Mutant DNA quantification by digital PCR can be confounded by heating during DNA fragmentation

Qing Kang¹, Brian Parkin¹, Maria D. Giraldez¹, and Muneesh Tewari^{1,2,3,4}

¹Department of Internal Medicine, Division of Hematology/Oncology, ²Department of Biomedical Engineering, ³Center for Computational Medicine and Bioinformatics, and ⁴Biointerfaces Institute, University of Michigan, Ann Arbor, MI

BioTechniques 60:175-185 (April 2016) doi 10.2144/000114401

Keywords: droplet digital PCR; DNA mutation; fragmentation; heating

Supplementary material for this article is available at www.BioTechniques.com/article/114401.

Digital PCR (dPCR) is gaining popularity as a DNA mutation quantification method for clinical specimens. Fragmentation prior to dPCR is required for non-fragmented genomic DNA samples; however, the effect of fragmentation on DNA analysis has not been well-studied. Here we evaluated three fragmentation methods for their effects on dPCR point mutation assay performance. Wild-type (WT) human genomic DNA was fragmented by heating, restriction digestion, or acoustic shearing using a Covaris focused-ultrasonicator. dPCR was then used to determine the limit of blank (LoB) by quantifying observed WT and mutant allele counts of the proto-oncogenes *KRAS* and *BRAF* in the WT DNA sample. DNA fragmentation by heating to 95°C, while the simplest and least expensive method, produced a high background mutation frequency for certain *KRAS* mutations relative to the other methods. This was due to heat-induced mutations, specifically affecting dPCR assays designed to interrogate guanine to adenine (G>A) mutations. Moreover, heat-induced fragmentation overestimated gene copy number, potentially due to denaturation and partition of single-stranded DNA into different droplets. Covaris acoustic shearing and restriction enzyme digestion showed similar LoBs and gene copy number estimates to one another. It should be noted that moderate heating, commonly used in genomic DNA extraction protocols, did not significantly increase observed *KRAS* mutation counts.

Absolute nucleic acid quantification using limiting dilution end-point PCR began in the 1990s, when this method was applied to detect HIV variants (1) and genetic rearrangements in acute lymphoblastic leukemia (AML) (2). After end point PCR, Poisson statistical analysis of the number of positive and negative reactions permitted calculation of the starting template concentration (3). This initial method was time- and resource-intensive because partitioning of PCR required large numbers of 96-well plates. Advances in nanofabrication and microfluidic technologies (4,5) have made digital PCR (dPCR)

routinely accessible through a number of platforms, including droplet-based instruments (e.g., Bio-Rad's Droplet Digital PCR System or Raindance Technologies' RainDrop Digital PCR System, which was used in this study). This technology has many applications, including detection of single nucleotide variants (SNVs), copy number variations (CNVs), and exogenous targets such as bacteria and viruses. One application for which dPCR has gained popularity is the detection of rare mutant alleles (6).

In dPCR, DNA concentration and size are determining factors in uniform droplet formation, because the viscosity

of highly concentrated intact genomic DNA can change the average volume of droplets, which in turn could affect the accuracy of DNA quantitation (7). Therefore, fragmentation of intact genomic DNA samples and quality control of fragment sizes are critical pre-analytical steps in dPCR. Depending on the platform, DNA shearing has been recommended for input DNA concentrations >66 ng/20 µL reaction (using the Bio-Rad QX100/200 system) (4) or when using >75 ng of DNA per 25 µL reaction (using the RainDrop system) (8). For the RainDrop system, it is recommended that DNA be fragmented to 3–4 kb to

METHOD SUMMARY

Wild-type (WT) human genomic DNA was fragmented by heating, restriction digestion, or acoustic shearing using a Covaris focused-ultrasonicator. Digital PCR (dPCR) was used to determine the limit of blank (LoB) by quantifying observed WT and mutant allele counts of the proto-oncogenes *KRAS* and *BRAF* in the WT DNA using a droplet-based dPCR system.

ensure uniform droplet generation and amplifiable targets (8).

