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Sidstedt, M., Romsos, E., Hedell, R. et al (2017). Accurate Digital Polymerase Chain Reaction Quantification of Challenging Samples Applying
Inhibitor-Tolerant DNA Polymerases. Analytical Chemistry, 89(3): 1642-1649.
http://dx.doi.org/10.1021/acs.analchem.6b03746

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Accurate Digital Polymerase Chain Reaction Quantification of Challenging Samples Applying Inhibitor-Tolerant DNA Polymerases

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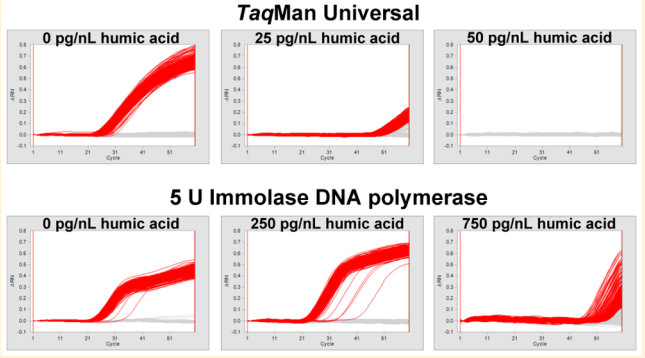
Supporting Information

ABSTRACT: Digital PCR (dPCR) enables absolute quantification of nucleic acids by partitioning of the sample into hundreds or thousands of minute reactions. By assuming a Poisson distribution for the number of DNA fragments present in each chamber, the DNA concentration is determined without the need for a standard curve. However, when analyzing nucleic acids from complex matrixes such as soil and blood, the dPCR quantification can be biased due to the presence of inhibitory compounds. In this study, we evaluated the impact of varying the DNA polymerase in chamber-based dPCR for both pure and impure samples using the common PCR inhibitor humic acid (HA) as a model. We compared the *Taq*Man Universal PCR Master Mix with two alternative DNA polymerases: Ex*Taq* HS and Immolase. By using Bayesian modeling, we show that there is no difference among the tested DNA polymerases in terms of accuracy of absolute quantification for pure template samples, i.e., without HA present. For samples containing HA, there were great differences in performance: the *Taq*Man Universal PCR Master Mix failed to correctly quantify DNA with more than 13 pg/nL HA, whereas Immolase (1 U) could handle up to 375 pg/nL HA. Furthermore, we found that BSA had a moderate positive effect for the *Taq*Man Universal PCR Master Mix, enabling accurate quantification for 25 pg/nL HA. Increasing the amount of DNA polymerase from 1 to 5 U had a strong effect for Ex*Taq* HS, elevating HA-tolerance four times. We also show that the average Cq values of positive reactions may be used as a measure of inhibition effects, e.g., to determine whether or not a dPCR quantification result is reliable. The statistical models developed to objectively analyze the data may also be applied in quality control. We conclude that the choice of DNA polymerase in dPCR is crucial for the accuracy of quantification when analyzing challenging samples.



Digital PCR (dPCR) enables absolute quantification of nucleic acids by partitioning of the sample into hundreds or thousands of minute reactions.[1,2] The method relies on a limiting dilution approach where some of the chambers or droplets do not contain any target DNA fragments and thus will not give an end-point signal. The DNA concentration is determined by counting the fraction of positive reactions and assuming a Poisson distribution for the number of DNA fragments present in each chamber. As opposed to real-time quantitative PCR (qPCR), dPCR does not rely on a standard curve for quantification, which means that dPCR analysis is less affected by differences in amplification kinetics.[3,4]

Thus, several reports have proven dPCR quantification to be more tolerant to various types of PCR inhibitors than qPCR.[5−8] This makes dPCR an appealing technique when analyzing samples from complex background matrixes such as soil and blood. Nevertheless, inhibition issues are not eradicated by using dPCR. For example, sodium dodecyl sulfate (SDS) and heparin have been reported to affect the fluorescence intensity in droplet dPCR, creating a need to redefine the fluorescence threshold used to differentiate between positive and negative droplets.[6] Humic acid (HA) from environmental samples has been shown to cause complete inhibition in dPCR, although higher levels of HA were tolerated compared with qPCR.[9,10]

Received: September 22, 2016
Accepted: January 9, 2017
Published: January 9, 2017

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DOI: 10.1021/acs.analchem.6b03746
Anal. Chem. 2017, 89, 1642−1649

Elevating the number of PCR cycles has been suggested as a means to partly counteract the effect of HA and calcium inhibition.[11]

HA, used as a model inhibitor in this study, consists of a group of large, heterogeneous phenolic molecules originating from the decomposition of plants. HA is the major cause of PCR inhibition from soil and sediment samples. It has been found that the molecules inhibit amplification by directly affecting the DNA polymerase.[12,13] We recently revealed a second mode of inhibition: quenching of the fluorescence from various dsDNA-binding dyes.[13] HA molecules, being negatively charged and highly reactive, likely interact directly with DNA polymerase and dye molecules, thus disturbing both amplification and product detection. The choice of DNA polymerase greatly affects PCR inhibitor tolerance.[14,15] However, the impact of varying the DNA polymerase on the accuracy of dPCR quantification has not yet been studied.

