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REVIEW

Digital PCR: a powerful new tool for noninvasive prenatal diagnosis?

Bernhard G. Zimmermann[1], Simon Grill[2], Wolfgang Holzgreve[2], Xiao Yan Zhong[2], Laird G. Jackson[3] and Sinuhe Hahn[2]*

[1]*Fluidigm Corporation, South San Francisco, USA*
[2]*Department of Biomedicine, University Women's Hospital, Basel, Switzerland*
[3]*Division of Obstetrics and Gynecology, Drexel University School of Medicine, Philadelphia, Pennsylvania, USA*

Recent reports have indicated that digital PCR may be useful for the noninvasive detection of fetal aneuploidies by the analysis of cell-free DNA and RNA in maternal plasma or serum. In this review we provide an insight into the underlying technology and its previous application in the determination of the allelic frequencies of oncogenic alterations in cancer specimens. We also provide an indication of how this new technology may prove useful for the detection of fetal aneuploidies and single gene Mendelian disorders. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

In recent years great strides have been made in the analysis of cell-free fetal DNA in maternal blood for the analysis of fetal genetic loci completely absent from the maternal genome, such as the presence of a Y chromosome for the determination of fetal sex or the fetal Rhesus D gene in Rhesus D negative pregnant women. Consequently, these are already being offered as a clinical service for these loci by a number of European and US American centers (Chiu and Lo, 2004; van der Schoot *et al*., 2008).

Although recent reports have indicated that it may be possible to noninvasively detect fetal aneuploidies such as Down syndrome (trisomy 21) (Lo *et al*., 2007) or Mendelian single gene disorders, such as the hemoglobinopathies (Ding *et al*., 2004; Li *et al*., 2005; Galbiati *et al*., 2008), no test has yet been implemented in a clinical setting.

The major reason for this is that cell-free DNA preparations from maternal plasma contain a vast proportion of maternal DNA sequences (>95%) and a minority of fetal DNA sequences (<5%) (Lo *et al*., 1998). This makes it difficult to discern how many copies of a particular chromosome the fetus has inherited, e.g. chromosome 21 in Down syndrome, or whether the fetus has inherited a maternal mutation of a Mendelian disorder (Hahn and Holzgreve, 2002).

Hence, current analysis of Mendelian disorders is restricted to the detection of paternally inherited mutant loci which differ from those of the mother (Hahn and Chitty, 2008). For the detection of both fetal aneuploidies, alternate strategies have been proposed employing single nucleotide polymorphisms (SNPs): For trisomy 21, the analysis of placenta-derived cell-free mRNA (PLAC4) species has been proposed (Lo *et al*., 2007), whereas for trisomy 18 an epigenetically modified placenta-derived cell-free DNA sequence (*maspin*) was suggested (Tong *et al*., 2006). Currently, these assays largely depend on the use of mass spectrometric devices, such as the Sequenom MassArray. Although this device permits a degree of accuracy superior to that of real-time PCR for relative quantification, it has become evident that a further technical development is necessary if these long sought after goals of noninvasive prenatal diagnosis are to be attained.

DEVELOPMENT AND PRINCIPLES OF DIGITAL PCR

In 1992, Sykes and colleagues described the PCR-based quantification by the use of limiting dilution and Poisson statistics (Sykes *et al*., 1992). Using a two-stage PCR they were able to detect the rearranged immunoglobulin heavy chain (IgH) gene derived from a leukemic clone against an excess of normal rearrangements from normal lymphocytes. The salient feature of this study was that the authors described a system for determining the number of input templates by simply counting the number of positive PCR results in a limiting dilution. Thereby, they paved the road for 'Digital PCR' by the use of a plus/minus readout per reaction.

In 1997, Kalinina and colleagues described a novel nanoliter scale PCR method using glass capillaries for the PCR reaction; amplification was detected in the reaction capillaries by fluorescence (Kalinina *et al*.,

*Correspondence to: Sinuhe Hahn, Laboratory for Prenatal Medicine and Gynecological Oncology, University Women's Hospital/Department of Biomedicine, Hebelstrasse 20, CH 4031 Basel, Switzerland. E-mail: shahn@uhbs.ch

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1997). In their analysis, the detection of single template molecules diluted into genomic DNA was possible with a simple PCR protocol because of the small reaction volumes and the use of TaqMan probes.