Current DNA fragmentation methods include double-strand breakage by heating (9), site-directed restriction enzyme digestion (4), digestion with an enzyme that generates random double-strand breaks (e.g., Fragmentase) (10), acoustic shearing based on focused-ultrasonication (e.g., using the Covaris focused-ultrasonicator), fragmentation with a biopolymer-based shredding system (QIAshredder, Qiagen) (11), and fragmenting by electromagnetic energy from microwave irradiation (12). Of these methods, high temperature is the simplest and least expensive approach. We adopted high temperature as a fragmentation method in developing dPCR assays for detecting SNVs using the RainDrop system, but found that heating had unanticipated effects on the results of DNA quantification: increased background mutation detection for certain alleles and greater than expected copy number quantification. These effects were not observed using either Covaris acoustic shearing or restriction enzyme digestion. We also found that moderate heating during genomic DNA extraction from whole blood did not cause an increase of false positive mutations, providing reassurance that the moderate heat incubation commonly used during DNA extraction is free from any significant mutagenic confounding effect. Here, we present a rationale for selecting an optimal DNA fragmentation strategy when performing dPCR, especially for clinical research applications such as minimal residual disease (MRD) monitoring.

Materials and methods

Genomic DNA fragmentation and quality control

Heating fragmentation was performed by incubating human genomic DNA (Promega, Madison, WI) at 95°C for 10 min, followed by pipetting 20 times using a 20- μ L pipette tip. The concentration of the DNA input was measured using a NanoDrop ND-1000 spectrometer (Wilmington, DE). Covaris acoustic shearing of 200 μ L genomic DNA (16 μ g) in blue miniTUBEs (Covaris, Woburn, MA), which generated DNA fragments centered on 3 kb, was done using a S220 Focused-ultrasonicator (Covaris) with duty cycle 20%, intensity 0.1, and

cycles per burst 1000, according to the manufacturer's instructions. Five units of restriction enzyme (*Hae*III or *Mse*I) (0.5 μ L) (New England BioLabs, Ipswich, MA) was added to a 25- μ L dPCR (see below), unless otherwise stated, and incubated at room temperature for 10 min before applying the sample to a RainDrop source chip (RainDance Technologies, Billerica, MA) for droplet generation. DNA fragment sizes were analyzed using Genomic DNA ScreenTape on an Agilent 2200 TapeStation (Santa Clara, CA).

Primers, probes, and synthetic DNA fragments

Primers were obtained from IDT (Coralville, IA) with standard desalting purification. *KRAS* forward primer: 5'-AAATGACTGAATATAAACTTGTGG-3'; *KRAS* reverse primer: 5'-TAGCTGTATC-GTCAAGGCACTC-3'. It should be noted that it is possible that a *KRAS* pseudogene (*KRAS*P1) could also be targeted by those primers; however, this would not change our conclusion that heat induces a higher false positive observed mutant frequency. Hydrolysis probes were synthesized by Life Technologies (Grand Island, NY) with wild-type (WT) and mutant sequences conjugated to VIC (Thermo Fisher Scientific, Waltham, MA) and 6-carboxyfluorescein (6-FAM) fluorophores, respectively. WT *KRAS* probe: 5'-TTGGAGCTGGTGGCGTA-3'; *KRAS* p.G12D (c. 35G>A) probe: 5'-TTGGAGCTGATGGCGTA-3'; *KRAS* p.G12V (c. 35G>T) probe: 5'-TTGGAGCT-GTTGGCGTA-3'; *KRAS* p.G12S (c. 34G>A): 5'-TTGGAGCTAGTGGCGTA-3'. *BRAF* primers and probe sequences are described elsewhere (4). Synthetic *KRAS* and *BRAF* mutant DNAs were synthesized as 225- or 200-bp gBlocks Gene Fragments by IDT with the SNV site located in the center of the synthetic gene sequence. The gBlock Gene Fragments (200 ng) were resuspended in TE buffer to a final concentration of 40 ng/ μ L in 5 μ L aliquots. Aliquots were serially diluted with nuclease-free water to 4000 copies/ μ L, and 1 μ L was spiked into normal WT human genomic DNA in dPCR assays as a positive control.

