Clinical Chemistry 54:10
1664–1672 (2008)

Microfluidics Digital PCR Reveals a Higher than Expected Fraction of Fetal DNA in Maternal Plasma

Fiona M. F. Lun,[1,2] Rossa W. K. Chiu,[1,2] K. C. Allen Chan,[1,2] Tak Yeung Leung,[3] Tze Kin Lau,[3] and Y. M. Dennis Lo[1,2*]

BACKGROUND: The precise measurement of cell-free fetal DNA in maternal plasma facilitates noninvasive prenatal diagnosis of fetal chromosomal aneuploidies and other applications. We tested the hypothesis that microfluidics digital PCR, in which individual fetal-DNA molecules are counted, could enhance the precision of measuring circulating fetal DNA.

METHODS: We first determined whether microfluidics digital PCR, real-time PCR, and mass spectrometry produced different estimates of male-DNA concentrations in artificial mixtures of male and female DNA. We then focused on comparing the imprecision of microfluidics digital PCR with that of a well-established nondigital PCR assay for measuring male fetal DNA in maternal plasma.

RESULTS: Of the tested platforms, microfluidics digital PCR demonstrated the least quantitative bias for measuring the fractional concentration of male DNA. This assay had a lower imprecision and higher clinical sensitivity compared with nondigital real-time PCR. With the *ZFY*/*ZFX* assay on the microfluidics digital PCR platform, the median fractional concentration of fetal DNA in maternal plasma was ≥2 times higher for all 3 trimesters of pregnancy than previously reported.

CONCLUSIONS: Microfluidics digital PCR represents an improvement over previous methods for quantifying fetal DNA in maternal plasma, enabling diagnostic and research applications requiring precise quantification. This approach may also impact other diagnostic applications of plasma nucleic acids, e.g., in oncology and transplantation.

Conventional prenatal diagnostic methods for harvesting fetal materials for molecular analysis, such as amniocentesis, are invasive and constitute a finite risk to the fetus. Much research has therefore been devoted to the development of new noninvasive methods for safe prenatal diagnosis. The discovery of circulating cell-free fetal DNA in maternal plasma in 1997 offered such new possibilities *(1)*. A number of clinical diagnostic tests with impacts on clinical practice that have since been developed *(2)* include the determination of fetal RhD blood group status *(3, 4)* and fetal sex determination for sex-linked disorders *(5)*. Furthermore, increased circulating fetal-DNA concentrations have been observed in certain pregnancy-associated disorders, including preeclampsia *(6, 7)* and preterm labor *(8)*.

Real-time PCR has become the most commonly used technology for the detection of fetal DNA in maternal plasma *(9)*, including both qualitative [e.g., for fetal RhD genotyping *(3, 4)*] and quantitative [e.g., in preeclampsia *(6)*] applications. Because most of the DNA molecules in maternal plasma are derived from the pregnant woman, with only a minor proportion coming from the fetus, real-time PCR–based assays are generally directed toward fetal targets that either have no maternal counterparts [e.g., Y chromosome sequences *(5)* or the *RHD*[4] gene (Rh blood group, D antigen) in a RhD-negative woman *(3, 4)*] or differ from the maternal counterparts at multiple DNA base pairs [e.g., the 4-nucleotide codon 41/42 deletion in

[1] Centre for Research into Circulating Fetal Nucleic Acids, Li Ka Shing Institute of Health Sciences, [2] Department of Chemical Pathology, and [3] Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong.
* Address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, 30–32 Ngan Shing St., New Territories, Hong Kong SAR, China. Fax +852 2636 5090; e-mail loym@cuhk.edu.hk.
Received May 27, 2008; accepted July 25, 2008.
Previously published online at DOI: 10.1373/clinchem.2008.111385
[4] Human genes: *RHD*, Rh blood group, D antigen; *SRY*, sex determining region Y; *ZFX*, zinc finger protein, X-linked; *ZFY*, zinc finger protein, Y-linked; *HBB*, hemoglobin, beta.

β-thalassemia *(10)*]. The detection of fetal-DNA targets that differ from the maternal background by single bases has required more complex methods, such as size fractionation to enrich for the relatively shorter fetal-DNA targets *(11)* and mass spectrometry to detect the single-base variations *(12)*. These methods have disadvantages that include a susceptibility to contamination with current size-fractionation strategies *(11)* and the need for extensive optimization for mass spectrometry analysis of certain genomic targets *(13)*.