In this study, we applied three different DNA polymerase−buffer systems for performing absolute quantification with chamber-based dPCR, analyzing samples both with and without HA. The dPCR *Taq*Man Universal master mix[16−18] was compared with two alternative DNA polymerases: Ex*Taq* HS and Immolase. HA was chosen as a model inhibitor since it may act as an amplification inhibitor and/or detection inhibitor depending on the type of DNA polymerase and fluorophore used.[13] In addition, by using Bayesian modeling we show that the accuracy of dPCR quantification of impure samples is greatly affected by the choice of DNA polymerase. Finally, the possible use of the developed Bayesian models in quality control is discussed.

MATERIALS AND METHODS

Materials. The DNA examined in this study is a material derived from component no. 16 of the discontinued NIST SRM 2390 RFLP Profiling Standard.[19,20] Each unit of SRM 2390 provided 25 μL of 200 ng/μL extracted single-donor male DNA. The DNA was originally quantified using optical spectroscopy measurements where 1 optical density (OD) unit is approximately equivalent to 50 ng/μL of DNA (λ = 260 nm).[19] Following the discontinuation of the SRM in 2009, the solution remaining was pooled, diluted with TE buffer (10 mM Tris and 0.1 mM ethylenediamine-tetraacetic acid [EDTA], pH 8.0) to a DNA concentration of about 50 ng/μL. An aliquot of this pooled solution was then volumetrically diluted with TE buffer to approximately 25 ng/μL. Humic acid sodium salt (product no. H16752) was obtained from Sigma-Aldrich (Taufkirchen, Germany) and dissolved in TE buffer.

Digital PCR. The work in this study was performed using the Fluidigm BioMark 48.770 Digital Array real time/end point limiting dilution assay system (Fluidigm, San Francisco, CA). The Fluidigm Digital PCR Analysis tool provided by the manufacturer was used for all primary data reduction using assay specific global intensity thresholds and a quality score threshold of 0.1. Cycles 1 through 60 were analyzed with the user global analysis method for determination of the positive chambers. Detailed results were exported as .csv files for further data handling.

The primers and hydrolysis probe (purchased from Thermo Fisher Scientific, Waltham, MA) used for the principal part of this study target the retinoblastoma 1 (RB1) gene of human DNA.[21] The probe is labeled with 6-carboxyfluorescein (FAM), and the passive reference dye 6-carboxy-X-rhodamine (ROX) (Invitrogen, Thermo Fisher Scientific, Waltham, MA) was used for normalization. Unless otherwise stated, the following reagents were included in all master mixes for the RB1 assay: 0.3 μM of each primer (RB1_80F and RB1_235R[21]), 0.2 μM hydrolysis probe (RB1_212_MGB,[21]), 1.0 μL of 20X GE Loading Reagent (Fluidigm), and 2.0 μL of DNA. If needed, amplification grade water (Promega, Madison, WI) was added to reach a total volume of 20 μL. For the samples with inhibitor, humic acid was added instead of water to the following final concentrations in the reaction: 13, 25, 50, 88, 125, 188, 250, 375, 500, 625, 750, and 875 pg/nL (analyzed in triplicates).

1X *Taq*Man Universal PCR Master Mix, No Amperase UNG (*Taq*Man Universal) (Thermo Fisher Scientific, Waltham, MA), containing DNA polymerase, ROX dye, buffer, dNTPs, and possibly one or more not specified additives was mixed with DNA, primers, and the hydrolysis probe. The detailed DNA polymerase−buffer composition is proprietary. In the experiments investigating the effects of bovine serum albumin (BSA, Roche Diagnostics), 0.5 μg/μL BSA was added to the *Taq*Man Universal reactions (the same level as applied for Immolase and Ex*Taq* HS, see below). For Immolase (Bioline Reagents, London, U.K.) and Ex*Taq* HS (TaKaRa Bio Inc., Shiga, Japan), the following reagents were included in the master mix: 1× specific buffer (Immobuffer, Bioline Reagents or Ex*Taq* buffer, TaKaRa), 0.2 mM deoxynucleoside triphosphate (dNTP) (Roche Diagnostics, Basel, Switzerland), 4.0 mM $MgCl_2$ in total (Roche Diagnostics), 0.5 μg/μL of BSA, 1 U DNA polymerase (Immolase or Ex*Taq* HS), 1× ROX dye.