Droplet digital PCR

A RainDrop Digital PCR System (RainDance Technologies) was used for detection of *KRAS* and *BRAF* mutations. In a pre-PCR environment, 1 $\times$ TaqMan

Genotyping Master Mix (Life Technologies) was mixed with 900 nM forward and reverse primers, 200 nM 6-FAM and VIC hydrolysis probes (Life Technologies), 1 $\times$ Droplet Stabilizer (RainDance Technologies), and DNA template to a final volume of 25 μ L (unless stated otherwise) in 8-strip PCR tubes (Axygen, Tewksbury, MA). Reaction mixtures were pipetted into 8-well source chips (RainDance Technologies) and applied to a RainDrop Source instrument for droplet generation. Droplets were produced into new 8-strip PCR tubes (final volume 75 μ L), sealed with opaque flat caps (RainDance Technologies), and PCR amplified to end point in a C1000 Touch thermal cycler with a 96-deep well reaction module (Bio-Rad, Hercules, CA). The thermal cycling conditions were: 10 min at 95°C, followed by 45 cycles of 15s at 95°C, 1 min for annealing and extension (at 64°C for *KRAS* and 60°C for *BRAF* assay, with 0.5°C/sec ramp rate), followed by 10 min at 98°C and storage at 12°C or immediate application to a RainDrop Sense instrument for droplet analysis.

Droplet data analysis

Calculation of WT copy numbers in genome equivalents (GE) assumed a haploid genome mass equivalent of 3.23 pg and used the equation $GE = \text{DNA mass (pg)} / 3.23 \text{ (pg)}$. Due to droplet loss in the RainDrop Sense instrument, the number of measured target droplets was corrected using the equation: measured target droplets = (total droplets generated / total intact droplets) $\times$ (counted droplets with target of interest). The mutant DNA false-positive percentage was calculated from dPCR results obtained when using normal WT human genomic DNA without spiked mutant DNA as input, following the equation: false positive% = (measured positive droplets in the mutant channel) / (measured positive droplets in both the WT and mutant channels) $\times$ 100%. Limit of blank (LoB) was calculated with the formula $LoB = \text{mean}_{\text{false positive\%}} + 1.645 \times SD_{\text{false positive\%}}$ (12).

Blood genomic DNA extraction

Human whole blood was purchased from BioreclamationIVT (Westbury, NY). Whole blood was pooled from 3 healthy donors that included both men and women, preserved with K₂EDTA, and shipped at 4°C overnight. Whole blood was stored at -80°C until DNA extraction.

Figure 1. Heat fragmentation increases false positives of KRAS G12D (c.35G>A) in digital (dPCR) analysis. (A) Intact (Lane 1), heat-fragmented (Lane 2), and Covaris acoustically sheared (Lane 3) normal human genomic DNA obtained on an Agilent TapeStation 2200 instrument specifically detecting double-stranded DNA. Heat-fragmented DNA showed a decreased overall signal and was larger, which may be due to heat-induced melting of the genomic DNA to single-strand DNA with partial re-annealing of repetitive sequences resulting in higher molecular weight complexes. L: ladder. (B) KRAS G12D dPCR data using heat-fragmented or Covaris acoustically sheared genomic DNA. Panel 1: no template; Panel 2: intact genomic DNA, low DNA input (23,220 GE); Panel 3: heat-fragmented genomic DNA, low DNA input; Panel 4: heat-fragmented genomic DNA, high DNA input (200,000 GE); Panel 5: Covaris acoustically sheared genomic DNA, low DNA input; Panel 6: positive control, synthetic KRAS G12D DNA fragments spiked into heat-fragmented genomic DNA. GE: genome equivalents. (C) Tabulated dPCR results for the conditions described in (B). Predicted wild-type (WT) copies = Input DNA mass (pg) / 3.23 (pg), where 1 haploid human genome is estimated as 3.23 pg. Efficiency of WT allele recovery% = measured WT droplets / predicted WT copies × 100%. Mutant false positive% = measured mutant droplets / (measured WT droplets + measured mutant droplets) × 100%. NA: not applicable.

Blood genomic DNA was extracted from 200 μ L whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). Purification protocols were modified from the manufacturer's instructions to test the effects of temperature during protease incubation and RNase treatment on dPCR performance: (i) Blood lysates were incubated at room temperature, 56°C, or 70°C for 10 min after supplementing with 20 μ L Qiagen Protease and 200 μ L AL buffer. (ii) At the elution step, columns were eluted in 50 μ L AE buffer with or without 0.4 μ L RNase A

solution (Promega) and incubated at room temperature for 10 min after elution. DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer and the Qubit dsDNA HS Assay Kit (Life Technologies). Isolated DNA was aliquoted and stored at -20°C until dPCR analysis.