For quantitative analyses, several investigators have reported higher imprecision with single-copy sequences, such as the *SRY* gene (sex determining region Y) on the Y chromosome, for fetal-DNA quantification in maternal plasma *(14)*. Furthermore, quantitative analysis with real-time PCR typically requires the use of calibrators that might vary between laboratories and between batches *(14)*. In addition, new diagnostic applications of plasma-DNA analysis, such as in detecting Down syndrome, require very precise quantification of circulating fetal DNA, which might be challenging with conventional technologies *(15)*.

In view of these limitations, an investigation of new approaches to plasma-DNA analysis would be of clinical and scientific interest. We explored the use of microfluidics digital PCR for the detection and measurement of cell-free fetal DNA in maternal plasma. Digital PCR is an analytical strategy in which a nucleic acid sample is diluted and subjected to multiple PCR analyses so that most of the reactions contain either a single or no target molecule *(16)*. The technology provides a "digital" readout because any of these multiple PCR analyses will be either positive or negative, corresponding to the presence or absence of the target molecule. Such presence/absence results are analogous to the "ones" and "zeros" in computer science. With appropriate statistical analyses, the proportion of positive and negative reactions would allow measurement of the number of target molecules in the input sample. Most published applications of this approach have been in the cancer-detection field *(17)*. Recently, digital PCR has been proposed as a possible strategy for the detection of fetal chromosomal aneuploidies in maternal plasma *(15)*.

The main limitation of digital PCR is the labor-intensiveness of performing hundreds to thousands of reactions for each sample, but recent advances in microfluidics technology have made possible the automation of digital PCR *(18, 19)*. Microfluidics permits nanoliter aliquots of a nucleic acid sample to be channeled into nanoliter-scale amplification chambers where hundreds or thousands of real-time digital PCRs could be carried out (Fig. 1A). In this study, we compared the performance of microfluidics digital PCR with existing methods for detecting fetal DNA in maternal plasma.

Materials and Methods

STUDY PARTICIPANTS

Women with singleton pregnancies were recruited at the Prince of Wales Hospital, Hong Kong, with informed consent and Institutional Review Board approval. Maternal peripheral blood samples were collected into EDTA-containing tubes during the first, second, and third trimesters before chorionic villus sampling, amniocentesis, and elective cesarean section, respectively. We recruited 10 pregnancies with male fetuses for each trimester and 5 first-trimester pregnancies with female fetuses. As a positive control in the DNA-mixing experiments, we collected placental tissue from a healthy male baby at term immediately after elective cesarean section. Samples were processed as described in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue10.

COMPARISON OF DIFFERENT ANALYTICAL PLATFORMS

We compared 3 platforms in this study: real-time quantitative PCR *(9)*, mass spectrometry *(12, 20)*, and microfluidics digital PCR *(18, 19)*. We prepared artificial DNA mixtures consisting of 0%, 5%, 10%, 25%, 50%, 75%, and 100% male placental DNA in female blood cell DNA at a final concentration of 1 ng/μL (1 mg/L). These mixtures were prepared from 100-ng/μL (100-mg/L) stock solutions of female blood cell DNA and male placental DNA. We then measured the quantitative deviation of observed concentrations of male DNA from the expected concentrations by means of the 3 analytical platforms. To compare the analytical imprecisions of the real-time PCR and digital PCR platforms, we prepared an artificial mixture containing 7% male placental DNA in a background of female blood cell DNA and diluted the mixture to 100 pg/μL (100 μg/L) to mimic extracted maternal plasma DNA from early pregnancy *(9)*.

MICROFLUIDICS DIGITAL PCR ANALYSIS

Two 87-bp amplicons of the *ZFX* (zinc finger protein, X-linked) and *ZFY* (zinc finger protein, Y-linked) loci were coamplified with the same primer set and distinguished with chromosome-specific TaqMan probes (Applied Biosystems; Table 1). We carried out all digital experiments on the BioMark System (Fluidigm) using the 12.765 Digital Arrays (Fluidigm). Each Digital Array consists of 12 panels, each of which is further partitioned into 765 reaction chambers (Fig. 1B). The reaction for one panel was set up with the 2× TaqMan Universal PCR Master Mix Kit (Applied Biosystems) in