Apart from the RB1 assay, two more dPCR assays targeting other parts of the human genome were applied to test their respective humic acid tolerance. The assays, called ND6 and D5, were developed at NIST, and their respective primer and probe sequences, and concentration of these, can be found in ref 20. *Taq*Man Universal was used for these assays, as described above. The following final concentrations of humic acid were tested: 5, 10, 25, 50, and 125 pg/nL (analyzed in triplicates).

Four microliters of the prepared master mixes, including DNA, were added to the appropriate sample inlet for each panel of a 48.770 array. The arrays were filled using the BioMark IFC controller MX and placed into the BioMark System for amplification and detection. Amplification conditions were 95 °C for 10 min, followed by 60 cycles of 15 s at 95 °C, and 1 min at 60 °C. The ramp speed between temperature set points was 2 °C/s.

Data Analysis. Data was analyzed with the Fluidigm Digital PCR Analysis software (version 3.1.3, build 20120816.1505) to determine the number of positive chambers. The concentration of DNA (copies/μL), here denoted as [DNA], can be estimated using the equation suggested by Dorazio et. al:[22]

$$[\mathrm{DNA}] = \frac{-\ln(1 - (y/770))}{0.85 \times 10^{-3}}$$

where y is the number of positive chambers, 0.85×10^{-3} in the denominator is the nominal chamber volume (μL) reported by the manufacturer, and 770 the total number of chambers in each panel. Determination of original sample concentration (ng/μL) was performed by multiplying the number of copies/μL by 10 (dilution factor) and dividing by 311 (approximate number of copies per ng, assuming 6.436 pg of DNA per diploid human male cell).[23]

For determination of Cq values, data from amplification curves were exported as .csv files and analyzed with the

DOI: 10.1021/acs.analchem.6b03746
Anal. Chem. 2017, 89, 1642−1649

Table 1. Accuracy of dPCR Absolute Quantification for Three DNA Polymerase−Buffer Systems[a]

		humic acid (pg/nL)							
		0	13	25	50	88	125	375	500
*Taq*Man Universal	positive reactions	424 ± 19	434 ± 11	368 ± 7	0	0	0	–	–
	quantity (ng/μL)	30.30 ± 2.06	31.38 ± 1.20	24.59 ± 0.62	0	0	0	–	–
	prob. mass above zero	NA	0.19	1	1	1	1	–	–
ExTaq HS 1 U	positive reactions	418 ± 9	–	413 ± 8	398 ± 20	0	0	0	–
	quantity (ng/μL)	29.62 ± 0.99	–	29.16 ± 0.92	27.56 ± 2.08	0	0	0	–
	prob. mass above zero	NA	–	0.65	0.96	1	1	1	–
Immolase 1 U	positive reactions	417 ± 12	–	–	419 ± 12	–	406 ± 3	401 ± 5	0
	quantity (ng/μL)	29.55 ± 1.23	–	–	29.72 ± 0.57	–	28.38 ± 0.32	27.83 ± 0.47	0
	prob. mass above zero	NA	–	–	0.44	–	0.84	0.93	1

[a]*Taq*Man Universal, Ex*Taq* HS, and Immolase were challenged with increasing amounts of HA. Numbers of positive reactions and estimated DNA quantities are given ± the standard deviations. Probability mass above zero for the difference in probability of positive reactions $P(p_0 - p_i > 0)$, resulting from the Bayesian model, was used to identify at what amount HA affected the quantification. "–", not analyzed. "NA", not applicable.

software R[24] using the qpcR package.[25] The function pcrbatch was used to fit the data for all curves with a five-parameter log−logistic function. Cq values were determined using the second derivate maximum method (called cpD2 in the qpcR package). The amplification efficiency was calculated from individual amplification curves applying the method described in[26] (example of the R code used can be found in Table S-4). For graphical visualization, Graphpad Prism v. 6.0 was used. To determine the fluorescence intensity of the ROX dye, ImageJ[27] was used and intensity was measured within six chambers per panel.

Statistical Analysis. The following Bayesian models were developed for statistical analysis. For each dPCR sample, the number of positive chambers was modeled as having a binomial distribution with probability of a positive reaction p. It was investigated whether the posterior distribution of the differences in p were clearly separated from zero or not.

Bayesian statistical analysis for the positive reactions was performed in the following way. For two different dPCR samples with different HA levels, $i = 1,2$ let y_{ij} denote the number of positive partitions for replicate $j = 1,...,n$. These variables were modeled as independent binomial distributions given parameters p_i:

$$y_{ij}|p_i \sim \text{Bin}(m, p_i)$$

where m is the number of partitions. The parameters p_i were modeled as independent beta distributions:

$$p_i \sim \text{Beta}(\alpha_i, \beta_i)$$

for fixed values $\alpha_i = 1$ and $\beta_i = 1$, (i.e., all p_i have a vague, uniform prior distribution). It follows from conjugacy that the distribution of p_i given data $y = (y_{11},...,y_{2n})$ is

$$p_i|y \sim \text{Beta}(\alpha_i + \sum_{j=1}^{n} y_{ij}, \beta_i + mn - \sum_{j=1}^{n} y_{ij})$$

The posterior distribution of $p_1 - p_2$ can be approximated via Monte Carlo simulation. We performed the computations using R.[24] The posterior mean, the 2.5% and 97.5% quantile, and the amount of posterior probability mass greater than zero: $P(p_1 - p_2 > 0)$ were used as summary statistics to detect any important differences from zero. For the samples without HA index $i = 0$ was used.