Results and discussion

The LoB and the limit of detection (LoD) are part of the digital MIQE (Minimum Information for publication of Quanti-

tative dPCR Experiments) (13). The LoB determines the LoD (the lowest concentration of analyte that can be reliably detected above the LoB) and thus is a critical parameter for defining the sensitivity of rare mutant allele detection in a WT background. LoB is defined by the frequency of positive droplets detected in samples with no target DNA present (14). To determine the LoB of the dPCR assay for detecting KRAS G12D (c.35G>A), a common oncogenic mutation, we analyzed normal, WT human genomic DNA that had been

Figure 2. Restriction enzyme-digested and Covaris acoustically sheared genomic DNA show similar limits of blank (LoBs) for *KRAS* G12D (c.35G>A) assays. (A) Agilent TapeStation 2200 images of wild-type (WT) genomic DNA digested for varying times with *Hind*III-, *Hae*III-, or *Mse*I. (B) *KRAS* G12D digital PCR (dPCR) data generated using *Hae*III- or *Mse*I-digested or Covaris acoustically sheared genomic DNA samples (200,000 GE). GE: genome equivalents. Samples were analyzed as in Figure 1. VIC, excitation λ : 528 nm, emission λ : 554 nm. 6-carboxyfluorescein (FAM), excitation λ : 494 nm, emission λ : 522 nm. The x-axis and y-axis represent fluorescence units observed for FAM and VIC, respectively. (C) Table comparing *KRAS* G12D (c.35G>A) dPCR assay results from *Hae*III- or *Mse*I-digested or Covaris acoustically sheared genomic DNA. Results represent mean and SD of data from 5 dPCR replicates for each fragmentation method. LoB = $\text{mean}_{\text{mutant false positive}} + 1.645 \times (\text{SD}_{\text{mutant false positive}})$.

fragmented by heating to 95°C for 10 min. In dPCR assays for *KRAS* G12D using 75 ng of unfragmented input DNA (23,220 GE), zero mutant copies were detected (Figure 1, B and C, Panel 2). However, when using heat-fragmented DNA, the assay detected 8 copies of mutant DNA, with a fractional abundance of 0.024% for mutant alleles (Figure 1, B and C, Panel 3). When total DNA input was increased to 200,000 GE (646 ng), detection of mutant copies increased to 41, with a fractional abundance of 0.012% for mutant alleles (Figure 1, B and C, Panel 4). We next evaluated the same mutation using WT genomic DNA fragmented by Covaris acoustic shearing, which generated a range of DNA fragment sizes, with a peak at 3–4

kb (Figure 1A, Lane 3; Supplementary Figure S1, Lanes 3 and 4). Using 23,220 GE of Covaris acoustically sheared WT genomic DNA, the dPCR assay detected zero mutant copies, a result similar to intact DNA (Figure 1, B and C, Panel 5). At 200,000 GE of sheared input DNA, 5 mutant copies were detected, representing a fractional abundance of 0.00276% for mutant alleles (Figure 2B, Panel 4 and Figure 2C).

One possible explanation for the observed increase of LoB in *KRAS* G12D (c. 35G>A) assays when using heating for DNA fragmentation is the appearance of new heat-induced mutations. Heating at high temperature causes deamination of cytosine (C) to uracil (U) in WT DNA (15), which might enable the

PCR probe that detected the mutant DNA with thymine (T) (complementary to A) to recognize a heat-induced mutation site containing U. To test this hypothesis, we set up additional *KRAS* mutation assays targeting *KRAS* G12V (c.35G>T) and *KRAS* G12S (c.34G>A). The only difference among the three *KRAS* mutation assays was in a single nucleotide in the probe that detected the point mutation site. Using heat-fragmented genomic DNA (200,000 GE), we observed zero false positives in the *KRAS* G12V assay detecting a G>T mutation, but 50 false positives (representing a fractional abundance of 0.017%) in the *KRAS* G12S assay detecting a G>A mutation, similar to results from the *KRAS* G12D assay, which also targeted

Figure 3. Heat fragmentation increases false positives in digital PCR (dPCR) assays for G>A (*KRAS* G12S) but not G>T (*KRAS* G12V). (A) dPCR data from *KRAS* G12V (c.35G>T) and *KRAS* G12S (c.34G>A) assays applied to heat-fragmented genomic DNA. Panel 1: no template; Panel 2: heat-fragmented genomic DNA (200,000 GE); Panel 3: synthetic *KRAS* G12V DNA spiked into heat-fragmented genomic DNA (200,000 GE); Panel 4: no template; Panel 5, heat-fragmented genomic DNA (200,000 GE); Panel 6: synthetic *KRAS* G12S DNA spiked into heat-fragmented genomic DNA (200,000 GE). GE: genome equivalents. (B) Tabulated dPCR results for the conditions described in (A). NA: not applicable.