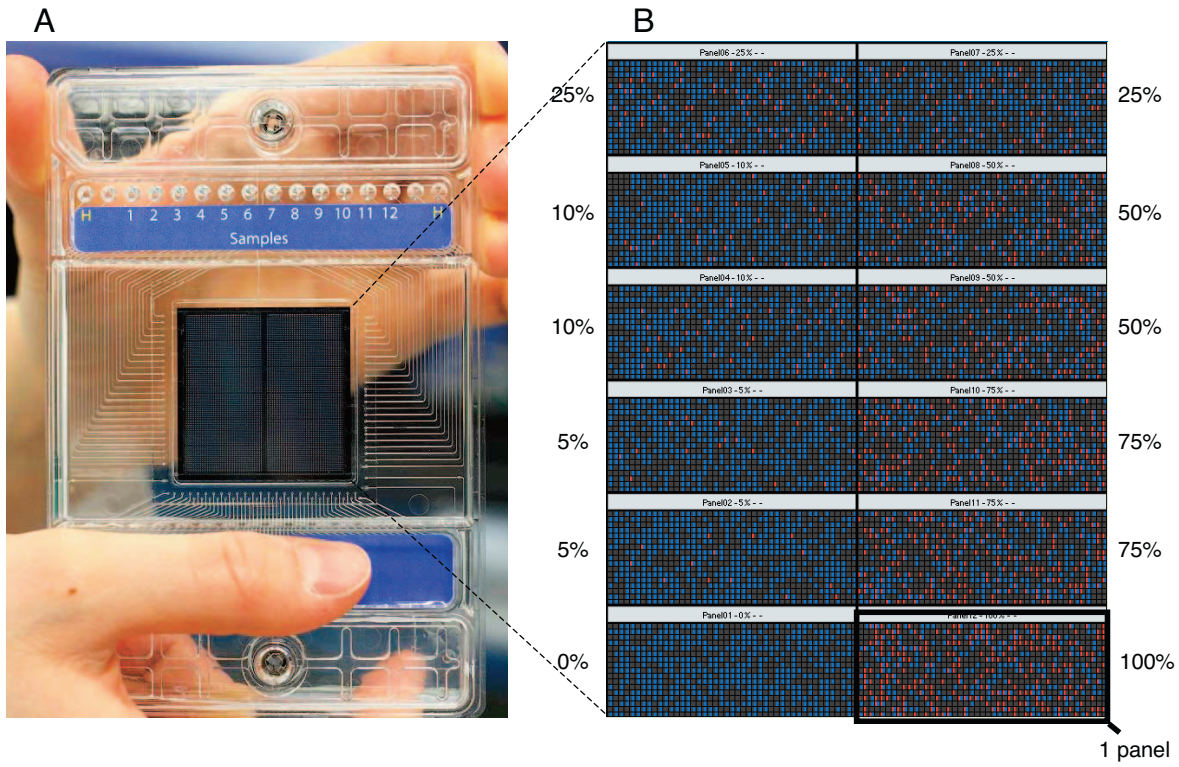

Fig. 1. Application of microfluidics chip for digital PCR analysis.

(A), Frontal view of a microfluidics digital array. The chip area in the center is divided into 12 panels, and each is connected to a sample inlet (on the top frame). (B), Digital readout of the accuracy experiment. Each panel is compartmentalized into 765 reaction wells. Red- and blue-colored dots represent reaction wells that are positive for *ZFY* and *ZFX* signals, respectively. Black-colored dots represent wells with no reaction. The percentage next to each panel denotes the fractional male-DNA concentration of the input mixture of artificial DNA in that particular panel.

Table 1. Oligonucleotide sequences for the *ZFX* and *ZFY* assays.[a]

Digital PCR and nondigital real-time PCR	
Forward primer	5′-CAAGTGCTGGACTCAGATGTAACTG-3′
Reverse primer	5′-TGAAGTAATGTCAGAAGCTAAAACATCA-3′
ZFX TaqMan probe	5′-(VIC)TCTTTAGCACATTGCA(MGBNFQ)-3′
ZFY TaqMan probe	5′-(FAM)TCTTTACCACACTGCAC(MGBNFQ)-3′
Mass spectrometry	
Forward primer	5′-**ACGTTGGATG**TCATTCCTGAGCAAGTGCTG-3′
Reverse primer	5′-**ACGTTGGATG**GCTAAAACATCATCTGGGAC-3′
Extension primer	5′-TCATCTGGGACTGTGCA-3′
ZFX extension product	5′-TCATCTGGGACTGTGCAA-3′
ZFY extension product	5′-TCATCTGGGACTGTGCAGT-3′

