChip chambers with different predetermined volumes.¹³

Statistical Power.

The power provides an estimate of the likelihood of a false negative. A reference and test concentration which differ by a given percentage difference were chosen and 200 simulations were performed using the P-method to determine the fraction of results which were too close together to satisfy a required confidence level (in this case 95%). The result was the fraction of results (β) which would yield false negatives, and the statistical power was $(1 - \beta)$. The results for this test are shown in Figure 3a–d for sets of 10,000 droplets. The different panels show the resulting power for the four different distributions of droplet diameter. Figure 3a shows the result for an even distribution with a diameter range of 4 to 190 μm , and includes both E_1 and E_2 measurement errors. For comparison, Figure 3b shows the results for a set of uniform sized droplets of 122 μm diameter and the same total volume (9.5 μL). This simulation included no error in measurement of droplet volume, to correspond to methods where droplet volume is rigorously controlled. The other parameters were the same as used in the P-Method for estimating the confidence. For each panel, sets of simulations were performed spanning the concentration range 1 to 6.3×10^7 molecules/ μL . Each concentration on the abscissa was tested against concentrations which were 50% and 200% larger (1.5-fold and 3-fold resolution, respectively).

In Figure 3a, the range of concentrations for which the power was >0.95 spanned a concentration range $>10^5$ for a 1.5-fold resolution and $>10^7$ for a 3-fold resolution. Figure 3 shows that using droplets with a range of sizes provides a significantly larger dynamic range than a set of uniform sized droplets. The set of 10,000 droplets used for the simulations in Figure 3b had the same total volume as in Figure 3a, but a smaller dynamic range. In Figure 3b, the range of concentrations for which the power was >0.95 was <1000 for a 1.5-fold resolution and $<10,000$ for a 3-fold resolution. The advantage of using a range of different volumes in digital as-says has been noted previously; these results provide another example.

These simulations were repeated for the clipped lognormal distribution from Figure 2b, and the results are shown in Figure 3c and 3d. Besides the distribution, the parameters were the same as in Figure 3a and 3b. Figure 3d shows the results for a set of uniform sized droplets of $22.6\text{ }\mu\text{m}$ diameter and includes no error in the measurement of droplet volume. Droplet sets in Figures 3c and 3d had the same total volume ($0.06\text{ }\mu\text{L}$).

In Figure 3c, the range of concentrations for which the power was >0.95 was $>10^4$ for a 1.5-fold resolution and $>10^5$ for a 3-fold resolution. For the uniform droplet size distribution in Figure 3d, the range of concentrations for which the power was >0.95 was <1000 and $<10,000$, respectively. These results provide another example of the improvement in dynamic range provided by using a distribution of volume sizes rather than a single size.

Details of the dynamic range depended on the shape of the droplet size distribution and the total volume (or total number of droplets) used. The clipped lognormal distribution of droplet diameters has a peak at $8\text{ }\mu\text{m}$. As a result, its dynamic range extends to slightly higher concentrations than for the even distribution, despite the latter's larger total volume. The clipped lognormal distribution has a larger number of small droplets (whose occupancy is more useful in determining higher concentrations) than the even distribution. The even distribution has more large droplets, so it is not surprising that it was more sensitive than the clipped lognormal distribution. Both the even and clipped lognormal distributions provided a larger dynamic range than uniform size distribution of droplets of the same total volume and number.

Fidelity of Measured Droplet Distribution.

The simulations described above suggest that unbiased error in droplet diameter has a negligible effect on the best-fit concentration. However, in experimental conditions, droplet sizes may change during PCR for various reasons, such as evaporation or fusion, leading to biased errors which the simulations fail to address. The amount of evaporation and fusion which might occur in this method depend on the properties of the emulsion used. Evaporation may occur within droplets during thermal cycling, altering the distribution of droplet diameters and volumes. Droplets in proximity to one another may fuse to reduce the overall surface tension of the system. For these reasons, we characterized the effects of heating and mechanically manipulating (e.g., pi-petting) the emulsion system on the accuracy of the droplet size measurements.

Droplet Fusion.

Two separate emulsions containing either FAM probe or ROX probe were prepared using the general PCR mix (Figure S1, Supporting Information). Briefly, the two emulsions were combined, aliquoted, and thermal cycled. Aliquots were collected at time zero, 1-cycle and 50-cycles for confocal imaging. The detailed procedure can be found in the Supporting Information.