a G>A mutation (Figure 3, A and B, Panels 2 and 5). In another dPCR assay detecting a non-G>A mutation, *BRAF* V600E (c.1799 T>A), heat-fragmented DNA gave zero false positives at 200,000 GE and only 1 mutant copy (0.00032% fractional abundance) measured in 400,000 GE (Supplementary Figure S2, Panels 2 and 3). These results supported the hypothesis that heat induces DNA mutations specifically at G/C sites and therefore increases the LoB for G>A SNVs, but not for G>T or T>A.

We also examined the efficiency of target DNA recovery, comparing the WT allele copy number measured by

dPCR with the expected input of WT DNA. Surprisingly, we found that heat-fragmented genomic DNA demonstrated a recovery efficiency ranging 148.8%–171.5% (Figure 1C, Panels 3, 4, and 6; Figure 3B, Panels 2, 3, 5, and 6), suggesting an over-estimation of WT DNA copy number. In contrast, both intact and Covaris acoustically sheared genomic DNA showed an amplification efficiency <100%, as expected, ranging 88.9%–93.5% (Figure 1, B and C, Panels 2 and 5). This result might be explained by heat denaturation of double-stranded DNA to single-stranded DNA with insufficient re-annealing to complementary

strands prior to incorporation into droplets for PCR. Initially, our result would seem to contradict a previous study using the Fluidigm BioMark system that reported heating can reduce the measured copy number of plasmid DNA by impairing DNA integrity (16). However, such an effect was only observed with heating to 95°C for >10 min, whereas with shorter heating times (30 s and 1 min), the measured copy number was increased compared with no heating (16). This is similar to our results but occurred on a different time scale, which may be explained by the reduced complexity of plasmid DNA in the prior study compared

Figure 4. Restriction enzyme digestion and Covaris acoustic shearing of DNA yields similar results in *KRAS* G12S and *BRAF* V600E assays. (A) Digital PCR (dPCR) data from *KRAS* G12S (c.34G>A) generated using *MseI*-digested or Covaris acoustically sheared genomic DNA as input. Panel 1: negative control, H₂O (no template control); Panel 2: *MseI*-digested genomic DNA (188,367 GE) (10 min digestion at room temperature); Panel 3: Covaris acoustically sheared genomic DNA (200,000 GE); Panel 4: synthetic *KRAS* G12S DNA fragment spiked into intact genomic DNA (14,570 GE). GE: genome equivalents. (B) Tabulated dPCR results for the conditions described in (A). (C) dPCR data from *BRAF* V600E (c.1799T>A) assays using *MseI*-digested or Covaris acoustically sheared genomic DNA: no template (Panels 1 and 5); *MseI*-digested genomic DNA (Panel 2, 188,367 GE; Panel 6, 419,628 GE); Covaris acoustically sheared genomic DNA (Panel 3, 200,000 GE; Panel 7, 448,173 GE); positive control, synthetic *BRAF* V600E DNA spiked into Covaris acoustically sheared genomic DNA (Panel 4, 14,490 GE; Panel 8, 448,173 GE). (D) Tabulated dPCR results for the conditions described in (C). NA: not applicable.

with the human genomic DNA analyzed in our study.

Our results suggested that DNA fragmentation by heating increases LoB and copy number quantification and is thus not ideal as a shearing strategy for dPCR, especially for assays measuring G>A mutations. Restriction enzyme digestion is an alternative DNA fragmentation method recommended for the Bio-Rad QX platform, but it has not been well-studied for the RainDrop dPCR system (4). We tested the fragmentation efficiency of 3 commonly used restriction enzymes by performing the digestions in 1× PCR master mix at room temperature for 10 min. The addition of 5 U of *HindIII* did not efficiently digest 1 or 2 μg of genomic DNA to 3–4 kb (Figure 2A). In contrast, the addition of 5 U of *HaeIII* efficiently digested 1 μg of genomic DNA to ~4 kb or smaller; however, the efficiency of *HaeIII* dropped substantially for 2 μg DNA. Finally, treatment with 5 U of *MseI* efficiently digested both 1 and 2 μg of genomic DNA to <4 kb. Based on these results, we selected *HaeIII* and *MseI* for further testing in both *KRAS* and *BRAF* dPCR assays after confirming that neither our targets nor our reference amplicons have *HaeIII* or *MseI* recognition sites and that both enzymes are insensitive to methylation.