[a] VIC and FAM denote the 2 fluorescent reporters; MGBNFQ, minor groove–binding nonfluorescent quencher. Boldfaced nucleotides indicate the 10-mer tags incorporated into the 5′ ends to ensure that the primers would not interfere with the subsequent mass spectrometry analysis.

a reaction volume of 10 μL, inclusive of a dead volume of 5.4 μL. A 3.5-μL volume of input DNA was loaded onto each panel. We used 2 reaction panels to measure the male-DNA concentration in each artificial DNA mixture in the experiment to measure the quantitative biases across the different analytical platforms. We used 12 reaction panels on all of the plasma samples and the artificial DNA mixture containing 7% male DNA to assess the imprecision of the digital PCR assay. For digital analysis of plasma DNA to measure fractional fetal-DNA concentrations, 12 reaction panels would allow a total of 19.32 μL of plasma DNA to be analyzed, after the dead volume of the chip had been taken into account. This volume was comparable to the total volume of plasma DNA analyzed for the conventional real-time *SRY/HBB* assay (see below) in which two 5-μL plasma-DNA aliquots were used for duplicate analyses for each of *SRY* and *HBB* (hemoglobin, beta), for a total of 20 μL of plasma DNA per reportable fractional fetal-DNA concentration *(9)*. For digital analysis of plasma DNA to qualitatively determine the fetal sex, we scored only the first 6 reaction panels, amounting to 9.66 μL of plasma DNA. This volume of maternal plasma DNA is comparable to the 10-μL total volume of plasma DNA used for the duplicate *SRY* real-time PCR *(9)*. Details of the digital assay are described in the online Data Supplement.

We counted the number of wells that were positive for *ZFY* or *ZFX* amplification for each sample. According to the Poisson distribution, the original number of molecules derived from chromosomes X and Y can be calculated with the following equations:

$$ZFY = -\ln[(N - Y)/N] \times N;$$

$$ZFX = -\ln[(N - X)/N] \times N,$$

where *ZFY* is the number of *ZFY* molecules, *N* is the total number of wells counted, *Y* is the number of Y-positive wells, *ZFX* is the number of *ZFX* molecules, and *X* is the number of X-positive wells. The fraction of *ZFY* molecules of the total zinc finger protein DNA sequences (i.e., *ZFY* plus *ZFX*) can be calculated as: $ZFY/(ZFY + ZFX)$.

Because each male fetal cell contains a copy each of *ZFX* and *ZFY* and each background maternal cell contains 2 copies of *ZFX* and no *ZFY*, the proportion of fetal DNA in a maternal plasma sample [i.e., the percentage of the total genome equivalents (GEs) of DNA in the maternal plasma that was fetus derived] is calculated as: $(2 \times ZFY)/(ZFY + ZFX) \times 100$.

REAL-TIME QUANTITATIVE PCR

Apart from the real-time *SRY* and *HBB* assays, which have been widely used in previous studies *(9)*, we also designed real-time PCR assays targeting *ZFX* and *ZFY* for comparison. The *SRY/HBB* and *ZFY/ZFX* assays were performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) with 5 μL of DNA sample per reaction. The *ZFX* and *ZFY* primer and probe sequences were the same as on the digital platform, but the probes were used separately. Reaction conditions are summarized in the online Data Supplement.

We ran duplicate DNA samples and reported the mean in the results. For absolute quantification, we ran a calibration curve consisting of serially diluted male blood cell DNA (1–1000 GE per reaction) in parallel and in duplicate with each analysis. We used a conversion factor of 6.6 pg DNA/cell. Amplification data were analyzed with Sequence Detection Software (version 1.2.3; Applied Biosystems). The same calibration curve was used for the real-time *SRY*/*HBB* assay and the nondigital *ZFY*/*ZFX* assay.

For the real-time *SRY*/*HBB* assay, the percentage of male DNA per reaction was given by: (*SRY* GE)/(*HBB* GE)×100.

To calculate the percentage of male DNA in the real-time *ZFY*/*ZFX* assay, we used the same equation as for the digital version of the assay.

MASS SPECTROMETRY

We performed MALDI-TOF mass spectrometry analysis with a standard homogenous MassEXTEND assay (Sequenom). An 82-bp region in *ZFX* and *ZFY* was coamplified with one primer set (Table 1). The respective amplicons were identified by a primer-extension reaction that targets the base differences between *ZFX* and *ZFY* (Table 1). Further details of the assay are provided in the online Data Supplement. The extension products were dispensed onto a SpectroCHIP (Sequenom) by a MassARRAY Nanodispenser S (Sequenom). Data acquisition from the SpectroCHIP was done in the MassARRAY Analyzer Compact Mass Spectrometer (Sequenom).