The ROX and FAM signals of the emulsion mixture were used to identify fusion events. Droplets marked with a white circle contained both dyes (Figure S4a and S4b, Supporting Information). FAM and ROX fusion events caused by post-emulsification droplet instability, sample handling, and pipetting were shown to be minimal: fusion occurred at a rate of $6.2 \pm 2.1\%$ (Figure S4c, Supporting Information). This measurement did not include fusion events between droplets containing the same dye, which would be expected to be comparable in frequency. The distributions in Figure S4d (Supporting Information) have a peak at the smallest diameters and a tail extending to the larger diameters and so resemble the clipped lognormal in Figure 2. Thus, the statistical analysis of the clipped lognormal is illustrative of the results which can be obtained from samples with such a distribution. After a hot start and one thermal cycle, the emulsion sample still had a comparable total rate of fusion events, $6.1 \pm 1.4\%$. The FAM-ROX fusion events detected for 50-cycles, $5.8 \pm 1.5\%$, was similar to that of 1-cycle. Both heating conditions were well within the range of error of the control aliquot.

It is important to note that fusion events did not appear to progressively increase with the duration of the thermal cycling. This suggests that heating during thermal cycling induced little to no droplet fusion. The main factors contributing to droplet instability were related to the mixing of the two color emulsions and sample handling. This is significant because fusion events that occur before or early during thermal cycling will not skew the results of the assay, since merged droplets that contain at least one target molecule will undergo amplification and give a positive signal, whereas merged droplets that do not undergo amplification will give a negative signal. In contrast, fusion events occurring later during thermal cycling between an occupied droplet and an unoccupied droplet can result in a merged droplet falling below the binary detection threshold of an assay, resulting in the droplet being incorrectly characterized. The results of the experiment indicated that fusion should have a minimal effect on sample quantification when using this method. Furthermore, if sample handling were to be entirely eliminated from the process by performing emulsification and thermal cycling all on the same device, fusion can have even less of an impact on the results of the assay.

Droplet Volume Loss from Heat Exposure.

The initial volume of the droplet is critical for accurately quantifying the initial sample concentration. Here, the droplets are sized post-thermal cycling, and it is important to know whether the droplet volumes are affected by the heating element. An analysis of droplet shrinkage was carried out by comparing the droplet size distribution of a negative control and thermal cycled emulsion samples. Emulsions were prepared using the standard PCR mixing and emulsification process. The same thermal conditions as the fusion experiments

(Figure S1c1–c3, Supporting Information) were examined to quantify changes in droplet size during repetitive thermal cycling. Smaller volume droplets have larger surface-to-volume ratios than larger droplets and are more at risk to volume loss from evaporation. To track this possibility, z-stacks were collected with 1- μm spacing between optical slices to ensure the accuracy of tracking of the smallest droplet diameter (7 μm). Figure S3d (Supporting Information) compares the droplet diameter distribution of the emulsions subjected to no heating, one thermal cycle, or 50 thermal cycles. Diameter sizes were normalized to the total droplets in the distribution and sorted into 2 μm bins. The control set had a mean diameter of $15.4 \pm 0.1 \mu\text{m}$ (1857 droplets measured). The emulsions subjected to one thermal cycle had a mean diameter of $14.9 \pm 0.4 \mu\text{m}$ (1952 droplets measured) while those subjected to 50 thermal cycles had a mean diameter of $15.8 \pm 0.5 \mu\text{m}$ (1660 droplets measured). The distributions of droplet diameters for the three conditions were indistinguishable, indicating that thermal cycling did not cause significant changes in droplet volume. Therefore, it was not required to image the droplet populations prior to thermal cycling, and post-thermal cycling we can reasonably expect very little systematic error in the measurement of the droplet diameters.

Experimental Validation.

A Nanodrop 2000c UVVis spectrophotometer (Thermo Scientific, Waltham, MA) was used to measure absorbance at 260 nm (A_{260}) of the stock ERBB2 dsDNA solution. Concentrations (c) were calculated using the Beer-Lambert Law, $A_{260} = \epsilon c l$, with $\epsilon = 2,784,500 \text{ M}^{-1} \text{ cm}^{-1}$ for our DNA sequence and a path length of 1 cm. A serial dilution of template in TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) was added to the PCR mix and emulsified for thermal cycling. Two aliquots at each dilution concentration were collected to evaluate sampling consistency. Z-stacks of the amplified products were imaged with 3- μm spacing between optical slices and processed in LabView. The ratio of the FAM/ROX mean value of identified droplets was plotted against the frequency of droplets (Figure 4b1–d1). The FAM mean intensity was normalized to the ROX signal to account for non-PCR-related signal fluctuations.