In 1999, Vogelstein and Kinzler extended upon these reports to develop a version of this approach using a 96 well plate system for the PCR amplification (Vogelstein and Kinzler, 1999). In their study which set out to determine mutant to normal allelic ratios of the *ras* oncogene in stool samples of patients with colorectal cancer, the input DNA was diluted to such an extent that there was approximately one template molecule per two wells. The mutant and wild-type *ras* oncogene sequences were then detected in a second step using specific stem-loop probes labeled with red and green fluorescent dyes, respectively. The red-labeled probe was chosen in such a manner that its binding of the *ras* amplicon was hindered should an activating mutation in codons 12 or 13 be present, whereas the green-labeled probe simply detected the presence of the *ras* amplicon. Therefore, by simply counting the number of wells with both red and green signals in comparison to those with only green signals, it was possible to determine the allelic ratio of normal to mutant *ras* genes present in the sample examined.

Since this system was cumbersome and would not permit the analysis of a large number of samples, the advent of a digital PCR approach suitable for more complex analyses had to await the development of emulsion PCR (emPCR) and microfluidic devices in which it was possible to perform hundreds to several thousands of PCR reactions in parallel.

EMULSION PCR

Performing PCR in a water–oil emulsion basically provides millions of individual microreactions. For one, such reactions profit from the increased reaction stringency observed in small reaction volumes. Ge *et al.* used a multiplex of six Y chromosome sequences in emPCR and detection of PCR products by microarray or gel electrophoresis for the analysis of plasma from 76 pregnant women (Ge *et al.*, 2006). They showed high sensitivity of the method, being able to detect Y chromosome sequences in all 42 gestations with male fetuses and all 6 products were detected in samples as early as 31 days into pregnancy.

To convert the digital nature of emPCR into actual digital data, Vogelstein introduced a method called BEAMing (beads, emulsion, amplification and magnetics): Magnetic beads covalently bound with streptavidin are coated with biotinylated PCR primers and added to emPCR. After demulsification, beads are analyzed for allelic variation using flow cytometry. Alleles can be distinguished by hybridization to allele-specific probes with different dyes or single nucleotide extension. So far, BEAMing has been used for detection and relative quantification of sequence variants such as SNPs and mutations (Diehl *et al.*, 2005). In the latter report the sensitivity to detect mutations was increased by preamplifying DNA from plasma prior to emPCR. Thus, it is ideally suited for analysis of minority sequence variants. Samples below 1 μg of genomic DNA are preamplified by PCR; it is, however, questionable if it can be used for absolute quantification or for relative quantification of two distinct sequences, as for the detection of aneuploidies. While multiple steps have to be performed it is an elegant method which can be carried out with widely available laboratory equipment. In addition, beads containing variant alleles can be isolated through flow sorting and used for subsequent analysis, for example sequencing (Diehl *et al.*, 2006).

DIGITAL PCR ON A MICROFLUIDIC CHIP

The second solution to modern digital PCR is offered by microfluidic devices with the ability to perform highly parallel analyses in a single PCR step. The development of microfabricated valves and pumps by multilayer soft lithography (MSL) confers the ability to design integrated fluidic circuits (IFCs) and to manufacture microfluidic chips cost effectively (Unger *et al.*, 2000). They give one the ability to create complex integrated designs and will in future challenge the conventional paradigm of high-throughput automation by fluid handling robots. A number of functional modules have already been described (Chou *et al.*, 2001; Melin and Quake, 2007). MSL chips have already been used for a wide selection of biochemical and molecular biology applications such as DNA sorting and synthesis, single cell sorting and analysis (Wheeler *et al.*, 2003), cell culture (El-Ali *et al.*, 2006), or whole genome amplification (Marcy *et al.*, 2007). In principle, any molecular reaction that can be run in a tube can be performed in a microfluidic chip, for example, highly parallel protein crystallization (Hansen *et al.*, 2002).

In 2003, using the concept of a microfluidic matrix, Liu *et al.* were able to perform 400 independent 3-nL PCR reactions on an MSL chip, at the same time minimizing reagent consumption and the number of pipetting steps necessary (Liu *et al.*, 2003). Applying similar logic but a different design, IFC chips have been developed for high-throughput gene expression and SNP genotyping (Spurgeon *et al.*, 2008); the latest version performs 9216 real-time or end-point PCR reactions in a single experiment. These PCR chips' matrix design permits the parallel analysis of every pair wise combination of a large number of samples and a large number of targets.