An inherent property of mass spectrometry is that products with higher molecular masses are usually attenuated on the mass spectrum. To control for the peak skewing, we included a calibration curve consisting of mixtures of male and female blood cell DNA (0%, 10%, 20%, 30%, 40%, and 50% of male DNA) for peak frequency correction. We carried out 2 homogenous MassEXTEND reactions for each test sample and dispensed each homogenous MassEXTEND product twice onto the SpectroCHIP. We therefore obtained 4 mass spectra for each case. The percentage of fetal DNA in maternal plasma was quantified by the relative peak frequencies of *ZFY* to *ZFX* in the mass spectrum. The percentage of male DNA for each case was the mean of 4 data points: 2 × (observed *ZFY* frequency) × 100. This value was further corrected for peak skewing by dividing by the slope-correction value (1.024 in this

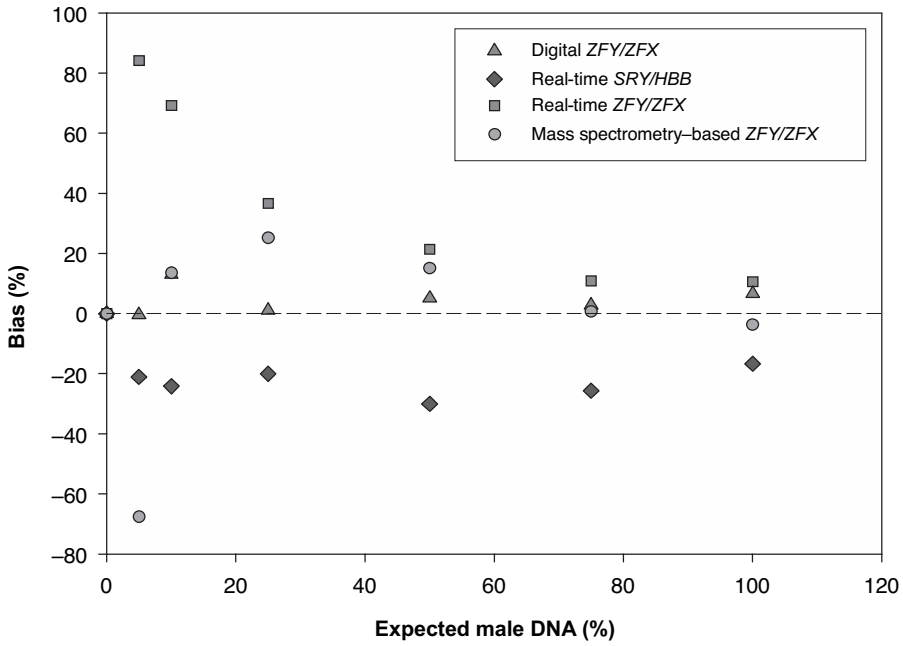

Fig. 2. Difference plot.

The *x* axis denotes the expected male-DNA percentage in the artificial DNA mixtures. The *y* axis denotes the degree of deviation of experimental results from expected values and is expressed as a percentage.

experiment): Percent reported male DNA = (Percent observed male DNA)/1.024.

Results

We determined whether biases existed with measurements made on the 3 tested analytical platforms by comparison with the expected concentrations of male DNA. The observed biases were expressed graphically in a difference plot vs the expected male-DNA concentration (Fig. 2) *(21)*. Of the 3 assays, the microfluidics digital PCR assay most correctly reflected the actual sample compositions and produced the smallest percentage bias. The nondigital real-time *SRY*/*HBB* assays *(9)*, which have been widely used in the field, showed a negative bias, underestimating the fractional male-DNA concentration by approximately 20%. The nondigital real-time *ZFY*/*ZFX* assay exhibited a positive bias that worsened as the fractional concentration of male DNA decreased. The mass spectrometry–based assay exhibited a positive bias at higher fractional male-DNA concentrations but had a negative bias as the fractional male-DNA concentration decreased.

We focused subsequent experiments on comparing the microfluidics digital PCR *ZFY*/*ZFX* system and the nondigital real-time PCR *SRY*/*HBB* assay *(9)*, because the latter has been one of the standard assays used in the field. We used an artificial sample mixture of 7% male DNA to compare the analytical imprecision of these 2 systems for measuring the fractional male-DNA concentration. We carried out 20 analyses of this sample with 20 different microfluidics chips (1 reportable result per chip) on the digital PCR platform. For the conventional nondigital PCR system, we measured this sample 20 times with 20 plates (1 reportable result per plate), with the *SRY* and *HBB* assays carried out in the same plate for each measurement. The CVs of the digital and nondigital assays were 16% and 49%, respectively (Fig. 3), indicating that the digital assay was 3.1 times more precise than the nondigital assay.

