Digital Droplet PCR for Monitoring Tissue-Specific Cell Death Using DNA Methylation Patterns of Circulating Cell-Free DNA

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Cell death involves the release of short DNA fragments into blood, termed circulating cell-free DNA (cfDNA). Sequencing of cfDNA in the plasma has recently emerged as a liquid biopsy for detecting fetal chromosomal aberrations, tumor DNA, and graft rejection. However, in cases where cfDNA is derived from tissues with a normal genome, its primary sequence is not informative regarding the tissue of origin. We developed a method of determining the tissue origins of cfDNA, allowing inference of tissue-specific cell death, based on tissue-specific methylation patterns. We have previously described a version of the method that uses next generation sequencing (NGS) to determine methylation patterns in specific marker loci. Here we describe a rapid and simple procedure for cfDNA methylation analysis using droplet digital PCR (ddPCR) on bisulfite treated cfDNA to accurately count the number of molecules carrying a specific methylation signature. Specificity and sensitivity of the assay increases by simultaneously interrogating four to six cytosines in the same molecule using two fluorescent probes. cfDNA methylation analysis using ddPCR can find multiple applications in the non-invasive study of human tissue dynamics in health and disease. © 2019 by John Wiley & Sons, Inc.

Keywords: bisulfite • blood test • cell-free DNA • cfDNA • ddPCR • DNA methylation

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BASIC PROTOCOL

We describe a procedure for cell-free DNA (cfDNA) methylation analysis using droplet digital PCR (ddPCR) on bisulfite treated cfDNA. The key steps of the procedure are as follows: Plasma is separated from blood, and cfDNA is extracted and treated with bisulfite to convert unmethylated but not methylated cytosines to uracils. Primers and probes are selected to amplify a genomic region (<150 bp) that has a tissue-specific methylation pattern. Two probes of different colors are used, each targeting adjacent, different methylation sites in the same molecule. Digital droplet PCR is performed and the number of molecules carrying the specific methylation pattern is estimated by counting the number of dual-color droplets. Digital droplet PCR-based cfDNA methylation analysis can be used for the study of human tissue dynamics in health and disease.

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STRATEGIC PLANNING

Biomarkers

Sequences of primers and probes to detect cfDNA derived from cardiomyocytes and hepatocytes are provided in the Illustrated Setup section below. Tissue-specific methylation markers for other cell types of interest can be selected by searching epigenetic data browsers and repositories, comparing the methylome of the tissue of interest to that of other tissues, and identifying loci with methylation patterns specific to the tissue of interest (that is, methylated in the tissue of interest and unmethylated elsewhere, or unmethylated in the tissue of interest and methylated elsewhere). Some useful databases include:

- IHEC Data Portal: The International Human Epigenome Consortium (IHEC) brings forth reference epigenomes relevant to health and disease.
- ROADMAP Epigenomics: The NIH Roadmap Epigenomics Mapping Consortium offers maps of DNA methylation.
- CEEHRC Platform: A reference epigenome project for human cells and not the typical stem cell lines.
- DeepBlue: Store and work with genomic and epigenomic data from a number of international consortiums.
- Epigenome Browser: For the UCSC genome browser fans.
- WashU Epigenome Browser: A web browser that offers tracks from ENCODE and Roadmap Epigenomics projects.
- Ensembl: Featuring ENCODE.
- The Epigenome Atlas: Human reference epigenomes.
- Gene Expression Omnibus (GEO): A public functional genomics data repository.
- The Cancer Genome Atlas Program (TCGA): Joint effort between the National Cancer Institute and the National Human Genome Research Institute.

Candidate marker loci are best defined as CpG sites with an average methylation value <0.4 in the tissue of interest, >0.9 in leukocytes (the main "contaminant" of cfDNA), and >0.8 in over 90% of tissues. Similar criteria can be used to identify sites that are methylated in the tissue of interest (>0.8) but unmethylated in all others (methylation <0.3, including in leukocytes).

TaqMan™ Probes

Having established a list of tissue-specific methylation biomarkers, we developed a ddPCR procedure to detect and quantify these markers. In this procedure, bisulfite treated cfDNA is interrogated using two methylation-sensitive TaqMan™ probes with different colors. The limited length of probes (up to 30 bp) dictates that they can cover only two or three informative CpG sites in the marker locus, predicting a relatively high level of noise (positive droplets) in DNA from non-specific tissue. In the sequencing-based assay we addressed this problem by documenting the methylation status of six adjacent cytosines in the same molecule (Fig. 1), which greatly increased specificity. To implement this concept in the ddPCR platform we designed two TaqMan probes, each recognizing lack of methylation in a different cluster of two to three CpGs, all within the same amplified ~100 bp fragment from the marker locus. Each probe was labeled with a different fluorophore, such that we could identify droplets in which both probes found a target (Fig. 1D,E, Fig. 2). Such droplets would be interpreted as containing a cfDNA fragment in which all four to six targeted cytosines are unmethylated. This results in a ddPCR assay with improved specificity, afforded by interrogating multiple cytosines on the same DNA molecule.