We analyzed the *KRAS* G12D mutation in the RainDrop dPCR system using WT genomic DNA fragmented by Covaris acoustic shearing or restriction enzyme digestion by *HaeIII* or *MseI* as input, with five replicates for each condition. The LoB and efficiency of recovery of target DNA were generally similar for all three fragmentation methods (Figure 2, B and C; Supplementary Figure S3) but modestly lower in the restriction enzyme-treated samples (i.e., 88.7% and 89.1% for restriction enzymes *HaeIII* and *MseI*, respectively, versus 92.7% for Covaris acoustic shearing). This could potentially be due to PCR inhibition by the restriction enzyme storage buffer since we added the restriction enzyme directly to the sample without further purification prior to PCR.

We then compared the false-positive rates when using restriction enzyme-digested DNA as input (using *MseI* because of its greater robustness to higher input of genomic DNA) versus Covaris acoustically sheared DNA

Table 1. Limit of blank (LoB) of *KRAS* G12D (c.35G>A) droplet digital PCR (dPCR) assay using whole blood genomic DNA template isolated without RNase treatment.

No RNase A	<i>KRAS</i> G12D false positive %			% efficiency of WT allele recovery		
	RT	56°C	70°C	RT	56°C	70°C
Extraction 1_replicate 1	0.00121%	0.00098%	0.00196%	58.2%	68.4%	65.1%
Extraction 1_replicate 2	0	0	0	55.6%	67.0%	64.7%
Extraction 2_replicate 1	0.00099%	0	0	68.8%	65.5%	51.3%
Extraction 2_replicate 2	0.00094%	0	0	67.8%	64.0%	50.9%
Mean (<i>n</i> = 4)	0.00078%	0.00024%	0.00049%	62.6%	66.2%	58.0%
SD	0.00054%	0.00049%	0.00098%	6.65%	1.93%	7.99%
LoB	0.00167%	0.00105%	0.00210%			
Whole blood genomic DNA template was isolated at varying temperatures without RNase treatment and then measured by a NanoDrop spectrophotometer. dPCR reaction volume: 30 μ L. DNA input: 200,000 genome equivalents (GE) / reaction digested with 0.5 μ L (5 U) <i>MseI</i> for 10 min at room temperature (RT). WT: wild-type.						

(Supplementary Figure S1). *MseI*-digested and Covaris acoustically sheared DNA showed similar false-positive rates and efficiencies of input target recovery for *KRAS* G12S assays (Figure 4, A and B, Panels 2 and 3). The performance was also similar for *BRAF* V600E assays, with no false positives detected in either *MseI*-digested or Covaris acoustically sheared genomic DNA (Figure 4, C and D, Panels 2, 3, 6, and 7). This result suggests that restriction enzyme digestion can be a simple and cost-effective alternative to Covaris acoustic shearing for dPCR assays using the RainDrop system. It is worth noting, however, that enzymes other than *HaeIII* or *MseI* may need to be chosen depending on the target mutation(s) being analyzed.

An important application of dPCR using genomic DNA is MRD monitoring in hematopoietic disorders such as AML. DNA extraction for MRD analysis commonly requires incubating blood cell lysates with protease at 56°C or 70°C (17). We investigated if moderate heating at 56°C or 70°C increases the LoB in G>A SNV dPCR assays. As contaminating RNA might serve as an additional template for dPCR assays,

we also evaluated the effect of RNase A treatment on the number of target DNA molecules measured by dPCR. Using *KRAS* G12D assays, we found no substantial difference in LoB when whole blood genomic DNA was extracted with protease incubation at room temperature, 56°C, or 70°C, and this was not affected by the optional RNase A treatment during DNA elution (Tables 1 and 2). A previous study suggested that cytosine deamination was 15 times slower at 70°C than at 95°C (15), which might explain why heating at moderate temperatures did not show a measurable mutagenic effect. We also found that efficiency of target DNA recovery by dPCR ranged from 50.9% to 65.1%, was similar at the 3 temperatures tested, and was not substantially influenced by RNase A treatment (Tables 1 and 2). The fact that the efficiency was not closer to 100% could be due to an overestimation of DNA concentration as measured by the NanoDrop ND-1000 spectrophotometer, which can be subject to absorption at 260 nm by co-purified impurities in the DNA sample (18). For a subset of samples, we re-measured concentrations using the Qubit assay and re-calculated targeted recovery efficiencies, which ranged from 68.3%

to 85.9% (Supplementary Tables S1 and S2). Although dPCR is more resistant to PCR inhibitors than real-time PCR methods, there is nevertheless some degree of inhibition in their presence (19). It is possible that co-purifying PCR inhibitors such as heme from whole blood (20) might also contribute in part to reducing target recovery efficiency.