Furthermore, the Cq values for the positive chambers were modeled as having a normal distribution with mean value μ, after removal of the "late starters" (defined as the first Cq value gap over 0.5 when all Cq values are sorted from lowest to highest), magenta colored in the curve plots (Figure S-1). Similar to above, it was investigated whether the posterior distribution of the differences in mean values μ between dPCR settings were clearly separated from zero or not.

Bayesian statistical analysis for the Cq values was performed in the following way. For two different dPCR settings (i.e., with different amounts of HA) $i = 1,2$, let y_{ij} denote the Cq value of replicate $j = 1,..., n$, after removal of both late-starters and results without positive reaction. These variables were modeled as independent normal distributions given parameters μ_i and σ:

$$y_{ij}|\mu_i, \sigma \sim N(\mu_i, \sigma^2)$$

To investigate how the mean value μ_i differs between groups, vague prior distributions were used. The mean parameters μ_i were modeled as independent uniform distributions over all possible values:

$$\mu_i \sim U(0, 60)$$

The standard deviation σ was modeled as uniformly distributed, excluding values that seem nonrealistic in this context:

$$\sigma \sim U(0, 20)$$

The posterior distribution of $\mu_1 - \mu_2$ can be approximated via Markov Chain Monte Carlo simulation. OpenBUGS 3.2.3[28] was used for approximation of the posterior distributions. Three chains with different starting values were used. The simulation was run long enough so the trace-plots did not exhibit any signs of nonconvergence.

RESULTS

Absolute Quantification Applying Different DNA Polymerases. The impact of varying the DNA polymerase on absolute quantification of pure template samples was investigated. The developed Bayesian models were applied to dPCR data to find any systematic differences in the probability of positive reactions between the DNA polymerases, with no systematic differences implying no differences in absolute quantification. There were no clear differences in the accuracy of absolute quantification for pure samples between *Taq*Man Universal PCR Master Mix (hereafter referred to as *Taq*Man Universal; estimated DNA concentration, 30.3 ng/μL), Ex*Taq* HS (29.6 ng/μL), and Immolase (29.6 ng/μL) (the posterior

DOI: 10.1021/acs.analchem.6b03746
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distributions of differences were not clearly separated from zero) (Table 1, Table S-1).

In the samples with HA, absolute quantification was greatly affected by the choice of DNA polymerase (Table 1). This was confirmed by the developed statistical model (Table S-2), from which it could be concluded at what amount HA affected the probability of positive reactions for each of the three DNA polymerases. *Taq*Man Universal gave accurate quantification up to 13 pg/nL HA with a clear decrease in the probability of positive reactions with 25 pg/nL HA (i.e., a result different from samples without inhibitor); posterior mean of difference equal to 0.07 and approximately 100% of posterior probability mass greater than zero. No positive reactions were recorded with 50 pg/nL HA or more. Ex*Taq* HS (1 U) gave reliable quantification up to 25 pg/nL HA with a decrease in estimated DNA quantity with 50 pg/nL; posterior mean of difference equal to 0.03 and 96% of posterior probability mass greater than zero. Amplification was completely inhibited by 88 pg/nL HA. For Immolase (1 U), quantification was accurate up to 375 pg/nL HA; posterior mean of difference equal to 0.02 and 93% of posterior probability mass greater than zero. Amplification was completely inhibited by 500 pg/nL. The concentration of HA that could be tolerated with the three different DNA polymerases without affecting quantification differed more than 25 times, ranging from 13 pg/nL for *Taq*Man Universal to 375 pg/nL for Immolase (1 U).

Elevated Cq values, signaling moderate inhibition effects, were observed for lower HA levels for all DNA polymerases. The statistical model was applied to determine at what HA concentration the Cq values differed from the samples without inhibitor (Table S-3). As it could be concluded that all groups were dissimilar, the posterior mean of the differences was used as a measure of inhibition. The dPCR Cq values confirmed impaired amplification for *Taq*Man Universal with 13 pg/nL HA, for Ex*Taq* HS with 50 pg/nL and for Immolase with 250 pg/nL (Figure 1). Amplification efficiency was calculated from individual amplification curves. No clear systematic decrease of amplification efficiency was observed with increasing HA concentration (Table S-4).