Alternatively, utilizing microfluidic valves, a single sample can be partitioned into many hundreds to thousands of reactions in an extremely convenient, automated manner to perform digital PCR. As the third embodiment of IFC PCR chips, the digital array performs 9180 reactions of 6 nL volume in parallel (Dube *et al.*, 2008). These are divided into 12 individual panels that can be used to analyze up to 12 different samples. Thus, a single sample is distributed to 765 discrete reaction compartments, and after PCR positive reactions can be counted. Owing to their widespread use, TaqMan assays are generally used, but other fluorescent probe based nucleic

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acid detection methods can be implemented. Positives can be determined by end-point analysis, where the final signal is determined to be positive or negative based on fluorescence intensity increase in comparison to the initial signal, or by analysis of real-time curves (Figure 1).

A positive reaction chamber contains at least one target molecule. In very dilute samples the count of positive wells equals the number of target molecules. Based on a poisson-based algorithm the template abundance (and 95% confidence interval) can be calculated even if a large proportion of the reactions is positive and contains on average, more than one target molecule (Dube *et al*., 2008). Thus, in order to perform absolute quantification of target sequences in a sample, microfluidic digital PCR does not rely on data collected during the exponential phase of PCR (as the gold-standard real-time PCR) nor does it require a competitor sequence nor a standard (curve) to achieve absolute quantification, and consequently data precision and accuracy are excellent. While most microfluidic digital PCR applications currently published utilize MSL chips, there are a number of other platforms on the market or emerging that could enable digital PCR in a convenient manner (Morrison *et al*., 2006).

The concept of digitalizing PCR by spreading a sample is very versatile and has found applications in the quantification of single cell gene expression (Ottesen *et al*., 2006; Warren *et al*., 2006), absolute quantification of point mutations (Oehler *et al*., 2008), and in a variety of relative quantifications, e.g. the determination of copy number variations (CNV) (Qin *et al*., 2008), loss of heterozygosity (LOH), or aneuploidy detection (Fan and Quake, 2007; Lo *et al*., 2007). Multiple color detection permits the analysis of, for example, SNPs or the measurement of a target and reference sequence in the same reaction.

But digital PCR on a chip also provides a tool to increase the detection sensitivity and specificity in samples with large background, which would otherwise mask the detection of a specific target or lead to unspecific amplification and false positives in approaches such as 'needle-in-a-haystack' detection of oncogenic point mutations in cancers (Vogelstein and Kinzler, 1999; Shih *et al*., 2001).

