Clinical Chemistry 59:5
000–000 (2013)

Multiplex Picoliter-Droplet Digital PCR for Quantitative Assessment of DNA Integrity in Clinical Samples

Audrey Didelot,[1†] Steve K. Kotsopoulos,[2†] Audrey Lupo,[1,3] Deniz Pekin,[1,4] Xinyu Li,[2] Ivan Atochin,[2] Preethi Srinivasan,[2] Qun Zhong,[2] Jeff Olson,[2] Darren R. Link,[2] Pierre Laurent-Puig,[1] Hélène Blons,[1] J. Brian Hutchison,[2] and Valerie Taly[1*]

BACKGROUND: Assessment of DNA integrity and quantity remains a bottleneck for high-throughput molecular genotyping technologies, including next-generation sequencing. In particular, DNA extracted from paraffin-embedded tissues, a major potential source of tumor DNA, varies widely in quality, leading to unpredictable sequencing data. We describe a picoliter droplet–based digital PCR method that enables simultaneous detection of DNA integrity and the quantity of amplifiable DNA.

METHODS: Using a multiplex assay, we detected 4 different target lengths (78, 159, 197, and 550 bp). Assays were validated with human genomic DNA fragmented to sizes of 170 bp to 3 kb. The technique was validated with DNA quantities as low as 1 ng. We evaluated 12 DNA samples extracted from paraffin-embedded lung adenocarcinoma tissues.

RESULTS: One sample contained no amplifiable DNA. The fractions of amplifiable DNA for the 11 other samples were between 0.05% and 10.1% for 78-bp fragments and ≤1% for longer fragments. Four samples were chosen for enrichment and next-generation sequencing. The quality of the sequencing data was in agreement with the results of the DNA-integrity test. Specifically, DNA with low integrity yielded sequencing results with lower levels of coverage and uniformity and had higher levels of false-positive variants.

CONCLUSIONS: The development of DNA-quality assays will enable researchers to downselect samples or process more DNA to achieve reliable genome sequencing with the highest possible efficiency of cost and effort, as well as minimize the waste of precious samples.

The assessment of DNA integrity and quantity in a sample remains a bottleneck for different molecular genotyping technologies, including next-generation sequencing. In particular, DNA extracted from formalin-fixed paraffin-embedded (FFPE)[5] tissue samples, a major potential source of tumor DNA *(1, 2)*, is subject to extensive fragmentation and damage that leads to unpredictable data quality *(3)*. DNA quality (or DNA integrity) is a loosely defined metric that describes the size distribution of fragmented DNA and the fraction of DNA that can be amplified by the PCR. DNA isolated from FFPE tissue samples is often highly fragmented and chemically degraded. Alterations occur during tissue collection, the fixation process, and sample storage *(4)*. The fixation process introduces damage by chemically modifying bases, denaturing double-stranded DNA, and cross-linking of cytosine residues *(5, 6)*.

Traditional tools for measuring DNA quantity are UV absorbance, gel electrophoresis, and assays with intercalating dyes. None of these tools measure the amount of amplifiable DNA specifically, and each of these tools has additional limitations *(7)*. UV absorbance does not allow detection of DNA fragment length. Capillary gel electrophoresis permits measurements of the length distributions of DNA fragments but requires a relatively large amount of DNA. Intercalating-dye assays require calibration. The current gold standard for measuring both DNA quality and quantity is to use a quantitative PCR (qPCR) assay. qPCR has a number of drawbacks, however, including the need for assay calibration with calibrators that are similar in quality to the samples being evaluated, which leads to an iterative process or a need for multiple calibration curves *(7)*. Furthermore, qPCR requires running multiple assays to evaluate different DNA lengths within a sample. For this reason, the assays are often

[1] Université Paris Sorbonne Cité, INSERM UMR-S775, Paris, France; [2] RainDance Technologies, Lexington, MA; [3] Assistance Publique – Hôpitaux de Paris, Paris, France; [4] Institut de Science et d'Ingénierie Supramoléculaires (ISIS), Université de Strasbourg, CNRS UMR 7006, Strasbourg, France.
* Address correspondence to this author at: Université Paris Sorbonne Cité, INSERM UMR-S775, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France. E-mail valerie.taly@parisdescartes.fr.
† These authors contributed equally to this work.
Received August 8, 2012; accepted January 29, 2013.
Previously published online at DOI: 10.1373/clinchem.2012.193409
[5] Nonstandard abbreviations: FFPE, formalin-fixed paraffin-embedded; qPCR, quantitative PCR; dPCR, digital PCR; FAM, 6-carboxyfluorescein; SNP, single-nucleotide polymorphism.

designed in a multiplexed format *(8–13)*, but bulk-phase multiplex PCR can be limited by poor compatibility of the multiple assays in the same volume. Finally, measurement of the absolute concentration of amplifiable DNA and the relative distribution of fragment lengths is limited by the imprecision (CV) of any qPCR assay, which is typically 10%–50% *(14–16)*.

The broad adoption of a pretesting work flow for an alternative sample hinges on 3 factors: The assay should be inexpensive compared with the ultimate genomic analysis, consume a small fraction of the sample, and, finally, be predictive of the quality of the data to be obtained in downstream genomic analyses. Digital PCR (dPCR) enables accurate quantification of amplifiable DNA with only a small amount of sample (i.e., hundreds of copies), and multiple amplicon lengths can be measured simultaneously without requiring a calibrator. This technology relies on compartmentalization of a sample into a large number of discrete volumes containing <1 copy of template DNA on average *(17–19)*, and it addresses the limitations of traditional and qPCR methods *(7)*. After amplification, intercalating dyes, TaqMan® probes, or other qPCR probes are used to enhance the fluorescence of target-containing compartments and in the counting of the absolute number of target molecules present in the compartmentalized sample. This approach has been demonstrated with microwell plates *(20)*, bulk emulsion droplets *(21)*, microfluidic compartments *(22, 23)*, and nanoliter- or picoliter-scale droplets produced with microfluidics *(24–26)*.