Materials

Blood samples
ddPCR™ Supermix for Probes (no dUTP; Bio-Rad Laboratories)

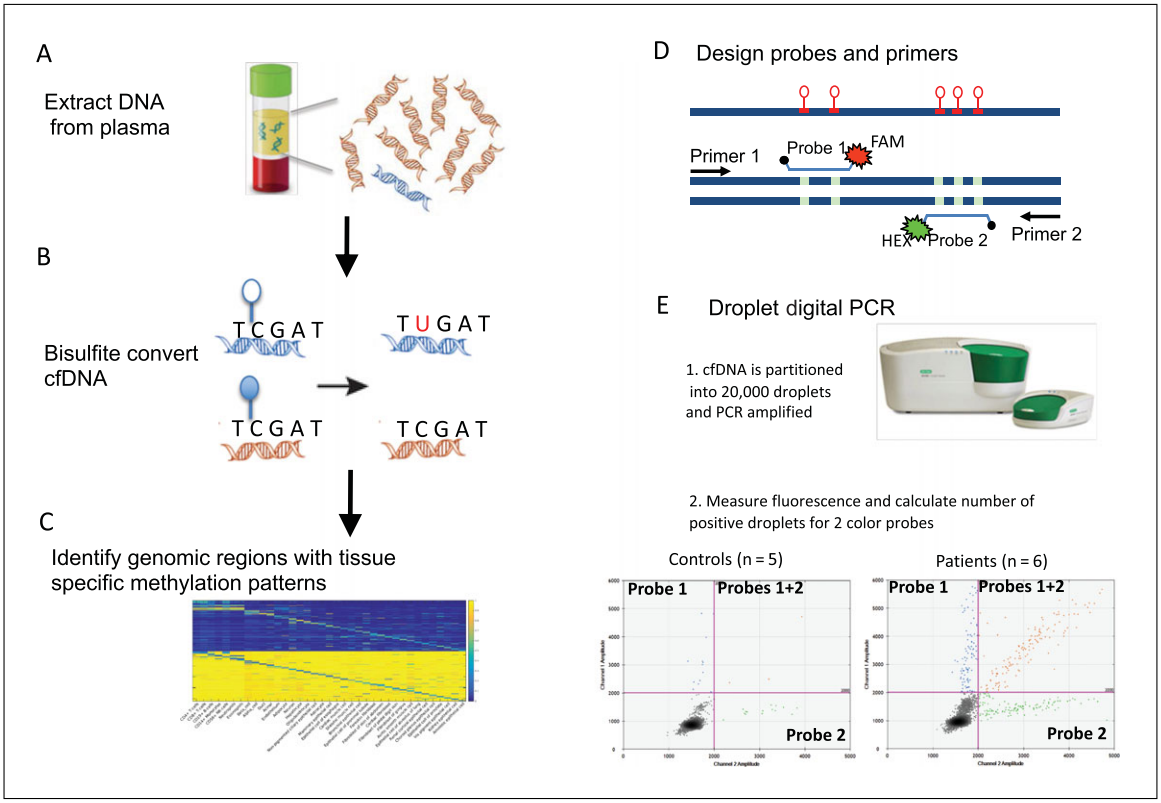

Figure 1 Flowchart of novel method for detecting cell death by ddPCR. (**A**) Collect plasma from relevant blood samples by centrifuging and then extracting cfDNA from plasma. (**B**) Bisulfite conversion of cfDNA. (**C**) Identify genomic regions with tissue-specific methylation patterns. Establish matrix of DNA methylation signatures of human tissues and cell types. DNA methylation was measured at >450,000 sites using the Illumina 450K platform. For each cell type, the 200 most specifically hypermethylated and 200 most specifically hypomethylated sites were selected by entropy. (**D**) Design probes and primers for a tissue-specific methylated region. (**E**) ddPCR. cfDNA is partitioned into 20,000 droplets and PCR. Measure fluorescence and calculate number of positive droplets for 2 color probes. ddPCR, droplet digital PCR; cfDNA, circulating cell-free DNA.