We next investigated the diagnostic sensitivity of the digital and nondigital assays for detecting male fetal DNA in the plasma of 10 first-trimester (12–14 weeks gestation) pregnant women carrying male fetuses. We scored the first 6 of the 12 panels of each chip to determine fetal sex by digital PCR and scored all 12 panels of each chip to quantitatively measure the fractional fetal-DNA concentration. We used this procedure to ensure that we fairly compared the digital and nondigital PCR systems with similar input volumes of plasma DNA (see *Materials and Methods*). For the nondigital PCR system, we carried out duplicate amplifications for each sample, as originally described *(9)*, with at least one *SRY*-positive signal for the 2 amplifications being

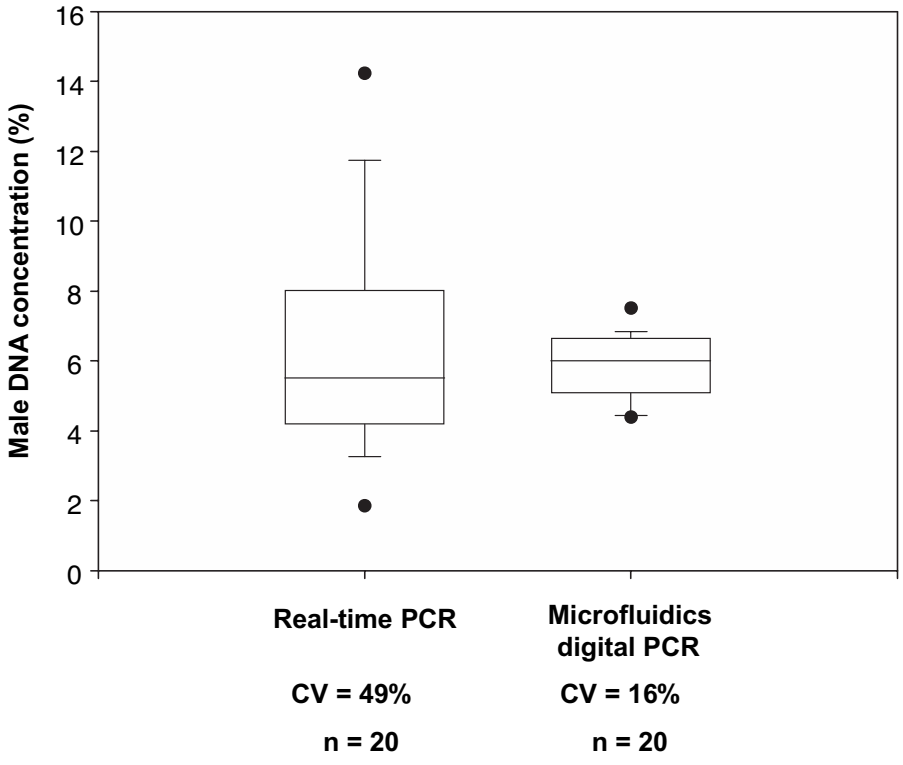

Fig. 3. Interplate and interchip imprecision of nondigital real-time *SRY*/*HBB* and digital *ZFY*/*ZFX* assays.
The horizontal line within each box denotes the median of 20 separate runs. The limits of each box denote the 25th and 75th percentiles. Whiskers denote the 5th and 95th percentiles. Filled circles depict the outliers.

scored as positive for a male fetus. The diagnostic sensitivities of the digital and nondigital assays for the detection of male fetuses were 100% and 90%, respectively. The diagnostic specificities of the digital (12 panels) and nondigital assays were confirmed with plasma samples from 5 first-trimester (12–14 weeks gestation) pregnant women carrying female fetuses. The digital and nondigital assays detected no *ZFY* and *SRY* signals, respectively.

