

## Video Article

# Simple Bulk Readout of Digital Nucleic Acid Quantification Assays

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## Abstract

Digital assays are powerful methods that enable detection of rare cells and counting of individual nucleic acid molecules. However, digital assays are still not routinely applied, due to the cost and specific equipment associated with commercially available methods. Here we present a simplified method for readout of digital droplet assays using a conventional real-time PCR instrument to measure bulk fluorescence of droplet-based digital assays.

We characterize the performance of the bulk readout assay using synthetic droplet mixtures and a droplet digital multiple displacement amplification (MDA) assay. Quantitative MDA particularly benefits from a digital reaction format, but our new method applies to any digital assay. For established digital assay protocols such as digital PCR, this method serves to speed up and simplify assay readout.

Our bulk readout methodology brings the advantages of partitioned assays without the need for specialized readout instrumentation. The principal limitations of the bulk readout methodology are reduced dynamic range compared with droplet-counting platforms and the need for a standard sample, although the requirements for this standard are less demanding than for a conventional real-time experiment. Quantitative whole genome amplification (WGA) is used to test for contaminants in WGA reactions and is the most sensitive way to detect the presence of DNA fragments with unknown sequences, giving the method great promise in diverse application areas including pharmaceutical quality control and astrobiology.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/52925/>

## Introduction

Digital assays for nucleic acid quantification (digital PCR)<sup>1-4</sup> and base order (sequencing) are strongly impacting the life sciences and medicine. Digital assays provide quantification of molecular counts on an absolute scale (not relative to a control), providing high sensitivity, enabling facile comparisons across experiments, and crucially, enabling the construction of large databases containing comparable data<sup>5</sup> (**Table 1**).

Over the last 15 years, whole genome amplification (WGA) emerged alongside PCR as a general tool for nucleic acid amplification. Like PCR, WGA is useful for analytical and preparative applications by amplifying minute sample elements up to a level that can be easily detected or used for subsequent analyses like base sequencing. Unlike PCR, WGA is not specific to a particular DNA locus, rather allowing amplification of all sequences in the sample, including unknown sequences. This fundamental difference between PCR and WGA makes the methods complementary to one another and gives rise to different challenges in their application.

The high yield of WGA reactions<sup>6</sup> enables routine amplification of genomic DNA from single molecules<sup>7</sup>, single cells<sup>8</sup> and other low-biomass samples<sup>9</sup> for quantification or further analysis. The major challenges associated with WGA chemistry are its extreme sensitivity to contaminants and the uneven amplification across individual template molecules<sup>9</sup>. However, WGA is gaining popularity as single-cell sequencing has emerged as the “killer application” of WGA technology<sup>10</sup>, and template quantification by WGA is important in many fields of application<sup>7</sup>.

Instrumentation for digital nucleic acid quantification has been previously described in a variety of valved and valveless microfluidic formats<sup>11-14</sup>, including droplet-based assays<sup>15,16</sup> (**Table 2**). However, commercial microfluidic systems for digital analysis require specialized equipment for reaction setup and product detection<sup>17</sup>. Custom valved microfluidics are flexible, but require precision microfabrication and pneumatic control systems<sup>18</sup>. While it is relatively simple to make monodisperse micro-droplets for emulsion-based digital assays<sup>19,20</sup>, digital readout is technically burdensome, requiring either large-scale wide-field imaging (similar to popular next-generation sequencing technologies<sup>21,22</sup> or high-speed flow-based droplet detection<sup>23-25</sup>). Ideally, a digital assay would be simple from set-up to readout, reducing the need for complex instrumentation and allowing large numbers of samples to be read out quickly. Here we describe a simplified method for readout of digital droplet assays that uses a conventional real-time PCR instrument to measure bulk fluorescence of droplet-based digital assays.

While the new approach can be applied to digital PCR assays, it is particularly advantageous for digital WGA assays for which analog real-time amplification assays (that give excellent results for PCR) are problematic. WGA is commonly applied to samples with template molecules that are heterogeneous in sequence, length, and base content. These different template molecules are amplified at different rates<sup>6</sup>, necessitating the use of a reference ("standard") sample with matching characteristics. Often, no such standard is available, or the characteristics of the incoming samples are unknown. The heterogeneity of the input material and length-dependent property of WGA chemistries<sup>26</sup> also complicate the interpretation of results by creating ambiguity in what is being quantified—the input mass, input number of molecules, a combination of the two, or neither. Finally, the sequence-non-specificity renders quantitative multiple displacement amplification (MDA) more sensitive to contamination than quantitative PCR since contaminant molecules of any sequence have a potential to interfere. Microfluidic digital assays address contamination by segregating template molecules and reducing reaction volumes such that fewer contaminants are sampled.