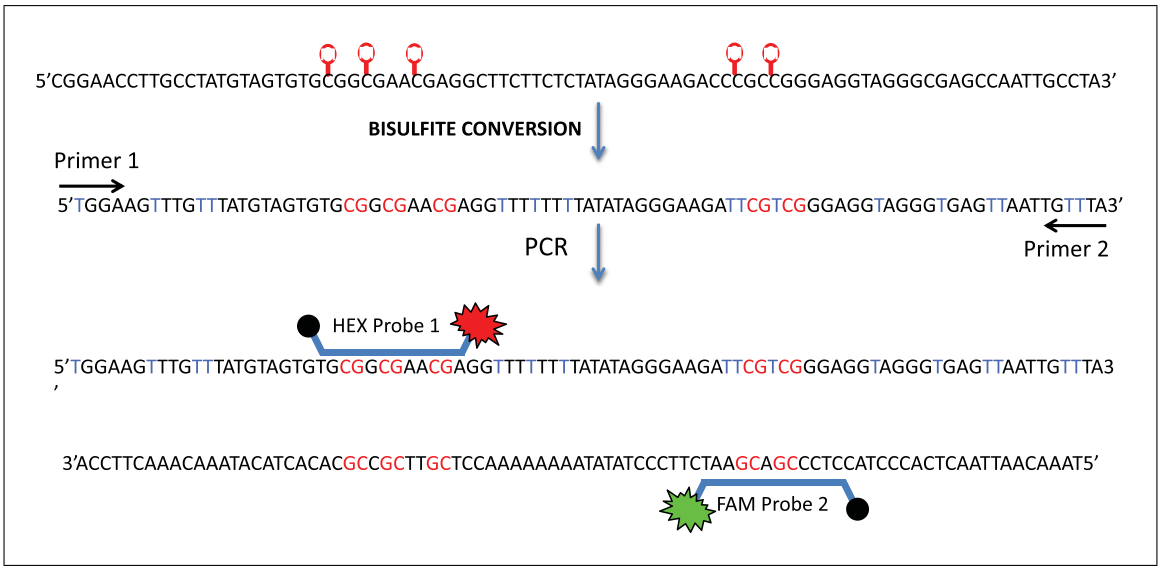

Figure 2 Schematic of approach for ddPCR-based detection of methylation status of multiple adjacent cytosines. A signal from two probes, labeled with FAM and HEX, in the same droplet reflects lack of methylation in five adjacent cytosines in the same original DNA strand. ddPCR, droplet digital PCR.

Droplet Generation Oil for Probes (Bio-Rad Laboratories)
EZ DNA Methylation-Gold™ (Zymo Research)

10-ml EDTA (purple cap) blood tubes
Streck® blood tubes
DG8 cartridges for QX100/QX200 Droplet Generator (Bio-Rad Laboratories)
QIAsymphony liquid handling robot (QIAGEN) or QIAamp Circulating Nucleic Acid Kit
QX200TM Droplet Generator (Bio-Rad Laboratories)
PX1 PCR Plate Sealer (Bio-Rad Laboratories)
T100 Thermal Cycler (Bio-Rad Laboratories)
QX200 Droplet Reader (Bio-Rad Laboratories)
QuantaSoft analysis software (Bio-Rad Laboratories)

NOTE: Sample preparation and DNA processing: It is important to isolate plasma from blood as quickly as possible to avoid contaminating DNA from leukocytes that were lysed during the procedure and wait time. Such contaminating genomic DNA will dilute cfDNA and reduce the chances of detecting rare signals from cfDNA.

1. Collect blood samples in 10-ml EDTA (purple cap) blood tubes or Streck® blood tubes and mix by gentle inversion. Do not shake or vortex tubes as cellular lysis may occur. Store tubes containing blood at room temperature.

   Centrifuge EDTA tubes (step 2) within 3 hr and Streck tubes within 5 days.

2. Centrifuge blood tubes 10 min at 1500 × *g*; centrifuge EDTA tubes at 4°C and Streck tubes at room temperature. Transfer supernatant to a fresh 15-ml conical tube without disturbing the cellular layer and centrifuge 10 min at 3000 × *g*. Collect supernatant, containing cell-free plasma, and store at −80°C (Fig. 1A).

3. Extract cell-free DNA from 1 to 4 ml of plasma using the QIAsymphony liquid handling robot. If plasma volume is <4 ml, add PBS to 4 ml.

   We typically obtain 4 ml plasma from 10 ml blood.

   As an alternative to QIAsymphony, use a manual cfDNA extraction kit such as QIAamp.