The recent progress in dPCR technology has included the demonstration of multiplex dPCR for the quantification of ≥5 DNA targets in picoliter droplets (i.e., the number of copies counted) in a single assay *(25)*. In addition to detecting multiple targets simultaneously, using picoliter volumes for dPCR has the major advantage of the ability to quantify targets across a relatively large range of input DNA concentrations *(24)*. Hence, multiplex dPCR using picoliter droplets is well suited for an assay that detects DNA quantity and quality.

We describe the development and application of a multiplex dPCR assay panel to measure the quality of a DNA sample, including the length distribution of DNA fragments and the amount of PCR-amplifiable DNA. This type of assay is valuable for assessing the quantity and integrity of DNA samples before potentially sensitive or precious samples are committed to expensive and/or sample-consuming genome analysis tools, such as sequencing. We applied the procedure to the analysis of 12 DNA samples extracted from FFPE tissues of lung adenocarcinomas. The results of this DNA-quality assay were verified with next-generation sequencing *(27)* of 4 samples that the dPCR assay had assessed as low, medium, or high quality.

Materials and Methods

TUMOR DNA EXTRACTION

Twelve paraffin-embedded tissues from lung adenocarcinomas were obtained from Hotel Dieu Hospital (Paris, France) in accordance with French law. Each tumor block was reviewed by a pathologist (A.L.), and 3 slides of 20-μm slices were prepared. Tumor cell content was assessed by hematoxylin-eosin-safran staining. All but 2 samples contained >60% tumor cells. The remaining 2 samples contained 30% and 40% tumor cells. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen), and DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

DNA-QUALITY ASSAY REACTIONS

All primers and TaqMan probes (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue5) were designed with the Custom TaqMan Assay Design Tool (Applied Biosystems). We mixed 12.5 μL TaqMan Universal Master Mix (Life Technologies) with the assay solution, which contained 0.75 μL of 40 mmol/L Deoxynucleotide (dNTP) Solution Mix (New England BioLabs), 0.5 μL 25 mmol/L $MgCl_2$, 2.5 μL Droplet Stabilizer (RainDance Technologies), 2.5 μL 10× DNA quality Assay Mix (8 μmol/L of forward and reverse primers and probe concentrations (see Table 1 in the online Data Supplement), and a minimum of 1 ng target DNA template. The final reaction volume was 25 μL.

FRAGMENTED DNA SAMPLES

Using the S2 Adaptive Focused Acoustics® S2 instrument (Covaris) and wild-type human genomic DNA (Promega), we fragmented DNA to mean sizes of 170 bp, 230 bp, 290 bp, 370 bp, and 570 bp. We used Nebulizers (Life Technologies) to generate 3000-bp fragmented controls. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer. Fragmented 100-ng DNA controls were used to assess the specificity of each biomarker in the DNA-quality assay.

SEQUENCE ENRICHMENT AND SEQUENCING

The 4 samples selected for sequencing were tested with TaqMan qPCR probes for *KRAS*[6] (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) and *EGFR* (epidermal growth factor receptor) mutations, as described previously *(28, 29)*. Three samples contain a mutation

[6] *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *EGFR*, epidermal growth factor receptor; *SPINT1*, serine peptidase inhibitor, Kunitz type 1; *INTS2*, integrator complex subunit 2; *MOCS2*, molybdenum cofactor synthesis 2; *PRP8*, pre-mRNA processing factor 8.