Here we use a popular isothermal WGA method, MDA<sup>27</sup>. Of note, several other WGA chemistries including PicoPlex and MALBAC<sup>28</sup> depend crucially on initial isothermal strand displacement steps. Isothermal steps exacerbate the challenge of applying analog real-time assays for quantitative WGA. WGA can neither be entirely prevented during setup, leading to unwanted variable pre-amplification, nor discretized ("cycled") in a way where replication of heterogeneous molecules can be driven to completion and stopped prior to the next cycle like PCR<sup>11</sup> (**Figure 1**). A digital assay format for WGA accommodates typical reaction setup procedures due to the segregation of each molecule for enumeration at the assay endpoint (so pre-amplification does not affect the results) original readout means accuracy would be largely independent of variation in amplification efficiency.

The assay depends on droplets produced in oil with uniform volumes, as the signal level per droplet at the assay endpoint will depend on the droplet volume, and we do not want the consistency of results to depend on averaging across a distribution of droplet sizes. Making monodisperse droplets is now a standard procedure (about 3,500 monodisperse droplet papers have been published since 2013), but requires microfluidic instrumentation<sup>25</sup>. In fact, more than six companies have developed independent commercial products that rely on production of such droplets, and droplet-making microfluidic chips are commercially available<sup>23,29</sup>. For this study, we used custom microfluidic devices produced in-house (see Protocol). Syringe pumps to drive flow through the devices are also commercially available, but alternatively, can be substituted with a single disposable syringe for vacuum-driven flow to reduce costs<sup>30</sup>.

## Protocol

Note: Fabricating the microfluidic device is not necessary for this assay, as droplets for this protocol can be formed with existing commercial droplet makers<sup>23,29</sup>.

### 1. Make the Droplet-forming Microfluidic Device

1. Prepare master mold for the channels with the SU-8 Master Fabrication protocol outlined previously<sup>31</sup>, but with a droplet generator mask pattern<sup>32</sup>.
2. Fabricate devices in PDMS using the techniques of soft lithography<sup>32,33</sup>.

### 2. Prepare Reaction Mix for Bulk Droplet Readout

Note: The protocol can be used to quantify nucleic acids using many types of amplification reactions. As an example, the reagents needed for MDA of several Lambda DNA concentrations are given. Reagent details are listed in the Table of Specific Reagents. The distribution of template within the droplets depends on sufficient mixing of the sample.

1. Obtain or Purify Phi29 DNA polymerase.
  1. Purify Phi29 as previously described<sup>7</sup>, or purchase from supplier. The Phi29 used for the shown experiments was purified.
2. Prepare 10 ml of Denaturation Buffer consisting of 65 mM KOH, 1.65 mM EDTA, and 14 mM DTT.
3. Prepare 10 ml of Neutralization Buffer consisting of 65 mM HCl, 0.21 M Tris-Cl pH 7.0, and 9 mM Tris-Cl pH 8.0.
4. Prepare two standards, one no template control (NTC) such as nuclease-free water, and one high template concentration (around 4 pg/ $\mu$ l Lambda DNA). Denature 3.3  $\mu$ l of each standard with 3.3  $\mu$ l of Denaturation Buffer in a qPCR tube. Incubate at RT for 3 min. Quench each with 3.3  $\mu$ l of Neutralization Buffer.
5. Denature 3.3  $\mu$ l of the template of interest with 3.3  $\mu$ l of Denaturation Buffer in a qPCR tube. Incubate at RT for 3 min. Quench with 3.3  $\mu$ l of Neutralization Buffer.
6. Prepare 11  $\mu$ l of master mixture per sample on ice. The 2x MDA reaction master mixture consists of 2x double-stranded DNA (dsDNA) binding dye, 2x qPCR reference dye, 50  $\mu$ M randomized oligo, 2 mg/ml BSA, 2x Phi29 DNA Polymerase Reaction Buffer, 4.8 mM dNTPs, nuclease-free water, and 40  $\mu$ g/ml Phi29 DNA polymerase. Add Phi29 DNA polymerase last to ensure the polymerase does not encounter pH levels much higher or lower than 7.5. Mix well.
7. Optional: For fewer false positives, expose master mix with UV light prior to forming droplets<sup>34</sup>.
  1. Prepare 10  $\mu$ l of pre-mixture per sample in a 0.5 ml or 1.5 ml clear tube on ice. The pre-mix consists of 55  $\mu$ M randomized oligo, 2.2 mg/ml BSA, 1.1x Phi29 DNA Polymerase Reaction Buffer, 5.3 mM dNTPs, and nuclease-free water.
  2. Place the tube in water on ice as described<sup>34</sup> and expose to UV light (254 nm) to an accumulated dose of 5.7 J/cm<sup>2</sup>.
  3. Add dsDNA-binding dye to 1x, reference dye to 1x, and Phi29 to 40  $\mu$ g/ml in the pre-mixture. Mix well.
8. Combine 10  $\mu$ l of denatured template or standard and 10  $\mu$ l of master mixture. Mix well.

### 3. Droplet Formation

Note: Droplets can be very sensitive to static electricity and shear stress. Remove clothes that may cause static electricity, and ground yourself before forming droplets. Handle tubes of emulsion from the top of the tube, as far from the emulsion as possible. Pipette emulsions very slowly, preferably with a wide bore pipet tip. When dispensing from a pipette tip, watch the emulsion on the side of the tip to determine pipetting speed.