We used both the digital and nondigital assays to measure the fractional fetal-DNA concentrations in 10 plasma samples each from pregnant women who were in their first trimester (as described above), second trimester (17–22 weeks gestation), and third trimester (38–39 weeks gestation) and carrying male fetuses. The median fractional fetal-DNA concentrations measured with the digital PCR assay were 9.7%, 9.0%, and 20.4% for the first, second, and third trimesters, respectively (Fig. 4). The corresponding values for the nondigital PCR assays were 4.8%, 4.1%, and 7.6%, respectively. Thus, the median fractional fetal-DNA concentrations measured with the digital PCR assay were 2.0, 2.2, and 2.7 times higher than those obtained with the nondigital PCR system for the respective gestational ages.

Discussion

We explored microfluidics digital PCR as a tool for the detection and measurement of fetal DNA in maternal plasma. Digital PCR is approximately 3.1 times more precise than conventional nondigital real-time PCR (Fig. 3). We also demonstrated that microfluidics digital PCR revealed the least bias in measuring the fractional concentration of male DNA, compared with assays based on conventional nondigital real-time PCR and mass spectrometry (Fig. 2). Digital PCR is expected a priori to be more correct and precise than nondigital PCR formats of PCR because by analyzing a sample in a multitude of aliquot volumes containing less than a single copy, digital PCR transforms the analog output of conventional PCR to an "all-or-nothing" (i.e., digital) readout of individual amplifications. This approach allows the counting of the number of individual positive amplifications and calculation of the concen-

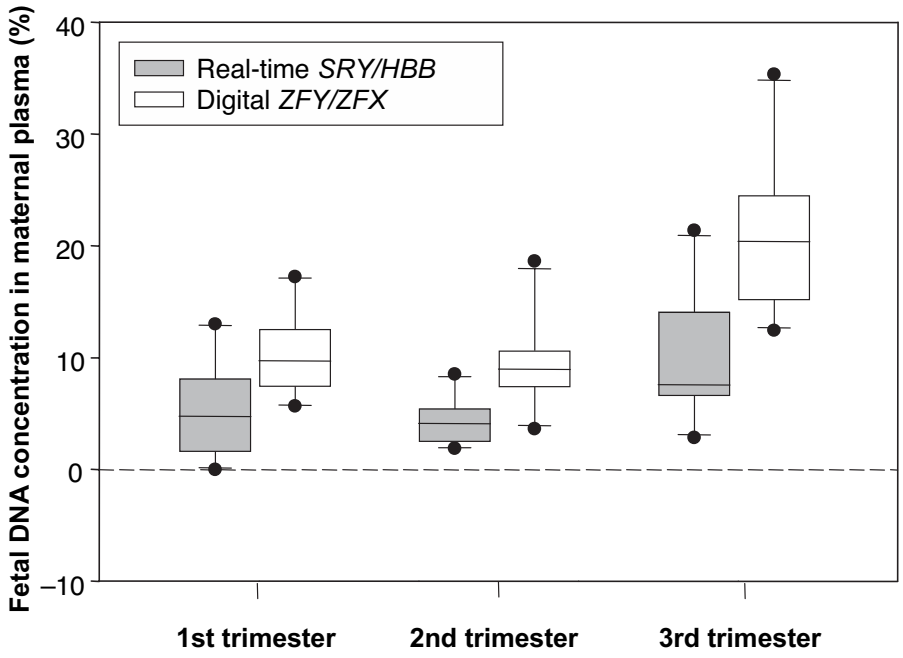

Fig. 4. Box plots of male fetal-DNA percentages measured with the nondigital real-time *SRY*/*HBB* assay and the digital *ZFY*/*ZFX* assay in first-, second- and third-trimester maternal plasma.

The horizontal line within each box denotes the median of 10 samples. The limits of each box denote the 25th and 75th percentiles. Whiskers denote the 5th and 95th percentiles. Filled circles depict the outliers.

tration on the basis of mathematical principles, i.e., the Poisson distribution. The physical nature of the quantitative data obviates the use of calibrator solutions. This fact is evident by the smaller bias value for the digital *ZFY*/*ZFX* assay than for the nondigital version of the assay (Fig. 2).

In contrast, the nondigital real-time PCR assays are dependent on the use of a series of calibrators to correlate the fluorescence signal with the nucleic acid concentration. One source of inaccuracy may occur when a new set of calibrators is instituted or when different laboratories prepare their own calibrator solutions. Furthermore, different assays may generate different dose–response curves for a series of calibrators, thus producing different degrees of bias. This point is illustrated by the difference plots for the *SRY*/*HBB* and *ZFY*/*ZFX* real-time PCRs in which the same calibrators were used for both systems (Fig. 2). The former assay showed a negative bias, whereas the latter showed a positive bias.