4. Treat cfDNA with bisulfite using EZ DNA Methylation-Gold™, according to manufacturer's instructions (Fig. 1B).

   Bisulfite treated cfDNA can be stored at −20°C.

Digital droplet PCR

5. Prepare a 20-µl volume reaction mix consisting of ddPCR™ Supermix for Probes (No dUTP), 900 nM primer, 250 nM probe, and 2 µl bisulfite treated cfDNA sample. Load mixture and droplet generation oil onto a droplet generator.

6. Transfer droplets to a 96-well PCR plate and seal. Run PCR on a thermal cycler as follows:

   a. 10 min activation at 95°C,
   b. 47 cycles of a two-step amplification protocol (30 sec at 94°C denaturation and 60 sec at 53.7°C),
   c. 10 min inactivation at 98°C.

   Note that annealing temperature has to be calibrated for each primer pair.

7. Transfer PCR plate to a QX100 Droplet Reader and analyze products with QuantaSoft analysis software. Discriminate between droplets that contain the target (positives)

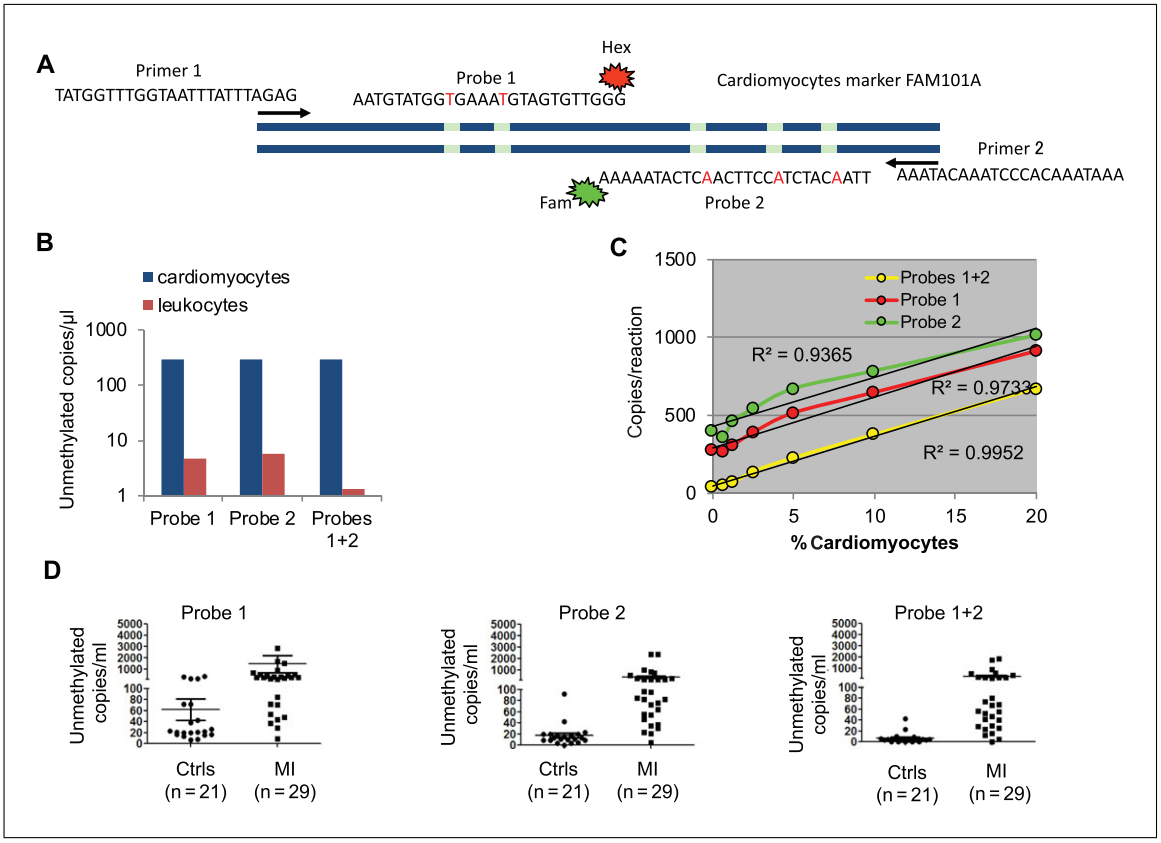

Figure 3 Detection of cardiac cfDNA using ddPCR. (**A**) Schematic of approach for ddPCR-based detection of methylation status of multiple adjacent cytosines in the *FAM101A* locus, identified as demethylated specifically in human cardiomyocytes. A signal from two probes in the same droplet reflects lack of methylation in five adjacent cytosines in the same original DNA strand. (**B**) Signal from cardiomyocyte and leukocyte DNA based on individual or dual probes. Scoring only dual probe signals reduces noise from leukocyte DNA. (**C**) Spike-in experiment assessing sensitivity and linearity of signal from cardiomyocyte DNA diluted in leukocyte DNA. The use of dual probe enhances linearity and reduces baseline signal; *x* axis shows the % of cardiac DNA diluted into blood DNA. (**D**) Measurement of cardiac cfDNA in plasma of healthy adult and patients with myocardial infarction (MI). The use of dual probes reduces the baseline signal in healthy plasma. Horizontal lines represent average and standard deviation of cfDNA values among the samples in each group. ddPCR, droplet digital PCR; cfDNA, circulating cell-free DNA (see Zemmour et al., 2018, *http://creativecommons.org/licenses/by/4.0/*).

and those that do not (negatives) by applying a fluorescence amplitude threshold based on the amplitude of reads from the negative template control.

Illustrated Setup: A digital droplet PCR procedure for measuring cardiac cfDNA

To define genomic loci that are methylated in a cardiac-specific manner, we compared the methylomes of human heart chambers (right atrium, left and right ventricle) to the methylomes of 23 other human tissues, all publicly available (Roadmap Epigenomics Consortium et al., 2015). A cluster of five cytosines adjacent to the *FAM101A* locus was selected for further analysis (Zemmour et al., 2018).

The following primers were used for the analysis of five cytosines located adjacent to the *FAM101A* locus: PCR primers 5′-TATGGTTTGGTAATTTATTTAGAG-3′ (forward) and 5′-AAATACAAATCCCACAAATAAA-3′ (reverse) in combination with probes that detected lack of methylation on three and two cytosines, respectively: 5′-AATGTATGGTGAAATGTAGTGTTGGG-3′ (FAM-forward probe) and 5′-AAAAATACTCAACTTCCATCTACAATT-3′ (HEX-reverse probe; Fig. 3A). ddPCR analysis of cardiomyocyte and leukocyte DNA revealed that each probe alone was able to discriminate between DNA from the two sources, with a signal-to-noise ratio of 50:1 to 58:1. However, when we scored only droplets positive for both probes, the cardiomyocyte/