1. Form droplets. For the experiments shown, the microfluidic device has an oil inlet and two aqueous inlets with a flow-focusing junction for generating droplets. Use two syringe pumps to control the flow rates of 55  $\mu\text{l}$  of oil with surfactant and a 20  $\mu\text{l}$  of reaction mixture through the microfluidic chip to generate 20,000 1 nl droplets.
2. Note: It is possible to produce droplets with many types of oil with surfactant, but the composition will greatly affect droplet stability at high temperatures and over time. One possible combination is fluorinated oil HFE 7500 with an anionic surfactant<sup>19,35</sup>.
3. Produce droplets at any size with any method<sup>13,36</sup>, but the size variability in the droplet population will affect the bulk fluorescence, and therefore the accuracy of the assay. For the experiments shown, droplets were monodisperse with a volume of 1 nl.
4. Collect all of the droplets and oil into PCR tubes. For each sample, aliquot 30  $\mu\text{l}$  of oil into fresh PCR tubes with optical caps, and transfer 20  $\mu\text{l}$  of droplets on top of the oil. The consistent volumes ensure the assay is as accurate as possible.
5. Close tube caps tightly, as loose caps will allow the oil to evaporate.

### 4. Isothermal Amplification

1. Using a PCR thermocycler, qPCR thermocycler, or hot plate, incubate the samples at 30 °C for 7 hr, and inactivate for 1 min at 75 °C. The tubes of droplets may be left in a fridge covered with foil for a few hours at this point.

### 5. Data Acquisition and Analysis

1. Immediately following reaction inactivation, measure dye fluorescence levels for all samples using the qPCR thermocycler. Optimal filter settings will depend on dye excitation and emission spectra. For this example, use the 492 nm-516 nm filter set for the dsDNA-binding dye, and the 585 nm-610 nm filter set for the reference dye. Other fluorescent measurement techniques may be used to quantify the fluorescence.
2. For each sample, divide the fluorescence intensity of the dsDNA-binding dye by the fluorescence intensity of the reference dye. Subtract the background fluorescence, or the normalized fluorescence of the no template control, from all samples.
3. Create a linear standard curve using the corrected fluorescence measurements from the standards. The NTC standard represents the fluorescence for a sample with 0% fluorescent droplets ("all negative"), and the high template concentration fluorescence measurement is the expected fluorescence for a sample with 100% fluorescent droplets ("all positive"). Using the corresponding standard curve, predict the intermediate ratios of positive and negative droplets based on their bulk fluorescence.

### 6. Producing Droplets for Proof-of-Principle Measurements

1. Make fluorescent (positive) droplets: Prepare a PCR reaction mixture consisting of 0.1 ng Lambda DNA, 1x dsDNA-binding dye, 1x reference dye, 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, and 0.5  $\mu\text{M}$  primers. Thermocycle the mixture 30 times (95 °C for 20 sec, 55 °C for 20 sec, 72 °C for 1 min), then form droplets as described above.
2. Make non-fluorescent (negative) droplets: Prepare a PCR reaction mixture consisting of 1x dsDNA-binding dye, 1x reference dye, 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, and 0.5  $\mu\text{M}$  primers. Thermocycle the mixture 30 times (95 °C for 20 sec, 55 °C for 20 sec, 72 °C for 1 min), then form droplets.
3. Forming pre-mixed droplet ratios
  1. Distribute 20  $\mu\text{l}$  of fluorinated oil into qPCR tubes, cover with an optical cap to prevent evaporation.
  2. Gently pipette 10  $\mu\text{l}$  of well-mixed droplets in desired positive:negative ratios on top of the oil. In the results shown, positive:negative droplet ratios were 1 (all positive), 0.8, 0.5, 0.2, 0.1, 0.01, 0.001, 0.0001, and 0 (all negative).
4. Measure dye fluorescence levels for all samples using the qPCR thermocycler. For this protocol, use the 492 nm-516 nm filter set for the dsDNA-binding dye, and the 585 nm-610 nm filter set for the reference dye.
  1. Normalize dsDNA binding dye fluorescence using reference dye fluorescence. Further normalize by the fluorescence of the "all positive" sample.
5. Create a linear standard curve using the normalized fluorescence measurements from the "all positive" and "all negative" samples. Using the corresponding standard curve, predict the intermediate ratios of positive and negative droplets based on their bulk fluorescence.
6. Repeat the experiment for each ratio ( $n = 3$ ) with independent droplet formation and mixing for each replicate.