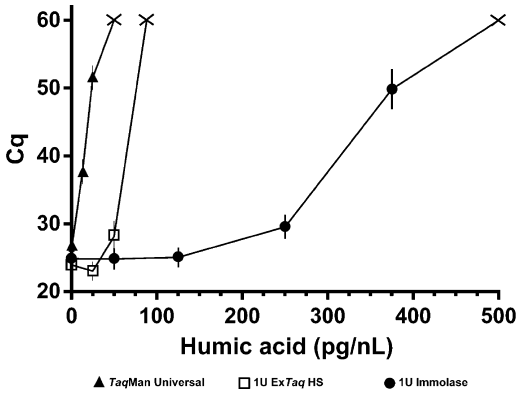

Figure 1. Average Cq values for three different DNA polymerases when challenged with increasing concentrations of HA. Average Cq values and standard deviation are presented for the *Taq*Man Universal DNA polymerase−buffer master mix (black triangles), 1 U Ex*Taq* HS (open squares), and 1 U Immolase (black circles). Complete amplification inhibition (i.e., no positive reactions) is represented by an average Cq value of 60 (crosses). All positive amplification curves were analyzed for three replicate panels and n varies depending on the number of positives (*Taq*Man Universal, n = 1104−1302; 1 U Ex*Taq* HS, n = 1194−1254; 1 U Immolase, n = 1203−1257).

Apart from investigating the effect of the choice of DNA polymerase, we evaluated three different digital PCR assays with respect to their tolerance to HA, using *Taq*Man Universal for all assays (Table S-5). One of the assays was completely inhibited by 125 pg/nL, although with affected amplification kinetics at 50 pg/nL (increased Cq values), and the other two were completely inhibited by the one-step lower dilution, 50 pg/nL.

Effects of Varying the Amount of BSA and DNA Polymerase. BSA is an established PCR amplification facilitator often applied to overcome inhibition.[29] In order to evaluate if addition of BSA could enhance the potential of the system, *Taq*Man Universal was tested with additional BSA (Figure 2). For the other DNA polymerases, we added BSA to

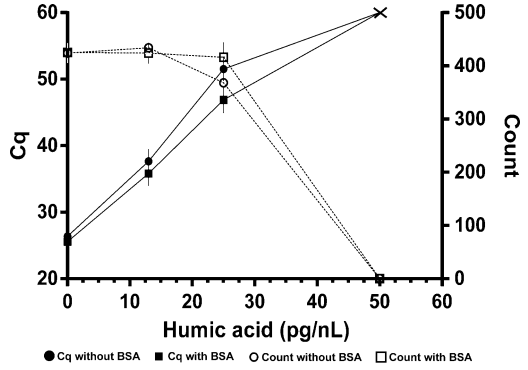

Figure 2. Effect of BSA on *Taq*Man Universal inhibitor-tolerance. Results are presented for *Taq*Man Universal without BSA added (black filled circles for Cq on the left y axis, open circles for count on the right y axis) and for *Taq*Man Universal with the addition of 10 μg of BSA (0.5 μg/μL) (filled squares for Cq on the left y axis, open squares for count on the right y axis). Complete amplification inhibition (i.e., no positive reactions) is represented by an average Cq value of 60 (crosses).

all reactions. With *Taq*Man Universal, the decrease in estimated DNA quantity with 25 pg/nL HA (368 ± 7 positive reactions, clear evidence of decrease compared with samples without inhibitor, see Table S-2) was counteracted by the addition of BSA (416 ± 28 positive reactions, no clear evidence of decrease, see Table S-2). Furthermore, the addition of BSA resulted in lowered Cq values with 25 pg/nL HA (46.9 ± 2.0 compared with 51.5 ± 1.8) showing that BSA had a positive effect. However, BSA did not aid in amplification with 50 pg/nL HA or more.

Increasing the amount of DNA polymerase from 1 U to 5 U resulted in improved HA-tolerance for both Ex*Taq* HS and Immolase (Figure 3). Applying 5 U of Ex*Taq* HS resulted in complete inhibition with 375 pg/nL HA (Figure 3A), as opposed to 88 pg/nL HA with the use of 1 U (Table 1). Underestimation of DNA quantity (i.e., a result clearly different from samples without inhibitor) occurred with 250 pg/nL of HA (Table S-2). The same trend was observed with 5 U Immolase, with complete inhibition with 875 pg/nL HA (Figure 3B), as opposed to 500 pg/nL with 1 U (Table 1). Underestimation of DNA quantity occurred with 750 pg/nL HA (Table S-2). For Ex*Taq* HS, an increase in Cq values could be observed with 250 pg/nL HA (Figure 3A), as opposed to 50 pg/nL with 1 U (Figure 1). For Immolase, elevated Cq values