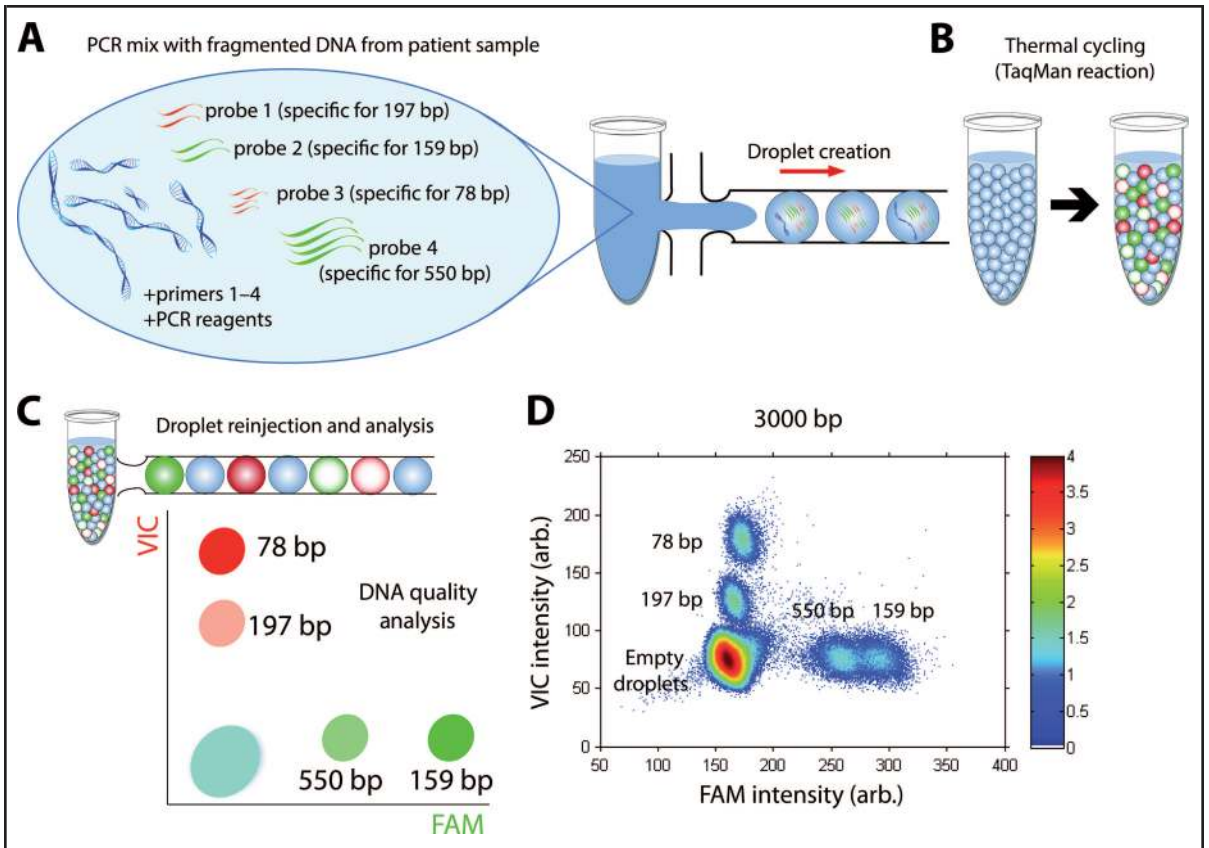

Fig. 1. Overview of the work flow used for the DNA-quality assay.

(A), An aqueous phase containing DNA extracted from paraffin-embedded tissues and PCR reagents is compartmentalized into droplets before thermocycling. (B), The mixture contains 2-color TaqMan® probes [FAM (excitation λ, 494 nm; emission λ, 522 nm) and VIC (excitation λ, 528 nm; emission λ, 554 nm)] at 2 concentrations (0.16 μmol/L and 0.2 μmol/L) to identify 4 kinds of DNA according to size (C, D). arb., arbitary.

in the *KRAS* gene (G13A, G12V, G12C), and 1 sample has a mutation in the *EGFR* gene (L858R). We used the Cancer HotSpot Panel sequence-enrichment panel and RDT 1000 instrumentation (RainDance Technologies) for amplifying 676 target regions (127 754 bases) in the template DNA. After sequence enrichment, each of the 4 DNA samples was sequenced with a PGM sequencer (Ion Torrent) and a 318™ Chip (Ambry Genetics).

SEQUENCING DATA ANALYSIS AND MICROFLUIDICS PROCEDURES

Method details for the sequencing data analysis and microfluidics procedures are provided in the online Data Supplement.

Results and Discussion

CONCEPT OF USING dPCR FOR A DNA-QUALITY ASSAY

We succeeded in developing a multiplex digital microfluidics procedure to generate and analyze picoliter-volume droplets. The goal was to measure the quality of a DNA sample, including the length distribution of DNA fragments and the amount of PCR-amplifiable DNA (Fig. 1). The DNA-quality assay was based on dPCR measurement of amplifiable DNA associated with 4 different amplicons with sizes of 78, 159, 197, and 550 bp, which corresponded to amplicons within the *SPINT1* (serine peptidase inhibitor, Kunitz type 1), *INTS2* (integrator complex subunit 2), *MOCS2* (molybdenum cofactor synthesis 2), and *PRP8* [PRP8 premRNA processing factor 8 homolog (*S. cerevisiae*)] genes, respectively (see Table 1 in the online Data Supplement). To overcome the fact that genetic alterations are highly variable from one tumor to another, we chose different markers from different chromosomal arms (*INTS2*, 17q23.2; *SPINT1*, 15q15.1; *MOCS2*, 5q11.2; *PRP8*, 17p13.3) to avoid systematic errors (gain or loss). An amplification or homozygous deletion that occurred in one of the markers would lead to an incoherent result that would be easily detectable (i.e., am-

plification of the 550-bp fragment without amplification of the 197-bp fragment). All reagents, probes, and primers were combined in a multiplex reaction with the sample DNA before droplet formation (Fig. 1A). Limiting the amount of input DNA, however, ensured that predominantly 1 or 0 target molecules were present within each droplet *(25)*. As we have previously demonstrated, the end point fluorescence intensity can be tuned by the concentration and nature of the TaqMan probe, which enabled the identification and counting of droplets containing each unique amplifiable target.

The amplification (Fig. 1B) of the *SPINT1* (78 bp) fragment produced a strong fluorescence signal on the VIC channel (VIC is a proprietary dye) because of the relatively high concentration of the VIC probe specific for the *SPINT1* gene, whereas amplification of the *MOCS2* (197 bp) sequences yielded a weaker signal on the VIC channel owing to the lower concentration of the VIC probe specific for the *MOCS2* gene. Similarly, amplification of the *INTS2* (159 bp) sequences yielded a strong fluorescence signal on the FAM (6-carboxyfluorescein) channel because of the higher concentration of the FAM probes specific for the *INTS2* gene, whereas amplification of the *PRP8* (550 bp) sequences produced a weaker signal on the FAM channel owing to the lower concentration of the FAM probes specific for the *PRP8* gene.