### 7. Proof-of-Principle MDA Experiment

1. Measure the concentration of Lambda DNA stock solution with a spectrophotometer.
  1. Calculate the template concentration necessary to average 10 template molecules per droplet. For this protocol, assume droplet volumes are 1 nl and 1 ng of Lambda DNA contains approximately  $1.9 \times 10^7$  copies. Therefore, 10 templates per droplet will be 10 copies per nl, or 526 fg Lambda DNA per  $\mu\text{l}$ . The template will be diluted ~6x when droplets are formed, therefore the initial highest template concentration will be 3.2 pg/ $\mu\text{l}$ .
2. Serially dilute the Lambda DNA to 3.2 pg/ $\mu\text{l}$  with Denaturation Buffer and equal volume Neutralization Buffer. For a dilution factor of 0.1, combine 10  $\mu\text{l}$  sample with 45  $\mu\text{l}$  of Denaturation Buffer and incubate at RT for 3 min. Add 45  $\mu\text{l}$  of Neutralization Buffer to quench.

3. Further dilute the sample as described in step 7.2 to make the rest of the samples for the experiment. The initial template concentrations in the reactions shown were 3.2 pg, 320 fg, 160 fg, 32 fg, 16 fg, 3.2 fg, and 1.6 fg per microliter.  
 Note: The final template concentrations after mastermix addition were 526 fg, 52.6 fg, 26.3 fg, 5.26 fg, 2.63 fg, 526 ag, and 263 ag per microliter. 10, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 expected Lambda copies per droplet, respectively.
4. Generate and analyze droplets from each sample as described in steps 2.5-5.3.
5. Acquire the fluorescence images shown (**Figure 2,3**) using a digital camera mounted to an epi-fluorescence microscope with a 10x objective at RT. Acquire twelve fields of view for each sample. Correct fluorescence images by a background image obtained with a fluorescent slide.
6. Use a contained chamber for imaging<sup>37</sup> as the emulsion oil will evaporate quickly.
7. Analyze individual droplet intensities using an ImageJ macro (Supplemental code file).
  1. Choose a threshold fluorescence intensity that best separates non-fluorescent droplets in the NTC images from the fluorescent droplets in the high template concentration images. For each unknown sample, calculate the fraction of droplets with a fluorescence intensity above the threshold.

## 8. PCR and MDA bulk reactions

1. Prepare PCR reaction.
  1. Serially dilute template DNA with a dilution factor of 0.1. For each dilution, combine 10  $\mu$ l of template with 45  $\mu$ l of Denaturation Buffer and incubate at RT for 3 min. Combine the dilution with 45  $\mu$ l of Neutralization Buffer.
  2. Prepare 20  $\mu$ l of PCR mastermix per sample. The 1.1x PCR reaction master mixture consists of 60 units/ $\mu$ l Taq DNA polymerase, 11 mM Tris-HCl, 55mM KCl, 1.65 mM MgCl<sub>2</sub>, 0.22 mM dNTP, 1.1x dsDNA-binding dye, 1.1x reference dye, and 550 nM each primer.
  3. Combine 2  $\mu$ l of diluted template and 20  $\mu$ l of mastermix.  
 Note: The final template concentrations in the PCR reactions shown were 50 pg, 5 pg, 500 fg, 50 fg, 5 fg, 500 ag, and 0 g Lambda DNA per microliter, and were performed in triplicate.
  4. Thermocycle PCR reaction in qPCR machine with the following program. Run 40 cycles of 95 °C for 30 sec, 57 °C for 20 sec, 72 °C for 30 sec before 2 min at 72 °C for 2 min and holding at 4 °C.
  5. Normalize dsDNA binding dye fluorescence using reference dye fluorescence prior to further analysis.
2. Prepare MDA reaction.
  1. Dilute samples as described in step 8.1.1.
  2. Prepare 11  $\mu$ l of master mixture per sample on ice. The 2x MDA reaction master mixture consists of 2x dsDNA-binding dye, 2x reference dye, 50  $\mu$ M randomized oligo, 2 mg/ml BSA, 2x phi29 DNA Polymerase Reaction Buffer, 4.8 mM dNTPs, nuclease-free water, and 40  $\mu$ g/ml Phi29 DNA Polymerase.
  3. Add the Phi29 DNA polymerase to 40  $\mu$ g/ml last to ensure the polymerase does not encounter pH levels much higher or lower than 7.5. Mix well.
  4. Denature 3.3  $\mu$ l of diluted template with 3.3  $\mu$ l of Denaturation Buffer in a qPCR tube. Incubate at RT for 3 min. Add 3.3  $\mu$ l of Neutralization Buffer.
  5. Combine 10  $\mu$ l of denatured template and 10  $\mu$ l of master mixture. Mix well.
  6. Run MDA reaction in qPCR machine with the following program.
  7. Hold at 30 °C for 4 hr, measure fluorescence every 7.5 min.
  8. Inactivate at 75 °C for 1 min.
  9. Normalize dsDNA binding dye fluorescence using reference dye fluorescence. Further normalize by the maximum fluorescence for each sample.

## Representative Results

While conventional bulk/real-time readouts can be used for both quantitative PCR and quantitative WGA assays (**Figure 1**), digital quantitative assays provide advantages (**Table 1**). In the method described, we read out digital assays in micro-droplet format with a simple bulk endpoint measurement (**Figure 2**). While this method is broadly applicable, we focus on quantitative WGA (MDA) because this method presents special challenges for conventional real-time assays.