DOI: 10.1021/acs.analchem.6b03746
Anal. Chem. 2017, 89, 1642−1649

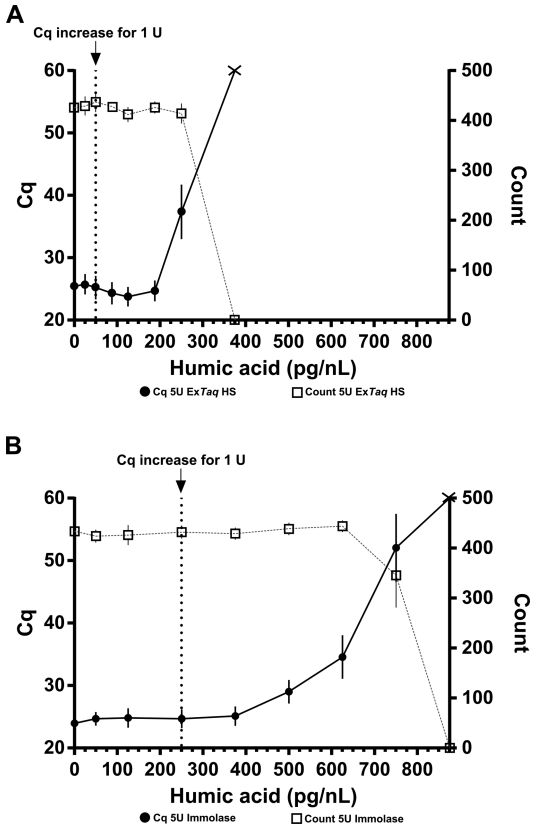

Figure 3. Excess DNA polymerase increase the HA-tolerance for (A) Ex*Taq* HS and (B) Immolase. Results are shown for 5 U of each polymerase. The inhibitory effect of HA is presented as Cq value on the left *y*-axis (black circles) with standard deviation and count of the positive reactions on the right *y*-axis (open squares) with standard deviation (3 panels/samples) (Ex*Taq* HS, n = 1236−1310; Immolase, n = 1036−1317). Vertical dotted lines represent the amount of HA that resulted in increased Cq values for 1 U of DNA polymerase (see Figure 1). Complete amplification inhibition (i.e., no positive reactions) is represented by an average Cq value of 60 (crosses).

were observed with 500 pg/nL HA, as opposed to 250 pg/nL with 1 U (Figure 1). Amplification efficiency determined from individual amplification curves was not systematically lowered by the addition of HA when applying 5 U DNA polymerase (Table S-4).

Fluorescence Quenching in Digital PCR. The samples with the highest concentration of HA demonstrated an increase in normalized fluorescence levels of the amplification curves (Figure 4A,B). This was determined to be due to quenching of the ROX dye fluorescence used for normalization of target signals. With high amounts of HA (375−750 pg/nL), the signal intensity of ROX was about half compared with reactions without HA (Figure 4C). The resulting amplification curves had similar sigmoidal shapes as the positive control and the fluorescence for the FAM-labeled hydrolysis probe was not apparently affected by increasing amounts of HA. At the tested levels of HA, the ROX quenching effect had no effect on the accuracy of absolute quantification.

DISCUSSION

In this study, the accuracy of dPCR-based absolute quantification was improved by applying inhibitor-tolerant DNA polymerases. We found that for impure samples, the choice of DNA polymerase has a great impact on dPCR accuracy. This has previously been shown to be of importance in conventional PCR and qPCR.[14,15]

Quantification of pure samples was not affected by the choice of DNA polymerase. This implies that the effects observed with different DNA polymerases are connected to PCR inhibitor-tolerance, meaning that without inhibitors present the three tested DNA polymerases amplify the target DNA with similar efficiencies. The observed differences in inhibitor-tolerance between the three enzymes were fairly large: applying the commonly used *Taq*Man Universal master mix,[16−18] problems with underestimation of DNA quantity occurred with 13 pg/nL of HA (Figure 1). With high amounts of Immolase (5 U),