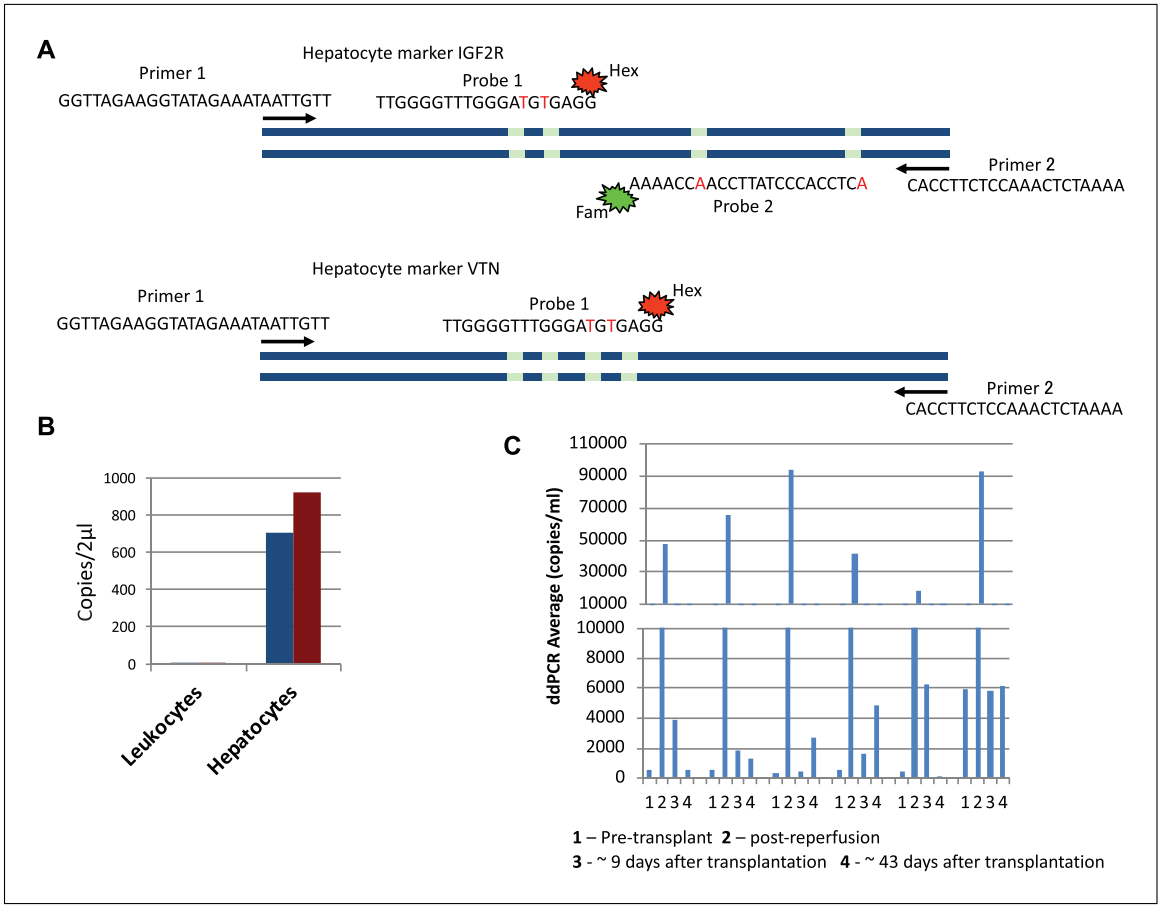

Figure 4 Digital droplet PCR for the identification of hepatocyte-derived cfDNA. (**A**) Schematic of approach for ddPCR-based detection of methylation status of two liver markers, IGF2R and VTN. In the case of IGF2R, two probes in the same droplet reflect lack of methylation in four adjacent cytosines in the same original DNA strand. In the case of the *VTN* locus, we used one probe for detecting four adjacent cytosines in the same original DNA strand. (**B**) Specificity: ddPCR signal for each marker in genomic DNA extracted from leukocytes and hepatocytes. (**C**) Hepatocyte-derived DNA in the plasma of six liver transplant recipients. Each patient was sampled at four time points as indicated. Graph shows the average values of the two liver markers in each sample. cfDNA, circulating cell-free DNA; ddPCR, droplet digital PCR.

leukocyte signal ratio increased to 258:1, affording a five-fold increase in specificity (Fig. 3B). ddPCR on cardiac DNA spiked into leukocyte DNA gave a signal that increased linearly with the amount of cardiac DNA; scoring only dual-labeled probes gave a lower baseline signal than scoring individual probes, better reflecting cardiomyocyte contribution to the mixture (Fig. 3C).

Finally, we tested the ddPCR strategy on plasma samples. ddPCR revealed a clear signal in the plasma of patients with myocardial infarction and was able to distinguish well between controls and patients. A lower baseline signal was observed in healthy individuals when scoring only dual-labeled probes, indicating increased specificity (Fig. 3D).

Illustrated Setup: A digital droplet PCR procedure for measuring hepatocyte cfDNA