To check whether our real-time PCR instrument could detect varying fractions of fluorescent microdroplets dispersed in oil, we measured the bulk fluorescence of synthetic mixtures of fluorescent and non-fluorescent droplets (**Figure 2**). The bulk fluorescence and positive droplet counts (independently assessed by fluorescence microscopy) scale linearly with the input fraction of fluorescent droplets as expected (**Figure 3A**). The experiment was performed three times, with independent droplet formation and mixing for each set.

To evaluate the theoretical quantification performance for "unknown" samples, we established linear standard curves using entirely positive and entirely negative control samples. With such droplet-lot-specific standard curves, we calculated the fraction of synthetic positive and negative droplet mixtures based on each sample's bulk fluorescence. The results show good performance in droplet ratio quantification across two logs of dynamic range ( $R^2 = 0.984$ ; **Figure 3B**). The input analyte concentration is easily calculated from the fraction of positive or negative droplets<sup>38</sup>.

To test our method in a real quantitative WGA assay, we measured fluorescence levels and fraction of positive droplets of a digital droplet MDA assay with Lambda DNA across a wide range of concentrations (**Figure 4**). In **Figure 4B**, representative fluorescent images of the droplets are shown. Both bulk fluorescence and positive droplet fraction from the digital MDA samples scale as expected with the average template per droplet, indicating that the bulk readout can faithfully capture the result of a digital assay ( $R^2 = 0.927$ ). We repeated the experiment for each concentration, independent forming droplets and carrying out WGA for each replicate.

assay type	description	measurement	quantitative	advantages	disadvantages
real-time	continuous bulk measurement of homogeneous assay	multiple time points, 1 analysis per sample	yes, relative to standard sample	large dynamic range; widely available instrumentation	relative quantification only; requires time-dependent measurement; interference from background limits sensitivity
digital	measures number of positive/negative reaction partitions	endpoint, many analyses per sample	yes, counts template molecules on absolute scale	no need for standards; single molecule sensitivity; low assay volume minimizes background	requires partitioning of each sample; conditions may need to be optimized for accurate end-point analysis; requires specialized readout instrumentation
bulk digital	bulk measurement of partitioned assay	endpoint, one analysis per sample*	yes, relative to standard sample	low assay volume minimizes background; widely available instrumentation; more robust than standard real-time in some applications	requires partitioning of each sample; relative quantification only; less sensitive than standard digital assays

\* in our implementation on instrumentation designed for qPCR, there is little drawback in recording time-dependent measurements from each sample

Table 1. Summary of real-time and digital assays. Please click here to view a larger version of this figure.

	method (vendor)	typical # samples	# partitions per sample	dynamic range (ideal template*)	sensitivity	cost: \$ capitial; \$ per sample	quantitation	standard required	readout format
qPCR	PicoGreen (Life Tech)	1	1	25 $\mu$ g - 1 $\mu$ g/mL	2 $\mu$ g/ $\mu$ L	\$20k; \$2	relative	yes	endpoint
	UV absorption (NanoDrop)	1	1	2 $\mu$ g - 15 mg/mL	2 ng/ $\mu$ L	\$15k; \$low	relative	yes	endpoint
	rtPCR (many)	96/384	1	2 fg - 20 ng/mL	0.2 - fold change	\$20k; \$0.5	relative	yes	real time
	comparative CI (many)	96/384	1	2 fg - 20 ng/mL	0.2 - fold change	\$20k; \$2	relative	internal	real time
	array dPCR (Fluidigm)	48	770	65 pg - 65 ng/mL	1 molecule	\$150k; \$15	absolute	no	endpoint
	droplet dPCR (BioRad)	8	20,000	0.4 fg - 0.2 $\mu$ g/mL	1 molecule	\$80k; \$3	absolute	no	endpoint
	array dMDA (Fluidigm)	48	770	1 fg - 20 ng/mL	1 molecule	\$150k; \$15	absolute	no	endpoint
	bulk ddMDA (described here)	96/384	10,000	100 fg - 10 $\mu$ g/mL	1 molecule/200 nL	\$20k; \$2	relative	yes	endpoint

\* is most favorable case for each measurement

Table 2. Summary of existing methods for quantitating nucleic acids. Please click here to view a larger version of this figure.