When fragmented genomic DNA was analyzed with this multiplex PCR assay, fluorescence signals were due to one of 5 possibilities. In addition to the cluster corresponding to empty droplets (no amplifiable molecule for any of the 4 targeted sequences within the droplet), there were 4 additional clusters that corresponded to the 4 targets appearing in a 2-dimensional histogram (Fig. 1, C and D). Counting the number of occurrences in each positive cluster revealed that 0.60% of all droplets contained a target fragment (Fig. 1D). This panel shows that the 4 clusters are well defined and separated for the sample composed of a mean of 3-kb fragments. Moreover, a triplicate of this experiment revealed that the mean (SD) percentages of droplets observed in each cluster (relative to the total number of droplets) were: 0.58% (0.01%) for the 78-bp *SPINT1* cluster, 0.50% (0.03%) for the 159-bp *INTS2* cluster, 0.57% (0.01%) for the 197-bp *MOCS2* cluster, and 0.53% (0.04%) for the 550-bp *PRP8* cluster. These values corresponded to the expected representation of each cluster (0.60%) for a sample in which most of the DNA was amplifiable and not highly fragmented or chemically damaged. In these experiments, the use of a limiting dilution of sample DNA ensured single-copy amplification according to defined Poisson criteria, as has previously been described *(24)*. On the assumption that the mass of a haploid genome is equivalent to 3.3 pg, an emulsion of 5-pL droplets prepared from a 25-μL sample containing 100 ng of DNA would have a mean DNA "loading" or "occupancy" of 0.006 genome equivalents per droplet (see Supplemental Methods in the online Data Supplement). The equal representation of each cluster demonstrated that each fragment was detected with equal efficiency, thereby indicating the performance of this assay.

EVALUATION OF THE DNA-QUALITY ASSAY

We fragmented DNA control samples by focused ultrasonication to simulate the wide range of fragmentation sizes that could be anticipated for clinical DNA samples and be tested with our assay. We progressively sheared 3-kb genomic DNA to prepare 5 highly fragmented control samples. Fig. 2A shows the distribution of fragment lengths determined by sample analysis with capillary gel electrophoresis. The mean lengths of the 5 samples were: 170 bp, 230 bp, 290 bp, 370 bp, and 570 bp.

Fig. 2, B–F, shows 2-dimensional histograms displaying the results of the DNA-quality assay applied to the control samples. As expected, analysis of the 170-bp sample revealed a highly degraded sample with only the *SPINT1* (78 bp) and *INTS2* (159 bp) clusters apparent on a histogram. Conversely, when we analyzed a relatively intact DNA target (570 bp), all 4 clusters appeared.

Fig. 2G summarizes the length distribution for the amplifiable fragments measured for each of the control samples. Individual data points represent dPCR measurements, and dashed lines indicate the distribution of fragment lengths calculated from the capillary electrophoresis data presented in Fig. 2A. The number of PCR-positive droplets detected for each DNA length reveals the level of amplifiable DNA available across a range of sizes. Fig. 2G shows that the quantitative dPCR measurement of the fraction of amplifiable fragments matched the prediction, which was based only on the size distribution presented in Fig. 2A. The size distributions of amplifiable targets (dashed lines) in Fig. 2G were determined by assuming that fragmentation points are randomly distributed throughout the DNA. The dPCR measurements at each length were consistent with the fractions expected from only the distribution of fragment lengths, confirming that physical shearing of DNA only fragments the DNA and that no chemical degradation occurs. The anticipated benefit of the dPCR DNA-quality assay is that it would reveal the length distribution of amplifiable DNA fragments for samples in which both chemical degradation and physical degradation are factors (e.g., DNA from FFPE samples).

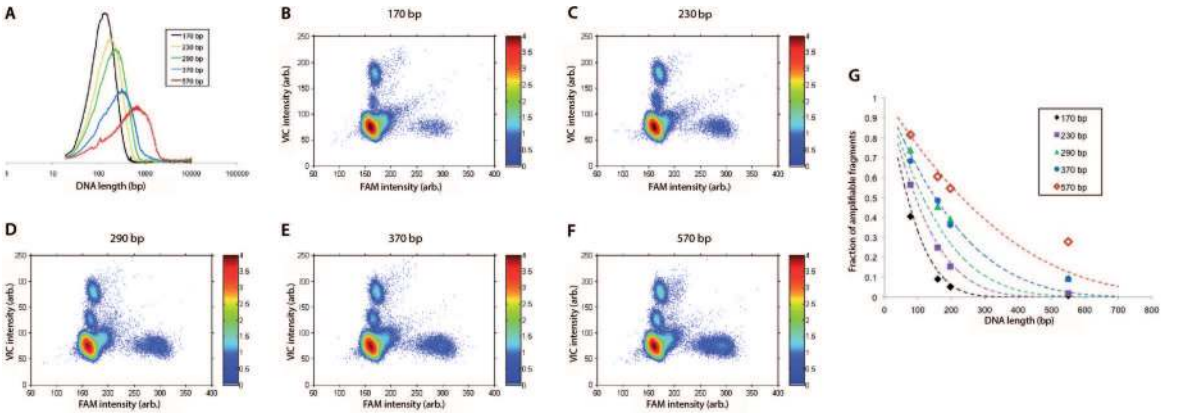

Fig. 2. dPCR-quality assay applied to human genomic DNA samples of various length.

(A), Distribution of fragment lengths in the samples as determined by gel electrophoresis. (B–F), Analysis of human genomic DNA fragmented to mean sizes from 170 bp to 570 bp. (G), Distributions of amplifiable DNA as measured by dPCR (data points) and standard capillary electrophoresis (dashed lines).