Here we demonstrated that heat-based DNA fragmentation caused DNA mutagenesis that confounded SNV analysis of G>A mutations in dPCR. Restriction enzyme digestion is an alternative DNA fragmentation approach that performed similarly to Covaris acoustic shearing. We should note that a limitation of our study is that we used spiked-in synthetic DNA of 200 or 225 bp in length, as opposed to fragmented genomic DNA. In future studies, cell line DNA harboring specific mutations could be used as an alternative. In conclusion, our study provides a rationale for choosing an optimal DNA fragmentation method for specific assay types and applications.

Author contributions

Q.K. and M.T. conceived the project. Q.K., B.P., and M.G. designed the experiments. Q.K. performed the experiments and analyzed the data. Q.K. and M.T. wrote the manuscript with contributions from all authors.

Acknowledgments

We thank William Kiffmeyer (RainDance Technologies) for advice on assay design, Andrew Rhim for use of the RainDrop dPCR platform, and Scott Tomlins for use of the Agilent TapeStation instrument. We also thank the DNA Sequencing Core Facility at the University of Michigan for use of their services. This project was supported by NIH R01DK085714 awarded to M.T. and UL1TR000433 from the National Center for Advancing Translational Sciences Postdoctoral Translational Scholar Program Fellowship to awarded to Q.K. The content is solely the responsibility of the authors and does not necessarily represent the official views of NCATS or the National Institutes of Health. This paper is subject to the NIH Public Access Policy.

Competing interests

The authors declare no competing interests.

Table 2. Limit of blank (LoB) of *KRAS* G12D (c.35G>A) droplet digital PCR (dPCR) assay using whole blood genomic DNA template isolated with RNase treatment.

Treated with RNase A	<i>KRAS</i> G12D false positive%			% efficiency of WT allele recovery		
	RT	56°C	70°C	RT	56°C	70°C
Extraction 1_replicate 1	0	0	0	68.8%	65.2%	63.6%
Extraction 1_replicate 2	0	0.00093%	0	69.5%	65.8%	64.0%
Extraction 2_replicate 1	0	0	0	64.1%	61.6%	56.8%
Extraction 2_replicate 2	0.00113%	0.00117%	0.00231%	57.9%	57.7%	58.4%
Mean (<i>n</i> = 4)	0.00028%	0.00052%	0.00058%	65.1%	62.5%	60.7%
SD	0.00057%	0.00061%	0.00115%	5.34%	3.74%	3.66%
LoB	0.00122%	0.00153%	0.00247%			
Whole blood genomic DNA template was isolated at various temperatures with RNase treatment and then measured by a NanoDrop spectrophotometer. dPCR reaction volume: 30 μ L. DNA input: 200,000 genome equivalents (GE) / reaction digested with 0.5 μ L (5 U) <i>MseI</i> for 10 min at room temperature (RT). WT: wild-type.						