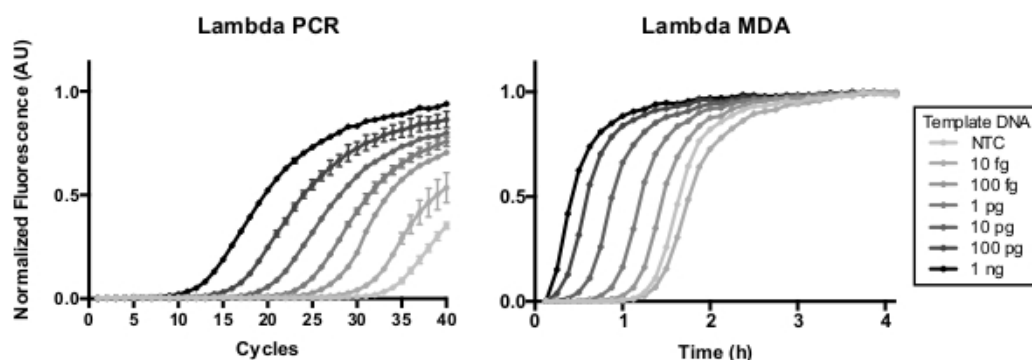
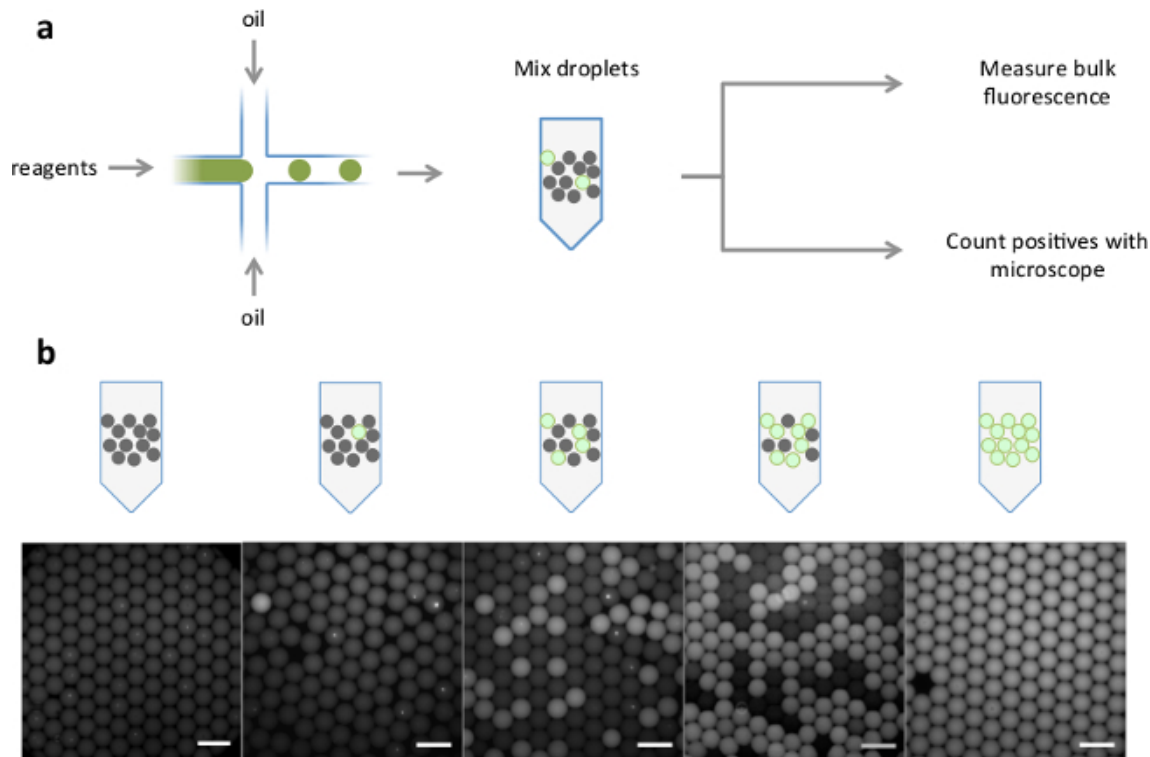
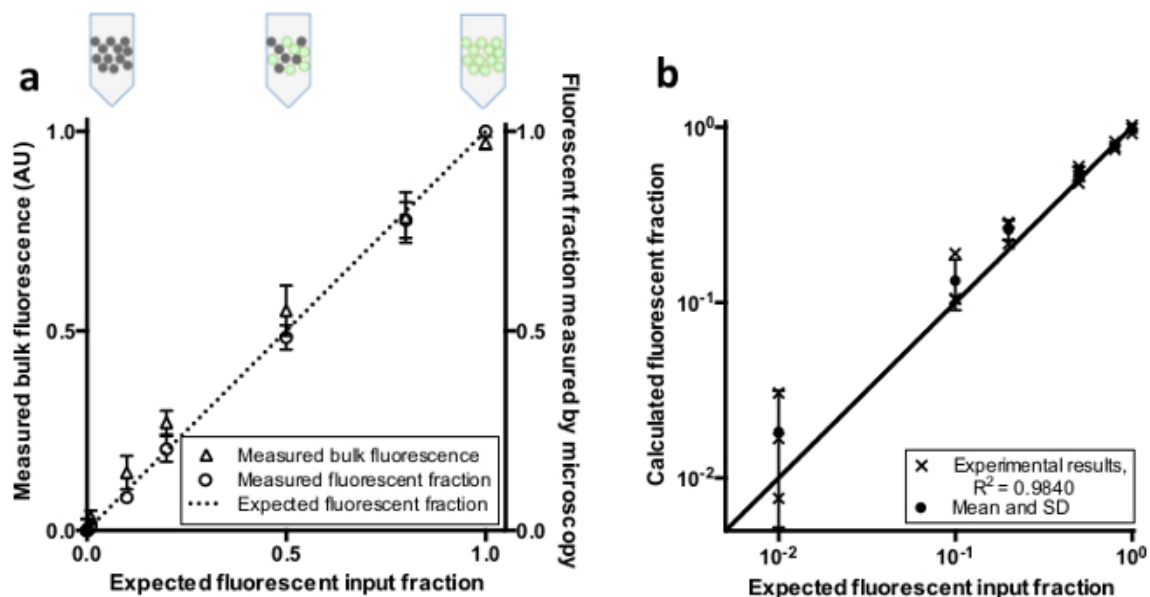