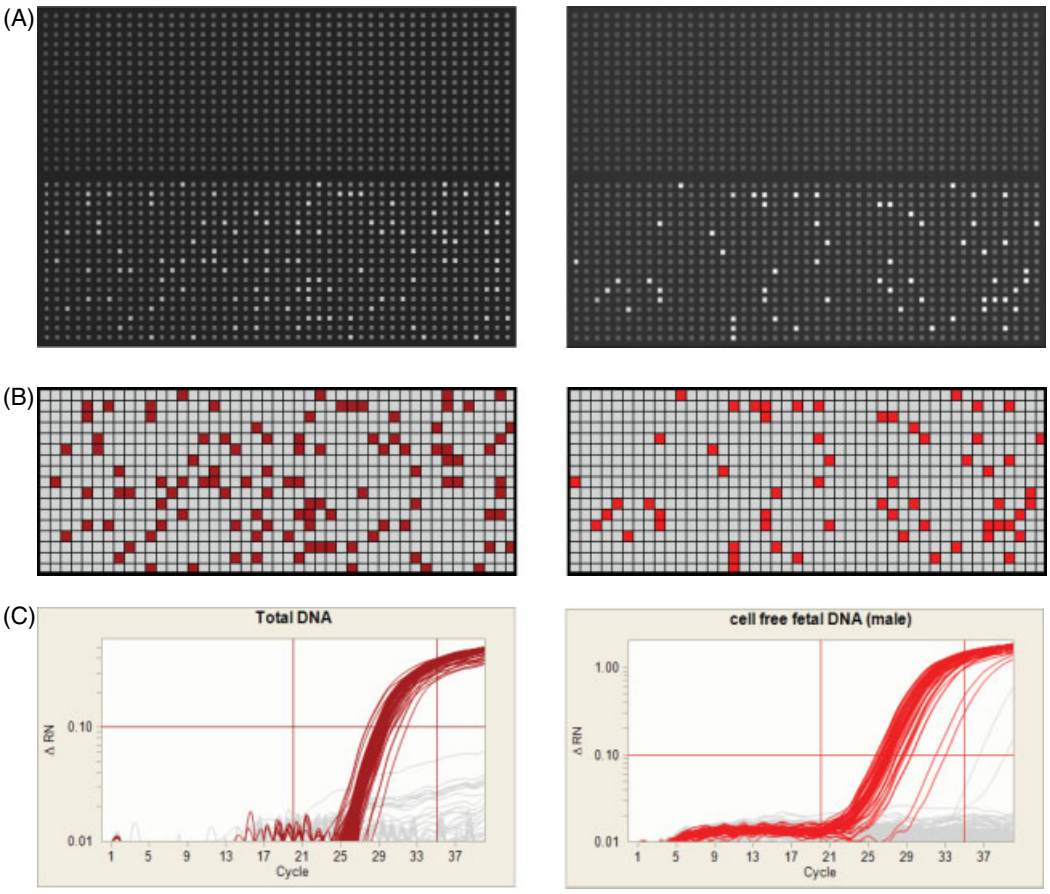

Figure 1—PCR amplification using the digital array Cell-free total (left) and fetal (right) DNA form maternal plasma were analyzed in duplex TaqMan PCR in a digital array chip panel. Cell-free fetal DNA from maternal plasma was quantified using an assay for the multicopy gene DYS14 on the Y chromosome (FAM dye); total cell-free DNA (left) was examined by an assay for chromosome 21 (ROX dye). (A) Illustration of the same panel after 1 (top) and 40 (bottom) PCR cycles. (B) Heat map of positive reactions. Positive chambers are represented by filled in squares. The number of positive chambers for total and fetal DNA is 105 and 90 which equals a copy number of 113 [CI (95%) 91–134] and 63 [CI (95%) 46–77] respectively. (C) Amplification plot of the panel in the exponential view. The window for target detection in real-time was set between 20 and 35 cycles (vertical lines) and the threshold to 0.1 ΔRn (normalized Fluorescence). Curves that cross this threshold after 35 cycles are scored as negative

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Approaches that have or may have an impact on noninvasive prenatal research in the future will now be discussed in more detail.

USE FOR LOH DETECTION

Since it was clear that digital PCR permitted the assessment of allelic ratios, it was a natural progression to determine whether this technology also permitted the detection of chromosomal instability, thereby allowing for the monitoring of loss or gain of defined genetic regions. In their studies of allelic imbalance in colorectal cancers, the Vogelstein group reported that over 90% of the tumors examined exhibited such chromosomal instability and that this frequently involved several chromosome regions, e.g. 5q, 1p, 15q, and 18q (Shih