Selecting the cutoff for the binary detection threshold of the FAM/ROX ratio required inspection of fluorescence signal intensities of amplified droplets with varying populations of occupancy. For this set of experiments, emulsion populations with mostly unoccupied droplets (such as in Figure 4b) averaged 0.351 FAM/ROX ($N=1701$ droplets). In mixed populations (Figure 4c), occupied droplets consistently displayed FAM/ROX signals >0.625 . This value was over 1.5 times greater than the mean for the unoccupied droplets, and was used as the binary detection threshold for amplification. The Poisson distribution-based best-fit concentrations (Table S2, Supporting Information) of the serial dilutions over the diameter range of 7 to 50 μm (1 to 500 pL volumes) were determined as described in the Data Fitting section of the Supporting Information. The best-fit concentrations were compared to the expected concentrations derived from the A_{260} measurement (Figure 4a). The diameter range of 7–60 μm (1 to 900 pL volumes) gave similar results. Fitted concentrations had a linear relationship of $y=0.771x$, $R^2=0.990$ in relation to the expected concentrations.

Estimates of the measurement error (σ) are obtained from a procedure described in the Supporting Information. In Figure 5a–c, error bars of $\pm 2\sigma$ are plotted for each aliquot. Aliquots 1 and 2 (filled and open symbols) from each serial dilution series (replicates a–c) agreed well with each other, indicating within each experiment the results were consistent. The greatest amount of variation in replicate measurements was observed at the lowest concentration (10^3 copies/ μL), most likely a result of the Poisson error being greatest at the extreme ends of a concentration curve.¹³ We can decrease the variation by increasing the number of z-stacks imaged and improving the sensitivity at lower concentrations with more droplets and/or a larger total volume sampled. Furthermore, at most concentrations, the fitted concentrations were slightly lower than expected concentrations derived from the UV-Vis A260 measurement. The A260/280 ratio for the stock solutions were on average ~ 2.0 , which is within acceptable purity levels for DNA. However, this method does not discriminate between measured nucleic sites for single- and double-stranded DNA. Thus, there is a possibility of over-estimating the concentration using the UV-Vis method.

The number of droplets imaged in our experimental test was limited by two factors. First, our droplet recognition algorithm has difficulties separating clusters of droplets which are touching; such droplets are ignored. Second, a long time was required to acquire a single z-stack. We are currently developing a revised version of the algorithm which will enable accurately distinguishing droplets in a cluster. Other imaging techniques, such as using a Nipkow disc or epi illumination combined with de-convolution, could increase throughput by enabling imaging of a larger number of z-stacks. The combined area scanned for a set of four z-stacks was $\sim 1 \text{ mm}^2$, and we were typically able to identify, size, and determine the occupancy of ~ 1500 droplets. Plates with flat-bottom wells for fluorescence imaging typically have an area of 10 mm^2 for 384-well plates and 31 mm^2 for 96-well plates. Improvements to the droplet recognition algorithm combined with imaging a larger area should provide an adequate number of droplets with sufficient total volume to obtain accurate determinations of concentration. Changing from scanning confocal microscopy to a different imaging technique should increase the throughput of this method so that it will provide a useful alternative to current methods.

CONCLUSIONS

We present a digital PCR assay for determining nucleic acid concentration in a sample using a nonuniform volume distribution. The method uses an emulsion to create separate volumes for PCR followed by imaging. No special equipment is required to create the emulsion, and the range of droplet sizes increases the dynamic range beyond what could be achieved with a uniform droplet size distribution. Simulations were used to estimate errors in best-fit concentrations and their dependence on the number of droplets, distribution of droplet sizes, and sample concentration. The effect of unbiased errors substantially larger than any that might reasonably be expected was modest.