Figure 1. Real-time quantitative PCR and MDA of Lambda DNA. MDA is not discretized through temperature cycling like PCR, and is prone to preamplification if not prepared carefully on ice. Here, quantitative PCR and MDA were performed with increasing concentrations of Lambda DNA. Please click here to view a larger version of this figure.

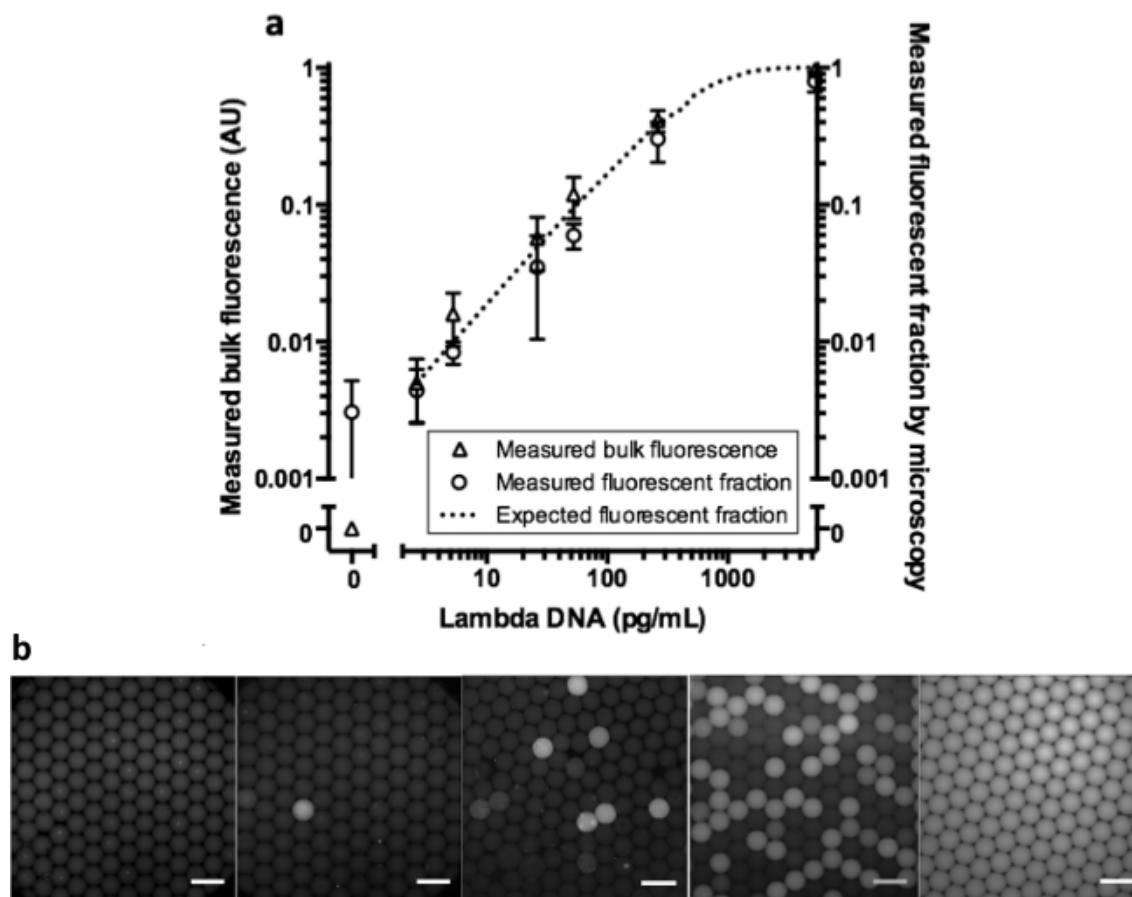




**Figure 2. Schematic of analog readout of droplets and representative images.** (A) Positive and negative droplets were generated from fluorescent and non-fluorescent reagents in a microfluidic device. The droplets were pre-mixed in different positive:negative ratios. Bulk fluorescence levels were measured with a standard real-time thermocycler, and fraction of positive droplets was determined by fluorescence microscopy. (B) Representative fluorescent overlaid images of increasing ratios of positive droplets (scale bar = 200  $\mu$ m). [Please click here to view a larger version of this figure.](#)