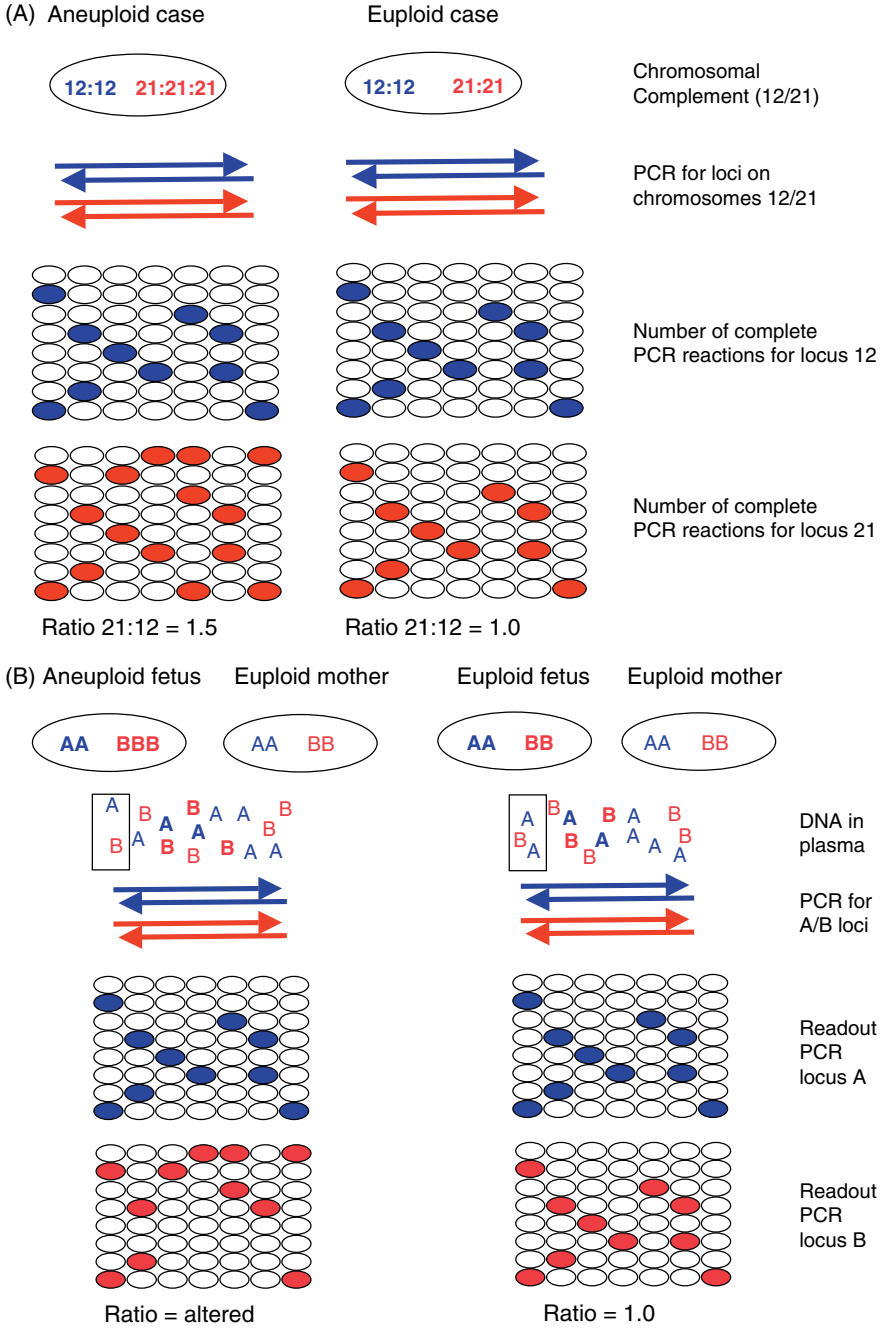

Figure 2—(A) Schematic representation of the method used by Fan and Quake for the determination of trisomy 21 by digital PCR. PCR was carried out using primers and TaqMan probes for loci on chromosome 21 and 12. Positive reactions for each locus were counted and the ratio assessed. In cases of trisomy 21, a ratio of 1.5 is expected, whereas in euploid cases this would be 1. (B) Possible scheme for the detection of fetal aneuploidy directly from cell-free DNA in maternal plasma by digital PCR. In a manner similar to that described above, PCR is carried out using primers and TaqMan probes for loci on chromosome A and B. Positive reactions for each locus were counted and the ratio assessed. In cases of trisomy for chromosome B, a ratio greater than 1.0 is expected, whereas in euploid cases this would be 1

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et al., 2001; Zhou *et al*., 2001, 2002). Of interest is that it was possible to perform these analyses on microdissected tissue samples, indicating that it was possible to use very small amounts of starting material (Morikawa *et al*., 2005, 2008). As these pioneering findings have since been confirmed in a number of other studies (Pohl and Shih, 2004; Diehl *et al*., 2005; Diehl and Diaz, 2007), they serve to reinforce the power of digital PCR to examine this complex genetic problem on small samples or limited amounts of input template.