We focused on the VTN and IGF2R markers, which emerged from our comparative methylome analysis as having a methylation pattern specific for hepatocytes and multiple CpGs in close proximity (Lehmann-Werman et al., 2018; Fig. 4A). We designed primers for ddPCR after bisulfite conversion of cfDNA and probes that recognize blocks of four unmethylated CpGs in the amplified marker regions of the *IGF2R* and *VTN* genes. To detect the methylation status of the *IGF2R* locus we used

two probes, each covering two or three CpG sites. To detect the methylation status of the *VTN* locus we used one probe that was able to cover all four CpG sites in this locus. The following primers were used for the analysis of four cytosines located in the *IGF2R* locus: PCR primers 5′-GGTTAGAAGGTATAGAAATAATTGTT-3′ (forward) and 5′-CACCTTCTCCAAACTCTAAAA-3′ (reverse) in combination with probes that detected lack of methylation on three and two cytosines, respectively: 5′-AAAACCAACCTTATCCCACCTCA-3′ (FAM-forward probe) and 5′-TTGGGGTTTGGGATGTGAGG-3′ (HEX-reverse probe; Fig. 3A).

The following primers were used for the analysis of four cytosines located in the *VTN* locus: PCR primers 5′-GGTTAGAAGGTATAGAAATAATTGTT-3′ (forward) and 5′-CACCTTCTCCAAACTCTAAAA-3′ (reverse) in combination with one probe that detected lack of methylation on four cytosines: 5′-TTGGGGTTTGGGATGTGAGG-3′ (HEX-reverse probe; Fig. 3A).

A ddPCR reaction using both amplicons showed no signal in leukocyte DNA and a strong signal in hepatocyte DNA (Fig. 4B). We then examined six sets of plasma samples from six patients before and after liver transplantation. Similar to results obtained by PCR and sequencing, the ddPCR assay revealed a strong and transient elevation of hepatocyte cfDNA in plasma shortly after transplantation, which declined thereafter, strongly suggesting the validity of the assay (Fig. 4C).