**Figure 3. Bulk fluorescence and fluorescent fraction of combinations of separately prepared positive and negative droplets.** (A) Fluorescent (positive) and non-fluorescent (negative) droplets were pre-mixed in different ratios. Comparison of the input fraction of positive droplets with the measured bulk fluorescence and measured positive droplet fraction ( $n = 3$ , bars represent  $\pm$  SD). The dotted line represents the expected fluorescent fraction given a linear relationship. (B) Comparison of predicted ratios based on standards to the expected fluorescent input fraction. The line indicates the expected value given a linear relationship. [Please click here to view a larger version of this figure.](#)



**Figure 4. Bulk fluorescence and fluorescent fraction of a droplet digital MDA assay.** Digital MDA was performed with increasing concentrations of template Lambda DNA ( $n = 3$ , error bars represent SD) and measured as described in **Figure 2A**. The line indicates the expected fluorescent fraction using the Poisson distribution to model the data from our experiment. **b**) Representative fluorescent overlaid images of droplets after reaction inactivation (scale bar = 200  $\mu\text{m}$ ) for increasing expected Lambda DNA molecules per droplet (NTC, 0.005, 0.05, 0.5, and 10). [Please click here to view a larger version of this figure.](#)

## Discussion

Digital assays are powerful methods that enable detection of rare cells and counting of individual of nucleic acid molecules. However, digital assays are still not routinely applied in analytical laboratories, due in part to the cost of specialized equipment associated with commercially available methods. Here we describe an endpoint digital assay for quantifying nucleic acids with a simplified analog readout using a standard real-time quantitative PCR machine. This method is quick to set-up, and readout is much simpler than droplet counting methods.

Our assay forfeits the single-molecule (single-droplet) sensitivity of individual droplet measurements for the simplicity of *en masse* endpoint measurement using standard instrumentation. In our digital MDA experiment, we could not distinguish less than 1 positive droplet per 200 negative droplets from background, despite confirmation by fluorescence microscopy that the actual fraction of positive droplets was as expected (**Figure 4**). The sensitivity and background in the bulk fluorescence measurement limit the sensitivity of the bulk measurement assay for low positive droplet fractions. If higher sensitivity is required, a digital readout is recommended. Differences in oil levels or bulk droplet volumes can greatly affect the sample fluorescence data. Even normalization with a reference dye cannot make up for the differences oil volumes make in fluorescence reads. It is possible that optimizing assay volume, instrument acquisition parameters, microwell geometry, or optical filters may improve assay performance.

The sensitivity and dynamic range of bulk digital assays can also be improved by optimizing the partition size. Larger droplets yield a greater quantity of fluorescent products from each template molecule, which can help overcome instrumental background and improve sensitivity for low input concentrations. On the other hand, smaller droplets allow the isolation of more molecules per volume assayed. If template concentration is highly variable across samples, both small and large droplet reactions can be carried out in parallel to expand the assay dynamic range<sup>39</sup>.

Consistent droplet volumes are also necessary for accurate measurement. Many custom microfluidic solutions exist for rapidly forming monodisperse droplets from a sample, both in series<sup>19,40</sup> and in parallel<sup>41,42</sup>. While few enable the droplet generation from several separate samples in parallel, simple modifications to existing designs would allow for simultaneous droplet generation of any number of samples. For example, the Bio-Rad ddPCR system<sup>23</sup> applies uniform pressure to the inlets of a series of independent droplet makers, enabling simultaneous droplet formation from multiple samples.

Our bulk readout methodology accesses key advantages of partitioned digital assays without the need for specialized readout instrumentation and is well-suited for quantitative WGA. Here, we focused on quantitative WGA, which is highly susceptible to background contaminants due to its lack of sequence specificity<sup>7</sup>. In addition, the unknown and potentially broad molecular weight distribution of products from real-time quantitative WGA assays make interpretation difficult in applications where the number of original templates is of interest. Digital assay formatting addresses both these problems. Contaminants are contained within individual reaction partitions, preventing the dominance of low-molecular-weight analytes by high-molecular-weight contaminants as would occur in a conventional real-time assay. In **Figures 2B and 4B**, possible contaminants, seen as bright spots within the droplets, are segregated and do not amplify or contribute substantially to overall fluorescence. Furthermore, the signal generated in digital assays is proportional to the number of templates, not the size of templates or their amplification rate. Although our method relies on standards to calibrate the bulk digital measurement, the requirement to match sample and standard characteristics can be relaxed significantly.

Quantitative WGA is commonly used to quantify contaminants in WGA reactions<sup>7,34</sup>, and is the most sensitive method known to quantify the presence of DNA of unknown sequence, giving the method great promise in application areas as diverse as pharmaceutical quality control, forensics, and astrobiology. Bulk digital readout can benefit other digital assay protocols such as digital PCR, by speeding up, parallelizing, and simplifying assay readout.

## Disclosures

The Broad Institute may move to file a patent application that includes aspects of this work.

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