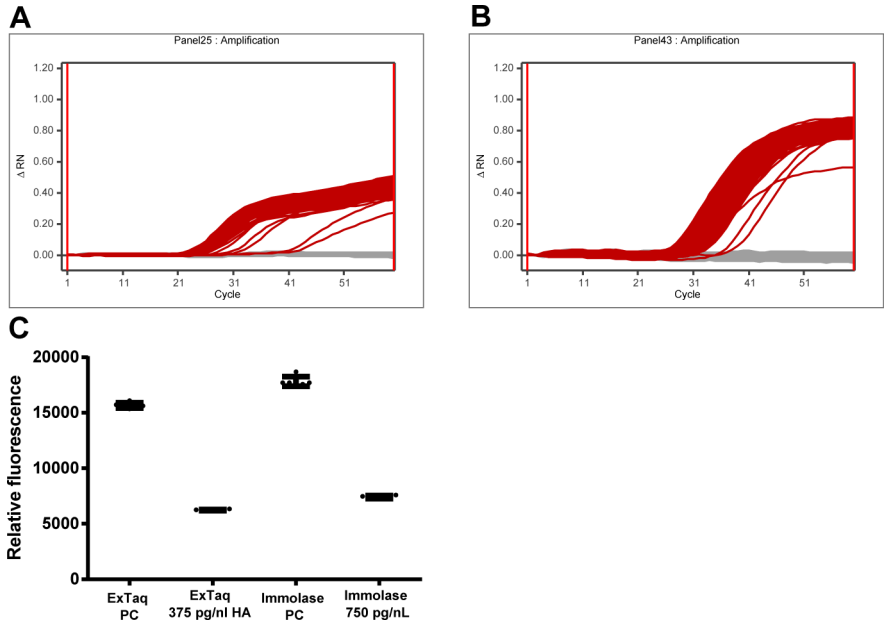

Figure 4. ROX reference dye is quenched by HA. Target amplification curves for (A) positive controls (PC) and (B) samples containing 750 pg/nL HA, showing an increase in normalized fluorescence due to HA. (C) ROX dye fluorescence intensity in the presence and absence of HA.

DOI: 10.1021/acs.analchem.6b03746
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quantification was accurate for up to 625 pg/nL HA, i.e., 48 times more (Figure 3).

For the commercial *Taq*Man Universal master mix, the detailed composition (buffer content and DNA polymerase) is proprietary. It is therefore not known if BSA or other facilitators are included. We showed that addition of BSA to the *Taq*Man Universal master mix resulted in a minor improvement in HA-tolerance (accurate quantification at 25 pg/nL) but far from the levels handled by 5 U Ex*Taq* HS or 5 U Immolase (Figures 2 and 3). Thus, the observed differences in inhibitor-tolerance are mainly related to the type and amount of DNA polymerase, not other additives. According to a published whitepaper from the manufacturer, *Taq*Man Universal contains Ampli*Taq* Gold DNA polymerase.[30] Ampli*Taq* Gold is derived from *Thermus aquaticus* (*Taq*) and has previously been shown to have low tolerance to inhibitors from blood and forensic DNA evidence, such as cigarette ends and chewing gum, when applying conventional PCR and qPCR.[14,15,31] Ex*Taq* HS is also derived from *Taq* but has higher resilience to inhibition as observed both previously[15,32,33] and in the present study. Differences between polymerases derived from the same organisms may be attributed to enzyme engineering, the manufacturing process, or the type of hot start inactivation applied (e.g., antibody attached to active site). However, because of the limited information released by the manufacturers, it is difficult to pinpoint the reasons. Immolase is isolated from a novel, proprietary organism and has in a previous study been found to be highly tolerant to HA in qPCR analysis.[13] Finding a DNA polymerase−buffer system that is tolerant to inhibitors present in the samples to be analyzed should preferably be one of the first steps when setting up a new DNA analysis process. For this purpose, we have developed a concept for overcoming PCR inhibition without impairing the detection limit, called pre-PCR processing.[34] The use of inhibitor-tolerant DNA polymerases is one option to overcome PCR inhibition without losing target DNA in time-consuming DNA purification steps.

It has previously been shown that different PCR assays, including different primers and target sequences, can be somewhat differently affected by inhibitors.[35−37] We found slight differences in HA-tolerance between three tested dPCR assays. However, the choice of DNA polymerase had a substantially stronger effect on inhibitor-tolerance compared with the choice of assay (Table 1, Table S-5, Figures 1 and 3). In a previous study, urine and the Mg^{2+} ion chelator EDTA were investigated in a real-time PCR setting and amplicon GC-content was suggested as a reason for the differences between assays, although this could not be verified.[35] Likewise, we were not able to find any clear correlations related to primer sequences, melting temperature, or GC content (data not shown). This may partly be explained by the fact that humic acid acts as a DNA polymerase inhibitor.[12,13] Substances directly affecting DNA polymerase activity will have a negative effect for any PCR assay, although the absolute amounts needed to disturb amplification may differ.

In this study, we varied the DNA polymerase−buffer system and the concentration of a model inhibitor, HA. By keeping the assay and input DNA amount fixed, we could systematically and statistically investigate the effects of DNA polymerase type and HA concentration on quantification accuracy. A Bayesian approach was chosen as it allows for direct probabilistic statements about the model parameters. The developed Bayesian statistical model aided in determining when the numbers of positive chambers could be statistically verified as different compared with the samples without inhibitor. A Bayesian model was also applied to investigate the differences in Cq values. The results showed that increased HA levels led to increased Cq values for all DNA polymerases. Average Cq values of around 50 coincided with a negative effect on quantification accuracy. We propose that monitoring changes in average Cq values can be applied to determine the reliability of dPCR quantification in routine analysis. False negative reactions can be expected over a certain critical Cq value level, leading to underestimated DNA concentrations. This critical level should be determined for each individual assay and sample type since different inhibitors can have different impacts. The developed Bayesian model may be applied as a practical tool for monitoring Cq value levels when applying dPCR instruments that allow for real-time analysis, e.g., Biomark Fluidigm and Quantstudio 12K Flex (Life Technologies). There is a need for improved quality control in dPCR, as previously noted by Dorazio and Hunter.[22] They suggest a class of statistical models for both the analysis and design of experiments for reliable dPCR measurements and argue that many published dPCR analyses fail to take into account the binomial nature of dPCR data.