COMMENTARY

Background Information

Dying cells are known to release fragmented DNA into the blood, termed circulating cell-free DNA (cfDNA; Schwarzenbach, Hoon, & Pantel, 2011; Wan et al., 2017). cfDNA fragments (on average nucleosome size, 165 bp fragments) have a half-life of 15 to 120 min, and thus can potentially serve as an indicator of instantaneous rather than cumulative cell death (Schwarzenbach et al., 2011). Normal plasma contains ∼5 ng of cfDNA/ml representing ∼1000 genome equivalents/ml, thought to originate mostly from dying leukocytes (Sun et al., 2015). The concentration of cfDNA increases in multiple physiological and pathological scenarios such as exercise, trauma, sepsis, and cancer, and can be used as a diagnostic tool to monitor disease progression and response to treatment. cfDNA analysis is broadly developed and used for monitoring conditions in which it contains mutations, for example fetal cfDNA in maternal plasma, cancer-derived cfDNA containing somatic mutations, and cfDNA from transplanted organs containing distinct single nucleotide polymorphisms (SNPs).

The utility of the cfDNA as a prognostic and diagnostic tool can increase dramatically if one can determine its tissue origins, that is the identity of dying cells that release cfDNA. Several approaches have been described to address this problem. For example, Snyder, Kircher, Hill, Daza, and Shedure (2016) have suggested that tissue-specific nucleosome positions can be mapped in cfDNA to infer the tissue origins. Similarly, Ulz et al. (2016) have used the fact that nucleosome-free regions are present in promoters of expressed genes, and are under-represented in cfDNA, to infer gene expression in the tissues giving rise to cfDNA. We and others have proposed an alternative approach for determining the tissue origins of cfDNA, based on identification of tissue-specific methylation markers that are retained in cfDNA (Akirav et al., 2011; Guo et al., 2017; Lehmann-Werman et al., 2016; Sun et al., 2015).

Despite having an identical nucleotide sequence, the DNA of each cell type in the body carries unique DNA methylation patterns correlating with its gene expression profile (Bergman & Cedar, 2013; Dor & Cedar, 2018). DNA methylation, taking place on cytosines adjacent to guanines (CpG sites), is an essential component of cell-type-specific gene regulation, and hence is a fundamental mark of cell identity. By defining the methylation patterns typical to specific tissues, multiple groups have attempted to infer the relative contributions of different tissues to cfDNA, using deconvolution of cfDNA methylation profiles from low-depth whole genome bisulfite sequencing (WGBS; Sun et al., 2015), via reduced representation bisulfite sequencing (RRBS; Guo et al., 2017), or by directed PCR and sequencing (Gala-Lopez et al., 2018;

Lehmann-Werman et al., 2018; Lehmann-Werman et al., 2016; Zemmour et al., 2018). Here we describe a version of a methylation assay for determining the tissue origins of cfDNA using droplet digital PCR (ddPCR).

We have recently shown that cfDNA molecules from loci carrying tissue-specific methylation can be used to identify cell death in a specific tissue (Gala-Lopez et al., 2018; Lehmann-Werman et al., 2018; Lehmann-Werman et al., 2016; Zemmour et al., 2018). We demonstrated, using 10 ml of blood, elevations of exocrine pancreas cfDNA in patients with pancreatic cancer and pancreatitis, beta cell DNA in islet graft recipients, neuronal/glial DNA in the circulation of patients after traumatic brain injury or cardiac arrest, oligodendrocyte DNA in the circulation of patients with multiple sclerosis during relapse, cardiomyocyte DNA in patients with myocardial infraction, and hepatocyte cfDNA in healthy individuals that increases upon liver damage (Gala-Lopez et al., 2018; Lehmann-Werman et al., 2018; Lehmann-Werman et al., 2016; Zemmour et al., 2018).

These experiments were performed using massively parallel sequencing, requiring access to a next generation sequencer and the application of a bioinformatics pipeline to interpret sequencing results, which takes considerable time (>24 hr from drawing blood to results) mainly due to sequencing run time, hindering utility in emergency medicine. In addition, access to next generation sequencing (NGS) platforms might be limiting for many researchers. We therefore translated the assay to a simpler and faster ddPCR format. We established a procedure using ddPCR to accurately count the number of molecules derived from a specific locus that carries a given methylation signature. In ddPCR, the sample is diluted such that each individual partition contains no more than one target sequence. Therefore by partitioning individual target molecules within distinct compartments prior to PCR amplification, ddPCR can detect rare sequences with unprecedented precision and sensitivity. Importantly, ddPCR provides an absolute value for the number of DNA molecules derived from the tissue of interest.