The results showed a significant improvement in dynamic range using an emulsion with a range of droplet diameters versus a uniform size distribution, as demonstrated previously.^{13,14} This is true for the dynamic range defined by LDL and ULQ, and for the range of concentrations for which the methods can resolve 1.5- and 3-fold differences in

concentration with >95% confidence and power levels. For an even distribution with 9.5 μL total volume, the dynamic range was $>10^7$; for a lognormal distribution with 0.06 μL total volume, the dynamic range was $>10^5$.

An experimental proof of concept is presented for our method. A DNA concentration range of 10^3 - 10^6 copies/ μL was assessed, and the results were comparable to those determined by using absorbance. The linearity of the results along with experimental tests for droplet shrinkage or fusion argue against any biased errors affecting the determined concentrations. This method should be applicable to other digital assays including isothermal and reverse transcription methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A PCR/oil mixture (a) was vortexed (b) to create an emulsion of non-uniform volume distribution. (c) Emulsion aliquots underwent PCR in a thermal cycler. (d) Aliquots were transferred to a well-plate for imaging using scanning confocal microscopy. The red fluorescence image displays signal from the ROX dye (present in all droplets); the green image displays signal from the FAM dye (significant only in occupied droplets).

Figure 2.

(a) The cumulative number of occupied droplets for a simulation of 10,000 droplets and a concentration of 2.0×10^{-6} molecules/fL, ordered by droplet size. Other details were the same as in Table 1. (b) Clipped lognormal distribution used for simulations in Table 2.

Figure 3.

(a, b) Estimated power for a set of 10,000 droplets with a total volume of 9.5 μ L. The green lines in each subplot are for a 1.5-fold resolution, and the blue lines are for a 3-fold resolution. The dotted line indicates a power of 0.95. (a) Simulated power with droplets from an even size distribution (4 to 190 μ m diameter). Dashed lines are for measurement errors of size E_1 , solid lines for measurement errors of size E_2 . (b) Simulated power for set of droplets with a uniform diameter of 122 μ m. Simulations assume droplet sizes are known exactly. (c, d) Estimated power for a set of 10,000 droplets with a total volume of 0.06 μ L from a clipped lognormal distribution (c) and from a uniform size distribution with a diameter of 22.6 μ m (d).

Figure 4.

(a) Best-fit concentration determined by emulsion PCR (ePCR) plotted against the concentration determined by measurement of absorbance at 260 nm. Samples were from a 10x serial dilution series (1×10^7 , 1×10^6 , 1×10^5 , and 1×10^4 dilutions) of purified ERBB2 DNA. Triangles: replicate A (2.26×10^{10} copies/ μL stock DNA concentration); Squares: replicate B (2.81×10^{10} copies/ μL); Circles: replicate C (3.08×10^{10} copies/ μL). For each replicate, two aliquots were collected at each dilution, denoted in the graph by either filled or open symbols. Best-fit concentrations were calculated from droplets with diameters in the range of 7–50 μm . The linear regression line through zero for all replicates is $y=0.771x$ and $R^2=0.990$. Measurement errors are not shown here, but can be seen in Figure 5. (b-d) DNA amplification was determined by FAM to ROX ratio histograms of amplified droplets containing (b1) 2.26×10^3 , (c1) 2.26×10^6 , and (d1) 2.26×10^7 copies of double-stranded ERBB2 DNA per μL ; bin size, 0.025. In the corresponding confocal imaged droplet insets (b2–d2), the ROX (red) and FAM signals (green) were colored and merged to depict droplet amplification (yellow). The inset scale bars are 50 μm .

Figure 5.
(a-c) The three serial dilutions described in Figure 4 (same symbols used). Error bars indicate $\pm 2\sigma$.

Table 1.Results for an even distribution (4–190 μm).