In addition to the criterion of being quantitative over a wide range of integrity levels, a DNA-quality test must be accurate when very small amounts of starting DNA are used, and the measured quality must be independent of input quantity. To demonstrate the feasibility of the assay with very small amounts of sample DNA (as expected in FFPE samples and/or with precious samples), we prepared 4 different amounts (1 ng, 5 ng, 10 ng, 50 ng, and 100 ng) of a control sample fragmented to 370 bp and assessed the preparations with the DNA-quality assay (Fig. 3). The number of droplets present in each of the 4 different clusters was counted and expressed as the fraction of the expected number of droplets for a nondegraded sample (as determined from the amount of control DNA, as described above). Fig. 3 provides evidence that the measurement of DNA integrity did not depend on the quantity of DNA processed and that the assay yielded a measurement of the length distribution of DNA fragments from an input of 1 ng amplifiable DNA.

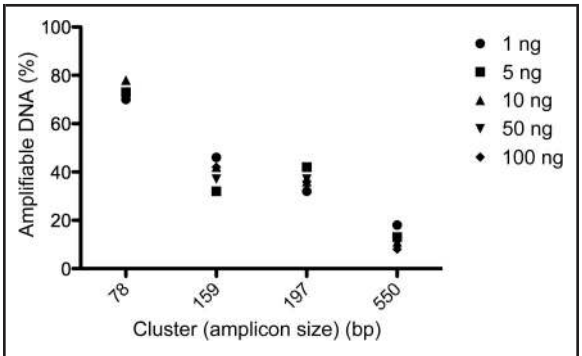

Fig. 3. dPCR-quality assay as applied to different quantities of human genomic DNA.

Human genomic DNA fragmented to a mean size of 400 bp and different quantities of the DNA test sample as analyzed with the multiplex dPCR-quality assay.

EVALUATION OF DNA INTEGRITY FOR PATIENT SAMPLES

We assessed FFPE samples from 12 lung adenocarcinoma tissues with the droplet dPCR assay. The data for all FFPE samples exhibited an increased level of fluorescence not associated with specific cluster locations (i.e., "noise") in the 2-dimensional histograms, compared with the control samples (Fig. 4). The "noise" in the histograms of patient samples interfered with quantification of the 550-bp assay component. In all cases, however, we were able to estimate that the number of true positives associated with the 550-bp amplicon was very low, because the amplicon amount at the next-largest size (197 bp) was very small.

The FFPE samples have 0.05% to 10.1% (mean values from 2 experiments, Fig. 5) of the expected amount of amplifiable DNA for the 78-bp target. The expected amount of amplifiable DNA was given by the theoretical occupancy in each cluster calculated from the quantity of input DNA (Table 1). Of the 12 samples, 2 had no or very little DNA detectable with the 159-bp and 197-bp assays. These results indicated that these samples (i.e., 8 and 12) were highly fragmented and/or chemically damaged. For these 2 samples, the fraction of droplets in the 78-bp cluster was also the lowest, with 0.05% and 0.95% of the expected number of droplets (calculated by assuming a nondegraded