More specifically, the *SRY*/*HBB* assay, which was the first real-time PCR system to be developed for measuring fractional fetal-DNA concentrations in maternal plasma *(9)*, underestimated the proportion of male DNA by approximately 20% in experiments involving artificial mixtures of male and female DNA (Fig. 2). In experiments with plasma samples from pregnant women carrying male fetuses, the digital PCR assay revealed median fractional fetal-DNA concentrations that were approximately 2-fold higher than previously reported *(9)*. Apart from the expected better quantitative performance of the digital PCR assay, another factor that might have contributed to the higher fractional fetal-DNA concentration with the digital assay is the fact that the 87-bp *ZFY* amplicon is shorter than the 137-bp *SRY* amplicon *(9)*. Previous work has indicated that fetal DNA in maternal plasma is shorter than the background maternally derived DNA *(22)*.

The enhanced analytical performance of microfluidics digital PCR could have an important impact on the use of fetal DNA in maternal plasma for noninvasive prenatal diagnosis. For example, many investigators have attempted to develop methods for increasing the fractional concentrations of fetal DNA extracted from maternal plasma, either through size fractionation *(11)* or suppression of the background maternal DNA through formaldehyde treatment *(23)*. The controversy surrounding the latter approach in particular has illustrated the importance of precise and correct measurements of circulating fetal-DNA concentra-

tions, because one area of dispute has concerned the imprecision of the serial-dilution approach used in the original version of the formaldehyde-treatment method *(23–25)*.

Furthermore, the number of counted molecules required to detect Down syndrome has been demonstrated to be inversely related to the fractional fetal-DNA concentration *(15)*. Our demonstration that the median fractional concentration of circulating fetal DNA obtained with digital PCR might be 2-fold higher than previously recognized (Fig. 4) suggests that the technical challenge for detecting Down syndrome via plasma-DNA analysis is less than has previously been assumed. Indeed, for every 2-fold increase in the fractional fetal-DNA concentration, the number of analyzed molecules required to diagnose Down syndrome decreases by a factor of approximately 4 *(15)*.

Microfluidics digital PCR also has benefits for applications requiring only the qualitative detection of circulating fetal DNA. For example, we have demonstrated that microfluidics digital PCR improves the detection of male fetal DNA in maternal plasma, compared with conventional real-time PCR. In addition, microfluidics digital PCR would have an advantage for detecting fetal-DNA targets that differ from maternal-DNA sequences by one or a small number of nucleotides, because digital PCR operates at target concentrations at which most positive reactions would contain only a single target molecule *(15, 16)*. Thus, fetal- and maternal-DNA targets that would be cross-amplified with the same primer set would now be separately amplified in different digital PCRs, with the corresponding fetal and maternal amplicons being identified by probes labeled with different reporters. Although we based this study on the quantification of male fetal DNA in maternal plasma, the advantage mentioned above also indicates that fetal-DNA concentrations for female fetuses could be measured via digital PCR quantification of fetus-specific, paternally inherited alleles of a panel of single-nucleotide polymorphisms.

One current drawback of microfluidics digital PCR is the cost of the chips. In this study, one chip was consumed for each maternal plasma sample. The enhanced analytical performance of digital PCR could be realized in nonmicrofluidics-based digital real-time PCR or in digital primer-extension reactions with mass spectrometry *(15)*. For investigators who prefer to use nondigital PCR approaches for cost reasons, there may be room for improvement in the quantitative performance of the nondigital platforms, for example via the addition of internal calibrators to each PCR reaction *(26)*.

In conclusion, this study has demonstrated that microfluidics digital PCR is a useful new tool that allows improved measurement of circulating cell-free fetal DNA and potentially other nucleic acid species in plasma, such as tumor-derived DNA *(27)* and donor-derived DNA *(28)* in the plasma of cancer patients and transplant recipients, respectively.

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 of Potential Conflicts of Interest: *Upon submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: Y.M. Dennis Lo, Sequenom
Stock Ownership: Y.M. Dennis Lo, Sequenom
Honoraria: None declared.
Research Funding: Rossa W.K. Chiu, University Grants Committee of the Government of the Hong Kong Special Administration Region, China, under the Areas of Excellence Scheme (AoE/M-04/06); Y.M. Dennis Lo, Sequenom.
Expert Testimony: None declared.
Other: Rossa W.K. Chiu, Patent applications have been filed on the detection of fetal nucleic acids in maternal plasma for noninvasive prenatal diagnosis. Part of this patent portfolio has been licensed to Sequenom.

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.

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