Simulation	Best-fit using V		Best-fit using $\hat{V}$	
	C_s	C	C	σ_c
E_1 ($\pm 1.0 \mu\text{m}$ or 8% error ^(b))				
1.00×10^1 ^(a)	1.00×10^1	1.08	1.06×10^1	1.07
1.00×10^2	1.00×10^2	3.48	1.05×10^2	3.47
1.00×10^3	1.00×10^3	1.72×10^1	1.02×10^3	1.90×10^1
1.00×10^4	1.00×10^4	2.38×10^2	1.00×10^4	2.67×10^2
1.00×10^5	1.00×10^5	3.54×10^3	1.00×10^5	3.95×10^3
1.00×10^6	1.00×10^6	5.35×10^4	9.97×10^5	5.62×10^4
1.00×10^7	1.00×10^7	7.96×10^5	9.26×10^6	8.30×10^5
E_2 ($\pm 2.0 \mu\text{m}$ or 15% error ^(b))				
1.00×10^1	9.96	1.08	1.20×10^1	1.21
1.00×10^2	1.00×10^2	3.37	1.18×10^2	3.79
1.00×10^3	1.00×10^3	1.82×10^1	1.05×10^3	1.96×10^1
1.00×10^4	1.00×10^4	2.42×10^2	1.01×10^4	2.65×10^2
1.00×10^5	1.00×10^5	3.42×10^3	1.01×10^5	3.77×10^3
1.00×10^6	1.00×10^6	5.39×10^4	9.74×10^5	5.53×10^4
1.00×10^7	1.00×10^7	8.23×10^5	7.40×10^6	6.27×10^5

^(a) All concentrations are in molecules/ μL .^(b) The larger of the fixed and relative errors for each droplet was used as the standard deviation of the Gaussian-distributed error. The simulated diameter (without error) and simulated measured diameter (with error) were used to calculate the simulated volume (V) and simulated measured volume ($\hat{V}$), respectively.

Table 2.Results for clipped lognormal distribution (5–61 μm)

Simulation	Best-fit using V			Best-fit using $\hat{V}$	
	C_s	C	σ_c	C	σ_c
E_1 ($\pm 1.0 \mu\text{m}$ or 8% error) ^(b)					
5.00×10^2 ^(a)	5.03×10^2	8.69×10^1	5.01×10^2	8.70×10^1	
1.00×10^3	1.01×10^3	1.27×10^2	1.01×10^3	1.26×10^2	
1.00×10^4	1.00×10^4	4.53×10^2	9.97×10^3	4.55×10^2	
1.00×10^5	1.00×10^5	2.25×10^3	9.98×10^4	2.28×10^3	
1.00×10^6	1.00×10^6	1.93×10^4	9.97×10^5	1.93×10^4	
1.00×10^7	1.00×10^7	2.81×10^5	9.53×10^6	2.70×10^5	
E_2 ($\pm 2.0 \mu\text{m}$ or 15% error)					
5.00×10^2	5.04×10^2	9.09×10^1	5.00×10^2	8.91×10^1	
1.00×10^3	9.95×10^2	1.29×10^2	9.90×10^2	1.25×10^2	
1.00×10^4	1.00×10^4	4.34×10^2	9.83×10^3	4.35×10^2	
1.00×10^5	1.00×10^5	2.31×10^3	9.90×10^4	2.37×10^3	
1.00×10^6	1.00×10^6	1.95×10^4	9.83×10^5	1.99×10^4	
1.00×10^7	1.00×10^7	2.65×10^5	8.58×10^6	2.46×10^5	

^(a) All concentrations are in molecules/ μL .^(b) The larger of the fixed and relative errors for each droplet was used as the standard deviation of the Gaussian-distributed error.

Table 3.

Quantitation limits from simulations of 10,000 droplets

Distribution (Range of droplet diameters)	Total Volume (μL)	LDL (molecules/ μL)	ULQ (molecules/ μL)	1.5-fold resolution (molecules/ μL)		3-fold resolution (molecules/ μL)	
				Absolute Range	Log(Range)	Absolute Range	Log(Range)
Even (4–190 μm)	9.5	0.3	6.5×10^7	$1.4 \times 10^1 - 6.3 \times 10^6$ (a)	5.65	$1.2 - 4.2 \times 10^7$ (a)	7.54
Uniform (122 μm)	9.5	0.3	8.5×10^3	$1.4 \times 10^1 - 6.3 \times 10^3$	2.65	$1.4 - 6.3 \times 10^3$	3.65
Lognormal (5–61 μm)	0.06	48	6.3×10^7	$2.0 \times 10^3 - 6.1 \times 10^7$ (a)	4.48	$2.2 \times 10^2 - 6.3 \times 10^7$ (a)	5.46
Uniform (22.6 μm)	0.06	50	1.3×10^6	$2.2 \times 10^3 - 1.0 \times 10^6$	2.66	$2.3 \times 10^2 - 1.0 \times 10^6$	2.64

(a) Range with simulated measurement error (E) included.