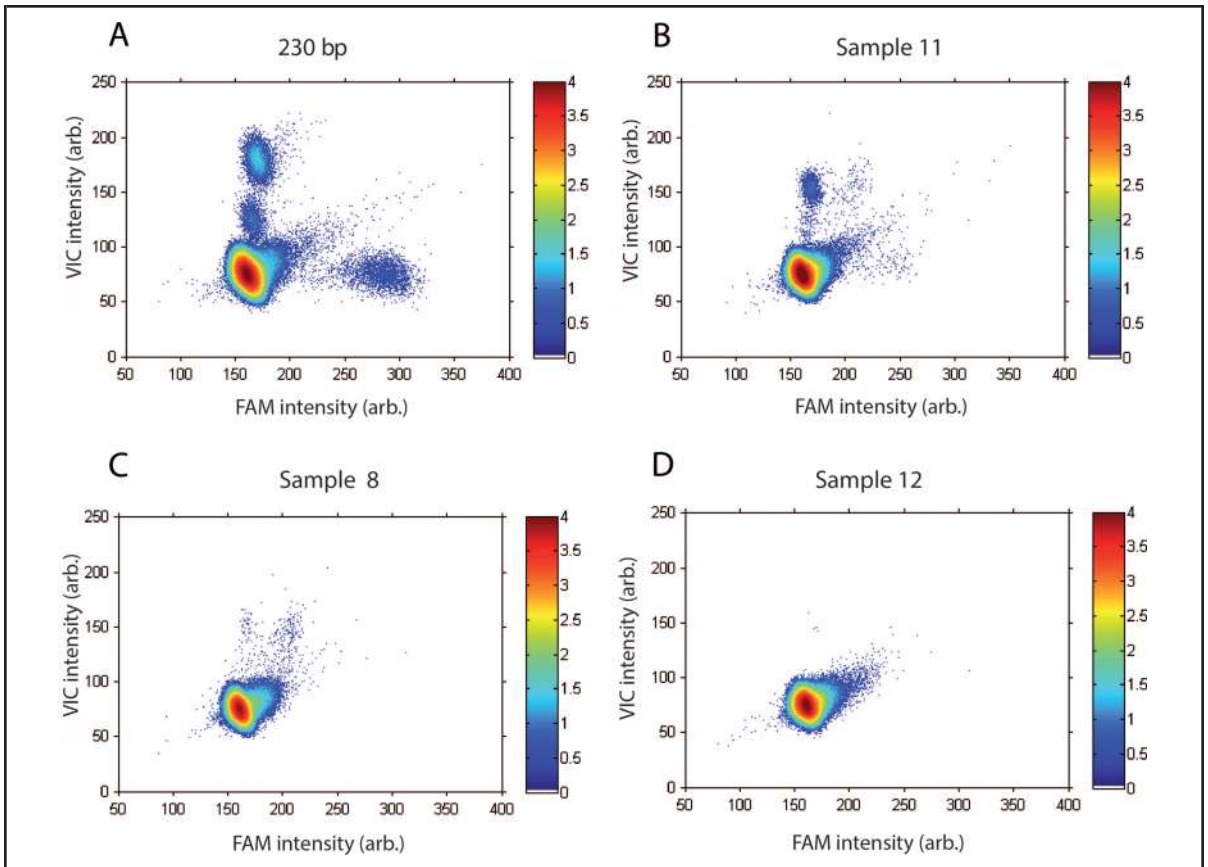

Fig. 4. DNA-quality assay comparison of fragmented DNA and FFPE samples.
(A), Control sample corresponding to genomic DNA fragmented to 230 bp. A sample of relatively high quality (B), a medium-quality sample (C), and a severely degraded sample of low quality (D). arb., arbitrary.

sample). Moreover, in addition to these samples, 6 others presented no droplets in the 550-bp cluster for at least one of the replicate experiments (samples 2, 4, 5, 6, 7, and 10). For the remaining samples, 0.2%–0.45% of the expected number of droplets were detected in the 550-bp cluster (samples 1, 3, 9, and 11). The samples in this study were ranked as poor, medium, or high quality on the basis of the amount of positive droplets in the 78-, 159-, and 550-bp clusters. Poor-quality samples were characterized by an absence of amplifiable fragments longer than 78 bp, medium-quality samples had amplifiable fragments in the 78-bp and 159-bp clusters, and high-quality samples were determined by the presence of amplifiable DNA of 550 bp in both replicate experiments. The high-quality samples were samples 1, 3, 9, and 11. The medium-quality samples were samples 2, 4, 5, 6, 7, and 10. The 2 remaining samples (8 and 12) were of poor quality. The designation of sample quality was used to organize and downselect samples for sequencing analysis. At this stage, the thresholds were not intended to be universal metrics for samples other than those examined in this study.

SEQUENCING OF FFPE SAMPLES

Four samples (samples 6, 8, 9, and 11) analyzed with the DNA-quality assay were further analyzed by targeted resequencing. These samples were selected according to the quantity of amplifiable 78-bp DNA, as well as the presence of amplifiable 550-bp DNA. One sample with low-quality DNA relative to almost all of the other samples (sample 8), 1 sample of medium quality (sample 6), and 2 samples of relatively high quality (samples 9 and 11) were selected for sequence enrichment and next-generation sequencing (Table 1, boldface text).

The samples chosen were analyzed by NanoDrop UV spectrophotometry and agarose gel electrophoresis to estimate the total DNA quantity and its fragmentation (see Fig. 1 and Table 2 in the online Data Supplement). These classic analytical procedures indicated a

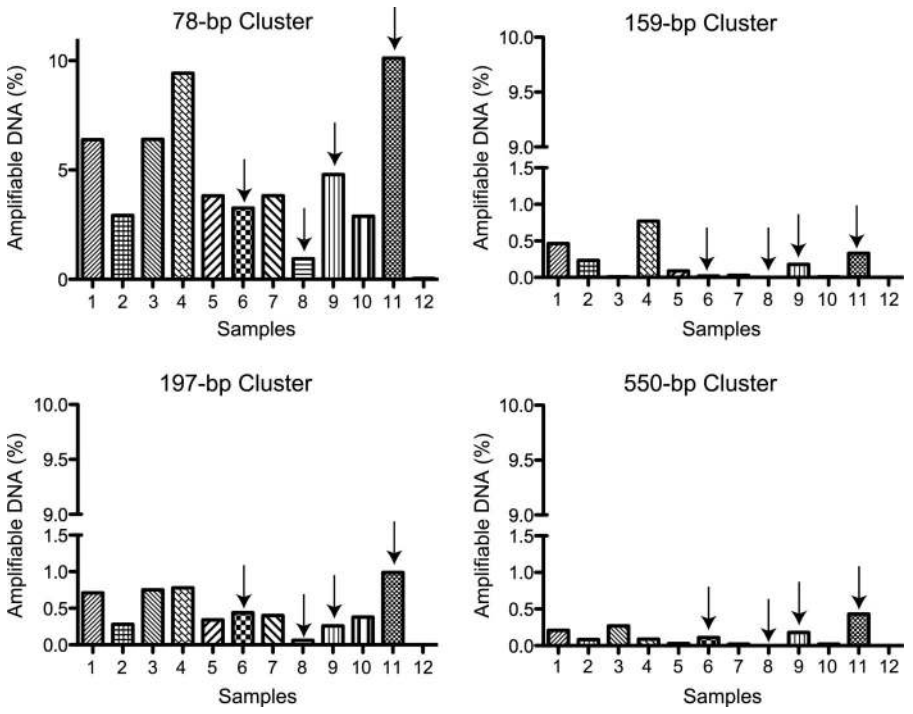

Fig. 5. Analysis of DNA extracted from 12 FFPE lung adenocarcinoma samples with the 4-plex DNA-quality assay. Results are expressed as the fraction of amplifiable DNA (calculated from the expected occupancy in droplets for a 3-kb fragmented sample for the 78-, 159-, 197- and 550-bp clusters). Black arrows indicate samples selected for sequencing.

quantity and quality of DNA sufficient to proceed to sequencing.