POSSIBLE USE FOR DETECTION OF FETAL ANEUPLOIDIES

Granted that it was possible to detect loss or gain of chromosomal regions reliably by digital PCR, it was a small, but pivotal, step to examine whether this approach could also be used to detect fetal chromosomal anomalies. In one report, Fan and Quake performed an examination of material obtained from a cell line with trisomy 21 and genetic material from cells with a normal genomic complement on a microfluidic chip (Fan and Quake, 2007). In their examination Fan and Quake used a PCR assay similar to that which we had previously established for the detection of trisomy 21 by TaqMan real-time PCR (Zimmermann *et al*., 2002), in that the dosage of an amyloid gene sequence on chromosome 21 was compared to that of the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) on chromosome 12 (refer to Figure 2(a)). Although preliminary, the data from their report clearly indicate that digital PCR could be used for a reliable discrimination between normal and aneuploid samples. Of particular interest is that this discrimination could be observed even when the aneuploid material only represented 10% of the total material being examined, far below the threshold which is possible when using other methods such as real-time PCR or fluorescent quantitative PCR (QF-PCR). This result implies that it may be possible to use this technology to detect fetal aneuploidies directly from cell-free DNA in maternal plasma, providing that the fetal cell-free DNA fraction is enriched using an approach like size-fractionation (Figure 2(b)).

In another report, Lo and colleagues demonstrated two digital PCR strategies for the noninvasive detection of fetal aneuploidy in microwell plates (Lo *et al*., 2007). The first being based on the PLAC4 mRNA SNP approach they had previously shown to permit the detection of fetal trisomy 21 with PCR linked to primer extension and mass spectrometry (Lo *et al*., 2007). By the use of digital PCR (dPCR) analysis of maternal plasma samples, they were able to distinguish four aneuploid fetuses from nine normal healthy ones. Unfortunately, this approach relies on the heterozygosity of SNPs that lie on chromosome 21 and are solely expressed by the placenta. As the number of suitable mRNA SNPs that are sufficiently high expressed and informative is limited, it will be important to perform clinical studies on population coverage in different ethnic groups. In their second strategy, a gene dosage approach similar to that used by Fan and Quake was used, in that the dosage of a locus on chromosome 21 was compared to a locus on chromosome 1. In this analysis they used mixtures of euploid and aneuploid (trisomy 21) DNA, and report being able to detect a fetal aneuploidy in artificial mixtures with as low as 25% trisomic DNA. Once again, while the approach was not applied to clinical samples, this concentration would be akin to what can be obtained using enrichment strategies for fetal cell-free DNA sequences such as size-fractionation. However, a very recent study to utilize microfluidic digital PCR by Lun and colleagues using dPCR suggests that fetal DNA concentrations may be higher than expected. Compared to conventional qPCR and mass-spectrometry based quantification of male DNA in artificial mixtures of male and female genomic DNA, dPCR produced the most accurate results (Lun *et al*., 2008).

Given the versatility of microfluidic digital PCR, it seems that a number of alternative approaches will also be described in the near future, such as for example the application of 'brute force' with a chip that has hundred thousands to millions of reaction compartments or maybe emulsion-based approaches.

The current barrier for using digital PCR for prenatal diagnosis of fetal aneuploidy is that the fraction of fetal DNA is a bit too low compared to what may be required. Comparing analyses from cited publications (Fan and Quake, 2007; Lo *et al*., 2007; Dube *et al*., 2008), a percentage of approximately 20% fetal DNA could be suitable. Enrichment by size separation can achieve these levels, so why is there not yet any successful report? For one, the amount of circulatory fetal DNA is limited. Sample processing methods for extracting large volumes of plasma efficiently need to be established. Analyzing several sequences per chromosome of interest is another possibility, but the establishment of multiplexed reactions is generally a major challenge and this is not possible for SNP-based approaches. Finally, size separation is a laborious method and leads to considerable loss of genetic material. As a consequence, while the critical fraction of fetal DNA may be achieved, the critical absolute amount of DNA is further removed.

USEFUL FOR THE NONINVASIVE DETECTION OF FETAL MENDELIAN DISORDERS

It is highly probable that similar strategies could be modified to permit the detection of fetal Mendelian disorders such as hemoglobinopathies or cystic fibrosis (outlined in Figure 3). The detection of the paternal mutation has already been achieved by other methods (Ding *et al*., 2004; Li *et al*., 2005; Galbiati *et al*., 2008) and should be straightforward using either of the digital PCR approaches, and an adequate size enrichment and precise allelic quantification similar to the one used by Lo *et al*. (2007) could even permit determining which maternal allele is passed on to the fetus.