ddPCR is already used routinely for detecting rare mutations in cfDNA. Applying it to the detection of methylation patterns, we had to take into consideration an important principle in DNA methylation, namely its regional nature: Adjacent cytosines are more likely to have a similar methylation pattern in a given cell type (Guo et al., 2017; Lehmann-Werman et al., 2016). In contrast, methylation noise—that is, rare occasions in which a specific cytosine has an altered methylation pattern (methylation or lack of methylation)—occurs typically in isolated nucleotides. Therefore, measuring the methylation status of several adjacent cytosines in the same molecule (a "haplotype block") provides greater confidence regarding the tissue source of a molecule, effectively increasing assay specificity (Gala-Lopez et al., 2018; Lehmann-Werman et al., 2018; Lehmann-Werman et al., 2016; Zemmour et al., 2018).

The probes used in standard ddPCR assays are capable of interrogating the methylation status of only one or two cytosines on a molecule. To overcome this limitation we designed the assay to simultaneously interrogate up to six cytosines in the locus using two adjacent probes, each with a different fluorophore, and with each capturing two to three distinct cytosines in their methylated or unmethylated form as needed. Consider a locus containing a stretch of six CpG sites, all of which are unmethylated in tissue X but not in all other tissues. A droplet containing an individual molecule from such a locus will be amplified in ddPCR and will fluoresce in two colors because both probes have captured their target. In contrast, droplets with molecules containing few CpG sites in their unmethylated form will have no fluorescence or will fluoresce in just one color. Thus, dual-color droplets reflect the methylation status of multiple cytosines in the same molecule, taking advantage of tissue specificity afforded by the information content of methylation haplotype blocks without the need for sequencing (Fig. 1; Lehmann-Werman et al., 2018; Zemmour et al., 2018).

Critical Parameters

In the separation of plasma from leukocytes and erythrocytes, it is critical to leave sufficient residual plasma in the tubes after centrifugation and to avoid disturbing the leukocyte layer when pipetting.

The second centrifugation is necessary to remove any residual cells carried over from the first centrifugation step. Cellular contamination can adversely affect downstream applications of cfDNA.

Troubleshooting

Table 1 provides a list of problems and suggested solutions.

Table 1 Troubleshooting Commonly Encountered Problems[a]

Problem	Possible cause	Solution
Abnormally high cfDNA levels	The time between blood draw and processing was too long, leukocytes have lysed and released genomic DNA that contaminates the sample	Separate plasma from blood as soon as possible after blood draw, preferably in <2 hr
Abnormally high cfDNA levels	Leukocytes were drowned with plasma during plasma separation	Transfer the supernatant without disturbing the cellular layer
Too few droplets in the ddPCR reaction	Breakdown of the droplets	Ensure careful and smooth pipetting when transferring droplets Pipet tips should be angled
Too few droplets in the ddPCR reaction	PCR was performed more than 1 hr after generating droplets	Process fewer samples per experiment, proceed more quickly to PCR
Extra cluster of positive droplets with fluorescence intensity that is higher than baseline but not reflecting true signal	Another DNA sequence that is not perfectly matched to the designed probe	Increase the threshold to exclude new cluster from target quantification Increase annealing temperature
High signal-to-noise ratio	TaqMan probe is not optimal	Design a probe that contains at least 3 CpG sites
No signal	Low amount of cfDNA	Increase amount of template in ddPCR reaction

[a]Abbreviations: ddPCR, droplet digital PCR; cfDNA, circulating cell-free DNA.

Understanding Results

QuantaSoft software measures the numbers of droplets that are positive and negative for both fluorophore (FAM and HEX) in a sample. The droplets are clustered into four groups: FAM-negative and HEX-negative; FAM-positive and HEX-negative; FAM-negative and HEX-positive; FAM-positive and HEX-positive. FAM-positive and HEX-positive are scored as positive. FAM-negative and HEX-negative are scored as negative. The fraction of positive droplets (FAM-positive and HEX-positive) is then fitted to a Poisson distribution to determine the starting concentration of the target DNA molecule in units of copies/μl input.

In other words, the level of positive droplets (FAM-positive and HEX-positive) determines the amount of cfDNA in the plasma sample originating from the dying cells of a specific tissue.

Time Considerations

In our hands, 32 plasma samples can be analyzed in 5 hr using the ddPCR procedure. We anticipate that further optimization of the assay will allow shortening the processing time even further, which might be important for time sensitive applications such as in an emergency medicine context.

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