We assessed the quality of the sequencing data by 4 metrics (see data in Tables 3, 4, and 5 in the online Data Supplement). The first metric is the base coverage of "C20," indicating the percentage of bases in the targeted regions with ≥20 reads. The second is the uniformity, which is the percentage of bases in the targeted regions with >20% sequencing reads, compared with the mean number of reads for all targeted bases. The last metrics are related to the number of detected false-positive signals for single-nucleotide polymorphisms (SNPs), which was determined by counting the detected sequence variants that are unknown in the dbSNP132 or Cosmic v.55 databases (see Supplemental Methods in the online Data Supplement). The total number of false-positive SNPs and the frequency at which each SNP was detected were used to calculate a cumulative false-positive SNP score. Although this parameter is expressed in arbitrary units, it is a single value that can be compared across samples to report the quality of the sequencing data to ensure accurate SNP calling.

Among the 4 sequenced samples (see Fig. 5), sample 11 yielded the highest-quality data with the highest level of coverage and uniformity, along with the lowest frequency of unknown variants. Sample 9 yielded moderate-quality sequence data according to all metrics, and sample 6 yielded relatively poor-quality sequencing data.. Finally, the sequencing for sample 8 failed because of unsuccessful preparation of the sequencing library. This failure is consistent with insufficient amplifiable DNA, which is indicated by the results of the quality assay, although other reasons for failed preparation of a sequencing library are possible. In all cases, the quality of the sequencing data correlated directly with DNA quality as measured by dPCR. Furthermore, use of the DNA-quality assay before sequencing could provide an opportunity to increase the amount of input DNA if necessary without wasting precious samples. For example, the DNA-quality assay revealed that samples 6, 8, and 9 contained 3.25%, 0.95%, and 4.8%, respectively, of the amplifiable copies of DNA with a length of 78 bp (mean of 2 values obtained from 2 separate experiments; Table 1). What may be of more importance to genomics researchers, however, is the high number of low-prevalence mutations found in sequencing reads of low-quality samples. Information regarding sample quality may be an important factor when it is used to exclude samples

Table 1. DNA-quality assay results for the analysis of 12 patient DNA samples extracted from FFPE lung adenocarcinoma tissues[a]

Sample no.	Fragment-size cluster							
	78-bp Cluster, %		159-bp Cluster, %		197-bp Cluster, %		550-bp Cluster, %	
1	5.8	7.0	0.6	0.4	0.7	0.7	0.2	0.3
2	2.9	2.9	0.3	0.2	0.3	0.3	ND	0.2
3	6.3	6.5	ND	ND	0.7	0.8	0.5	0.1
4	9.2	9.6	0.7	0.9	0.8	0.8	ND	0.2
5	3.9	3.8	0.1	0.1	0.4	0.3	ND	0.1
6	**3.5**	**3.0**	**ND**	**ND**	**0.3**	**0.6**	**0.2**	**ND**
7	4.0	3.7	0.1	ND	0.4	0.4	ND	ND
8	**0.9**	**1.0**	**ND**	**ND**	**ND**	**0.1**	**ND**	**ND**
9	**4.8**	**4.8**	**0.2**	**0.1**	**0.3**	**0.2**	**0.2**	**0.2**
10	3.0	2.8	ND	ND	0.5	0.3	ND	ND
11	**9.0**	**11.2**	**0.6**	**0.1**	**1.0**	**1.0**	**0.5**	**0.4**
12	0.1	ND	ND	ND	ND	ND	ND	ND

[a] The results of 2 separate experiments are shown for each sample. The results are expressed as the percentage of expected amplifiable DNA calculated for a nondegraded sample (see main text). Rows in boldface correspond to the samples selected for sequencing analysis. ND, no droplets for the cluster.

from further analysis, if it confirms that chemical damage, impurities, or other factors that cannot be overcome by increasing the starting amount of DNA will produce misleading and ambiguous sequencing results.

For the 3 samples that were successfully sequenced, the *KRAS* and *EGFR* mutations previously characterized by allelic discrimination were correctly identified by sequencing [see Supplemental Methods (for the analysis and complementary data) and Table 6 in the online Data Supplement].