In theory this system should be applicable to those situations where the parents carry different mutations or even if they both share the same mutant allele (Figure 3). In the former scenario, conventional PCR could be

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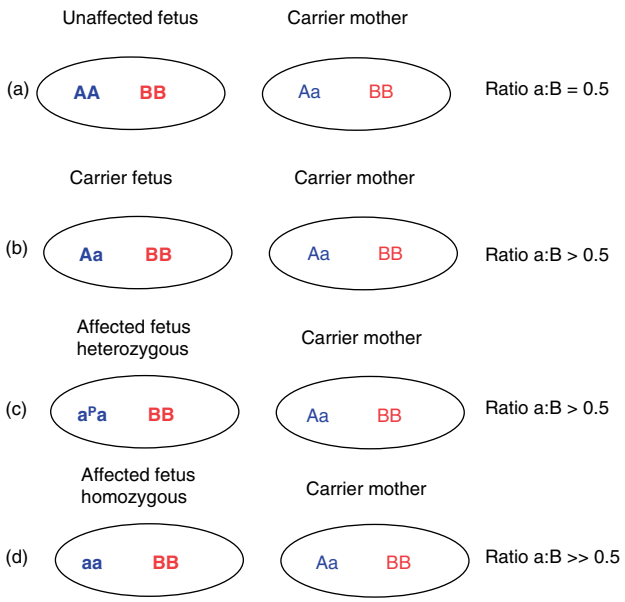

Figure 3—Possible scheme for the detection of fetal single gene disorders directly from cell-free DNA in maternal plasma by digital PCR. In this instance, PCR reactions specific for the maternal mutant allele would be used (a). (a) If the fetus is unaffected (AA), then only the maternal mutant allele would be detected and the ratio of this to the healthy allele or another locus could be assessed. (b) If the fetus is a carrier for the maternal mutant allele, then the ratio of a : B would be expected to increase. (c) The same would hold true if the fetus was affected due to the occurrence of a heterozygous constellation for the mutations examined. In this case the presence of the paternal allele would be detected by a separate specific PCR reaction. (D) In the case where the fetus is homozygous for the mutation, a further increase in the ratio a : B can be expected

used to determine whether the fetus has inherited the paternal mutant allele, as described in a number of recent publications (Ding *et al*., 2004; Li *et al*., 2005; Galbiati *et al*., 2008). Digital PCR would then be used to count the number of maternal mutant alleles relative to the wild-type allele or an unrelated locus: in essence, very similar to the analysis performed for the detection of trisomy 21.

FUTURE DEVELOPMENTS

Both emulsion-based and microfluidic digital PCR offer several advantages. Equipment for emulsion-based PCR is affordable and offers the potential of multiplexing. A preamplification could increase the sensitivity for certain applications (e.g. for Sequenom-MS approach). However, quantification is currently not absolute and multiple steps are necessary to perform an experiment. In contrast, microfluidics allows absolute quantification in a one-step analysis with a few thousand (or even 10^6) reactions performed simultaneously, even though special instrumentation is required.

As microfluidic devices become more dense and permit for very large numbers (in the order of several hundred thousand) of independent PCR reactions to be monitored, it will become possible to discriminate between even smaller differences in template. In this manner, it may be possible to use such new devices directly for the analysis of cell-free DNA in maternal plasma without the need for any selective enrichment of fetal cell-free DNA sequences. However, it remains to be seen to what point competing methodologies, such as the counting of single molecules by NanoString technology can be developed, as these offer the potential to detect single nucleic acids molecules without the need for any PCR amplification (Geiss *et al*., 2008).

DIAGNOSTIC OR SCREENING

As digital PCR relies on statistical analysis and a series of algorithms indicating probability that a fetus is normal or affected, the question arises whether this method will be relegated for use as a screening tool or whether it will indeed pass the necessary scientific and regulatory hurdles permitting it to be used diagnostically. As to date, only two potential proof-of-principle studies have been performed, it is too early to answer these questions.

SUMMARY AND CONCLUSIONS

The advent of digital PCR opens up new possibilities for the noninvasive assessment of fetal genetic loci, not yet possible by current means. These include the detection of fetal aneuploidies and Mendelian disorders, and it is likely that these developments will be widely reported on in the near future.

DISCLAIMER

B.G. Zimmermann is an employee of Fluidigm Corporation, manufacturer of instruments and chips for Microfluidic PCR analysis.

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