It has previously been reported that dPCR enables accurate quantification of samples with higher amounts of inhibitors compared with qPCR.[5,7,38] This partly depends on the fact that dPCR does not rely on standard curves and Cq values for quantification: partial inhibition causing somewhat increased Cq values lead to underestimated DNA concentrations with qPCR but can be handled in dPCR as the technique relies on end-point fluorescence measurements. Although we did not directly compare dPCR and qPCR results here, the same assay (RB1) with identical reagents and concentrations (Immolase 1 U) were used in a previous qPCR study.[13] In qPCR, quantification was affected by 50 pg/nL HA, compared with 375 pg/nL for dPCR. Complete inhibition was also reached at a lower HA concentration compared with dPCR, which implies that the difference in inhibitor-tolerance between the platforms is not fully explained by the use of end-point measurements in dPCR. Other reports have directly compared the performances of qPCR and dPCR, providing valuable information concerning the applicability of dPCR for quantification of nucleic acids in impure samples.[7,10,38] Previously, it was reported that 11.2 pg/nL HA completely inhibited the amplification in dPCR using the same instrument as in the present study but with a different commercial master mix (SYBR Premix Dimer Eraser, Takara Bio, Shiga, Japan) and using a dsDNA-binding dye for detection.[10] This level is low compared with the Immolase results and fairly close to our results for *Taq*Man Universal, showing the general need to identify a DNA polymerase−buffer system compatible with the inhibitors that may be encountered in unknown samples.

HA was used as a model inhibitor in this study due to its broad effect on PCR, acting both as a DNA polymerase inhibitor and fluorescence quencher.[12,13,39] Inhibitory compounds can disturb PCR-based analysis by affecting any of the components or subprocesses needed for amplification and detection of products. PCR inhibition is often a combination of several factors and it is challenging to pinpoint the exact mechanism. In this study, HA demonstrated a strong negative effect on amplification, lowering the number of positive reactions and elevating the Cq values. However, there was no clear systematic effect on amplification efficiency, as calculated

DOI: 10.1021/acs.analchem.6b03746
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from individual amplification curves. This implies that HA predominantly affects the initial PCR cycles, where the template is mostly genomic DNA. Once the amplification has been properly initiated, and the short amplicons are the dominating template, the samples containing HA are seemingly amplified with the same efficiency as pure samples. Amplification efficiency calculated from individual amplification curves has been proposed as a measure of inhibition (see ref 40 for a review of methods). For HA and other inhibitors with similar effects, amplification efficiency calculations are apparently not applicable to monitor inhibition.

By using Immolase and thus improving the inhibitor-tolerance of the reaction, we found a new possible limitation in dPCR quantification of impure samples: the quenching of fluorescence of ROX passive reference dye. An important note is that this quenching effect is "hidden" by the Biomark software since the user only sees the normalized fluorescence data. Although the quenching had no effect on quantification accuracy in our experiments, it may cause analytical problems when analyzing unknown samples since the fluorescence threshold distinguishing the negative chambers from the positive chambers will be inaccurate. Fluorescence quenching is a general phenomenon in dPCR and qPCR and may affect different assays in different ways depending on the inhibitor and the fluorophores applied. For example, HA interacts with DNA-binding qPCR dyes,[13] and DTT has been found to quench fluorescence of the passive reference dye Mustang Purple.[41] This often overlooked effect should be noted when troubleshooting PCR reactions.

For accurate dPCR quantification of impure DNA samples it is vital to identify an inhibitor-tolerant DNA polymerase. The final choice depends on the nature of the analyzed samples, as different polymerases have different abilities to function in different backgrounds.[14,42] Some dPCR instruments, including the commonly used droplet dPCR instrument QX200 (BioRad), come with closed chemistries, where the user is unable to replace the DNA polymerase in case of PCR inhibition. Other platforms give more flexibility in this aspect, e.g., Fluidigm BioMark, Quantstudio 12K Flex (Life Technologies) and RainDrop Digital PCR System (Raindance Technologies).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03746.

Posterior distributions of differences between DNA polymerases and between pure and impure samples; posterior means of Cq value differences for pure and impure samples; summary of numbers of positive reactions (count), Cq values, and amplification efficiencies for the different DNA polymerase−buffer systems with varying HA amounts; effect of inhibition by humic acid as visualized by dPCR amplification curves for different DNA polymerase−buffer systems; and humic acid effects on three dPCR assays targeting different parts of the human genome (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank David Duewer, NIST, for assistance with amplification curve graphics. This study was financially supported by the Swedish Research Council (Grant 621-2013-5999).

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DOI: 10.1021/acs.analchem.6b03746
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