In summary, we have presented a dPCR procedure that uses droplet microfluidics technology for evaluating DNA quality in biological samples. We used a multiplex assay to measure the amount of amplifiable DNA with 4 target lengths (78, 159, 197, and 550 bp) that are relevant for genotyping analysis. We validated the procedure with artificially fragmented human genomic DNA to mimic different levels of sample degradation. Moreover, we used this procedure to analyze FFPE samples of lung adenocarcinoma tumor tissues and correlated the sequencing results for a subset of samples for DNA-quality assessment. The assay procedure is simple, and no calibration is required. It also requires a small amount of input DNA but is robust within a range of input DNA concentrations. This is particularly important for FFPE samples that contain a wide range of amplifiable material. Future development of the DNA-quality assay should focus on the selection and optimization of fragment targets for universal use or for study-specific purposes. In addition, determination of universal thresholds and validation of the dPCR procedure for predicting the quality of sequencing data should be executed with substantially larger sample populations. Combined with screening for tumor markers in patient plasma samples *(30)*, an assay for DNA integrity might also be valuable as a cancer biomarker *(31)* for diagnostics and patient follow-up.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: S.K. Kotsopoulos, RainDance Technologies; X. Li, RainDance Technologies; I. Atochin, RainDance Technologies; P. Srinivasan, RainDance Technologies; Q. Zhong, RainDance Technologies; J. Olson, RainDance Technologies; D.R. Link, RainDance Technologies; J.B. Hutchison, RainDance Technologies.
Consultant or Advisory Role: P. Laurent-Puig, RainDance Technologies; V. Taly, RainDance Technologies.
Stock Ownership: S.K. Kotsopoulos, RainDance Technologies; P. Srinivasan, RainDance Technologies; J. Olson, RainDance Technologies; D.R. Link, RainDance Technologies.
Honoraria: P. Laurent-Puig, RainDance Technologies; V. Taly, RainDance Technologies.
Research Funding: Region Alsace, Ministère de l'Enseignement Supérieur et de la Recherche, Université Paris-Descartes, Université de Strasbourg, Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM), Institut National du Cancer (INCA) (no. 2009–1-RT-03-US-1), Association pour la recherche contre le cancer (ARC, no. SL220100601375), Agence Nationale de la Recherche (ANR Nanobiotechnologie), and the Biointelligence program; D. Pekin, fellowship from the Region Alsace.
Expert Testimony: None declared.
Patents: D.R. Link, United States patent US 13,460,762; J.B. Hutchison, United States patent US 13,460,762.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Michalik S. Overcoming poor quality DNA. Drug Discov Dev 2008;11:24–30.
2. Lewis F, Maughan NJ, Smith V, Hillan K, Quirke P. Unlocking the archive—gene expression in paraffin-embedded tissue. J Pathol 2001;195:66–71.
3. Yost SE, Smith EN, Schwab RB, Bao L, Jung H, Wang X, et al. Identification of high-confidence somatic mutations in whole genome sequence of formalin-fixed breast cancer specimens. Nucleic Acids Res 2012;40:e107.
4. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002;161:1961–71.
5. Ben-Ezra J, Johnson DA, Rossi J, Cook N, Wu A. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. J Histochem Cytochem 1991;39:351–4.
6. Williams C, Ponten F, Moberg C, Soderkvist P, Uhlen M, Ponten J, et al. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155:1467–71.
7. White RA 3rd, Blainey PC, Fan HC, Quake SR. Digital PCR provides sensitive and absolute calibration for high throughput sequencing. BMC Genomics 2009;10:116.
8. van Beers EH, Joosse SA, Ligtenberg MJ, Fles R, Hogervorst FB, Verhoef S, et al. A multiplex PCR predictor for aCGH success of FFPE samples. Br J Cancer 2006;94:333–7.
9. Wang F, Wang L, Briggs C, Sicinska E, Gaston SM, Mamon H, et al. DNA degradation test predicts success in whole-genome amplification from diverse clinical samples. J Mol Diagn 2007;9:441–51.
10. Walker JA, Kilroy GE, Xing J, Shewale J, Sinha SK, Batzer MA. Human DNA quantitation using Alu element-based polymerase chain reaction. Anal Biochem 2003;315:122–8.
11. Swango KL, Timken MD, Chong MD, Buoncristiani MR. A quantitative PCR assay for the assessment of DNA degradation in forensic samples. Forensic Sci Int 2006;158:14–26.
12. Siwoski A, Ishkanian A, Garnis C, Zhang L, Rosin M, Lam WL. An efficient method for the assessment of DNA quality of archival microdissected specimens. Mod Pathol 2002;15:889–92.
13. Umetani N, Kim J, Hiramatsu S, Reber HA, Hines OJ, Bilchik AJ, et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. Clin Chem 2006;52:1062–9.
14. Rutledge RG, Cote C. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res 2003;31:e93.
15. Karlen Y, McNair A, Perseguers S, Mazza C, Mermod N. Statistical significance of quantitative PCR. BMC Bioinformatics 2007;8:131.
16. White RA 3rd, Quake SR, Curr K. Digital PCR provides absolute quantitation of viral load for an occult RNA virus. J Virol Methods 2012;179:45–50.
17. Taly V, Pekin D, Abed AE, Laurent-Puig P. Detecting biomarkers with microdroplet technology. Trends Mol Med 2012;18:405–16.
18. Pohl G, Shih IeM. Principle and applications of digital PCR. Expert Rev Mol Diagn 2004;4:41–7.
19. VanInsberghe M, Heyries KA, Hansen CL. Should genetic testing go digital? Expert Rev Mol Diagn 2012;12:111–4.
20. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.
21. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc Natl Acad Sci U S A 2003;100:8817–22.
22. Ottesen EA, Hong JW, Quake SR, Leadbetter JR. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science 2006;314:1464–7.
23. Shen F, Du W, Kreutz JE, Fok A, Ismagilov RF. Digital PCR on a SlipChip. Lab Chip 2010;10:2666–72.
24. Pekin D, Skhiri Y, Baret JC, Le Corre D, Mazutis L, Ben Salem C, et al. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. Lab Chip 2011:2156–66.
25. Zhong Q, Bhattacharya S, Kotsopoulos S, Olson J, Taly V, Griffiths AD, et al. Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR. Lab Chip 2011;11:2167–74.
26. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 2011;83:8604–10.
27. Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet 2010;11:31–46.
28. Didelot A, Le Corre D, Luscan A, Cazes A, Pallier K, Emile JF, et al. Competitive allele specific TaqMan PCR for KRAS, BRAF and EGFR mutation detection in clinical formalin fixed paraffin embedded samples. Exp Mol Pathol 2012;92:275–80.
29. Lievre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol 2008;26:374–9.
30. Lecomte T, Ceze N, Dorval E, Laurent-Puig P. Circulating free tumor DNA and colorectal cancer. Gastroenterol Clin Biol 2010;34:662–81.
31. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.