References

1. Simmonds, P., P. Balfe, C.A. Ludlam, J.O. Bishop, and A.J. Brown. 1990. Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. *J. Virol.* 64:5840-5850.
2. Sykes, P.J., S.H. Neoh, M.J. Brisco, E. Hughes, J. Condon, and A.A. Morley. 1992. Quantitation of targets for PCR by use of limiting dilution. *Biotechniques* 13:444-449.
3. Vogelstein, B. and K.W. Kinzler. 1999. Digital PCR. *Proc. Natl. Acad. Sci. USA* 96:9236-9241.
4. Hindson, B.J., K.D. Ness, D.A. Masquelier, P. Belgrader, N.J. Heredia, A.J. Makarewicz, I.J. Bright, M.Y. Lucero, et al. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* 83:8604-8610.
5. Taly, V., D. Pekin, L. Benhaim, S.K. Kotsoopoulos, D. Le Corre, X. Li, I. Atochin, D.R. Link, et al. 2013. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. *Clin. Chem.* 59:1722-1731.
6. Oxnard, G.R., C.P. Paweletz, Y. Kuang, S.L. Mach, A. O'Connell, M.M. Messineo, J.J. Luke, M. Butaney, et al. 2014. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res.* 20:1698-1705.
7. Nie, Z.H., M.S. Seo, S.Q. Xu, P.C. Lewis, M. Mok, E. Kumacheva, G.M. Whitesides, P. Garstecki, and H.A. Stone. 2008. Emulsification in a microfluidic flow-focusing device: effect of the viscosities of the liquids. *Microfluid Nanofluid* 5:585-594.
8. Milbury, C.A., Q. Zhong, J. Lin, M. Williams, J. Olson, D.R. Link, and B. Hutchison. 2014. Determining lower limits of detection of digital PCR assays for cancer-related gene mutations. *Biomol Detect Quantif.* 7:8-22.
9. Wang, J. and B. McCord. 2011. The application of magnetic bead hybridization for the recovery and STR amplification of degraded and inhibited forensic DNA. *Electrophoresis* 32:1631-1638.
10. Knierim, E., B. Lucke, J.M. Schwarz, M. Schuelke, and D. Seelow. 2011. Systematic comparison of three methods for fragmentation of long-range PCR products for next generation sequencing. *PLoS ONE* 6:e28240.
11. Yuki, S.A., P. Kaiser, P. Kim, P.L. Li, and J.K. Wong. 2014. Advantages of using the QIAshredder instead of restriction digestion to prepare DNA for droplet digital PCR. *Biotechniques* 56:194-196.
12. Yang, Y. and J. Hang. 2013. Fragmentation of genomic DNA using microwave irradiation. *Journal of biomolecular techniques.* 24:98-103.
13. Huggett, J.F., C.A. Foy, V. Benes, K. Emslie, J.A. Garson, R. Haynes, J. Hellemans, M. Kubista, et al. 2013. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin. Chem.* 59:892-902.
14. Armbruster, D.A. and T. Pry. 2008. Limit of blank, limit of detection and limit of quantitation. *Clin. Biochem. Rev.* 29(Suppl 1):S49-S52.
15. Lindahl, T. and B. Nyberg. 1974. Heat-induced deamination of cytosine residues in deoxyribonucleic acid. *Biochemistry* 13:3405-3410.
16. Bhat, S., J.L.H. McLaughlin, and K.R. Emslie. 2011. Effect of sustained elevated temperature prior to amplification on template copy number estimation using digital polymerase chain reaction. *Analyst.* 136:724-732.
17. Bosch, L.A. 2014. A Proposal for an Individualized Pharmacogenetic-Guided Warfarin Dosage Regimen for Puerto Rican Patients Commencing Anticoagulation Therapy. *J Pharmacogenomics Pharmacoproteomics.* 5:pii:T-001.
18. Simbolo, M., M. Gottardi, V. Corbo, M. Fassan, A. Mafficini, G. Malpeli, R. Lawlor, and A. Scarpa. 2013. DNA Quantification Workflow for Next Generation Sequencing of Histopathological Samples. *PLoS ONE* 8:e62692.
19. Dingle, T.C., R.H. Sedlak, L. Cook, and K.R. Jerome. 2013. Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances. *Clin. Chem.* 59:1670-1672.
20. Akane, A., K. Matsubara, H. Nakamura, S. Takahashi, and K. Kimura. 1994. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. *J. Forensic Sci.* 39:362-372.

Received 12 September 2015; accepted 05 January 2016.

Address correspondence to Muneesh Tewari, Department of Internal Medicine, University of Michigan, 109 Zina Pitcher Place, 1502 BSRB, SPC 2200, Ann Arbor, MI 48109. E-mail: mtewari@med.umich.edu

To purchase reprints of this article, contact: biotechniques@fosterprinting.com

The BMG LABTECH All Stars

Innovative, high-performance microplate readers for all assay needs

CLARIOstar®

The most sensitive monochromator-based microplate reader.

PERAstar® FSX

The new gold standard for High Throughput Screening.

Omega series

Upgradable filter-based microplate readers for any life science application.

SPECTROstar® Nano

Absorbance reader with ultra-fast detection of UV/Vis absorbance spectra.

Find all our microplate readers on www.bmglabtech.com

BMG LABTECH
The Microplate Reader Company