Exhibit 3

Review

Principle and applications of digital PCR

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Digital PCR represents an example of the power of PCR and provides unprecedented opportunities for molecular genetic analysis in cancer. The technique is to amplify a single DNA template from minimally diluted samples, therefore generating amplicons that are exclusively derived from one template and can be detected with different fluorophores or sequencing to discriminate different alleles (e.g., wild type vs. mutant or paternal vs. maternal alleles). Thus, digital PCR transforms the exponential, analog signals obtained from conventional PCR to linear, digital signals, allowing statistical analysis of the PCR product. Digital PCR has been applied in quantification of mutant alleles and detection of allelic imbalance in clinical specimens, providing a promising molecular diagnostic tool for cancer detection. The scope of this article is to review the principles of digital PCR and its practical applications in cancer research and in the molecular diagnosis of cancer.

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Digital PCR was first developed by Vogelstein and Kinzler to extend the applications of conventional PCR [1]. This technology is based on applying optimal PCR conditions to amplify a single template and is followed by detection of sequence-specific PCR products (alleles) for allelic counting. Digital PCR has proven useful in detecting rare mutations in a bulk of wild type sequences and in assessing allelic imbalance in tumor tissue and in plasma DNA samples. Therefore, this article will primarily focus on reviewing the principles of digital PCR and its applications in mutational analysis and assessment of allelic imbalance (TABLE 1).

Principles of digital PCR

The principle of digital PCR is illustrated in FIGURES 1 & 2. This new experimental approach involves two components [1]. First, the DNA to be analyzed is diluted into multi-well plates with one template molecule per two wells (on average) and PCR is performed in optimal conditions designed to amplify a single copy of PCR template. The amplicons are hybridized with fluorescence probes, such as molecular beacons, that allow detection of sequence-specific products using different fluorophores. Thus, digital PCR is employed to directly count, one by one, the number of each of the two (paternal vs. maternal or wild type vs. mutant) alleles in the samples. Second, several statistical analyses, including Bayesian-type likelihood methods, can be applied to measure the strength of the evidence for the allele distribution being different from normal [1]. This approach imparts a rigorous statistical basis to analyze allelic status and is expected to provide more reliable information than heretofore possible in allelic studies of tissue or body fluid samples. Therefore, digital PCR transforms the exponential and analog signals of conventional PCR to linear and digital signals.

To perform digital PCR, genomic DNA samples from tissue or body fluid are diluted in 384-well PCR plates so that there will be, on average, approximately 0.5 template molecules (genomic equivalent) per well. The optimal dilution of DNA samples can be achieved by DNA quantification kits to determine the amount of genomic equivalents in the original samples. As the PCR products from the amplification of single template molecules are homogeneous in sequence, a variety of conventional techniques could be used to assess their presence. Fluorescent probe-based reagents, which can be

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Table 1. Examples using digital PCR for molecular analysis in clinical samples.

Application	Findings	Ref.
Detection of *KRAS* mutations in stool	*KRAS* mutations can be detected and quantified in stool DNA samples from colorectal cancer patients	[1]
Detection of *KRAS* mutational status	Low- and high-grade ovarian serous carcinoma develop through independent pathways	[18]
Analysis of *KRAS* and AI in APC genes	Mutations in *KRAS* and AI of APC occur in appendiceal adenomas	[12]
Detection of AI and *KRAS* mutations	High-grade ovarian serous carcinoma contain wild type *KRAS* and a high frequency of AI, even in small primary tumors	[17]
Detection of LOH in APC locus	Development of adenomatous polyps may proceed through a top-down mechanism	[37]
Detection of AI of chromosomes 1p, 8p, 15q and 18q	Evidence of AI occurs in early colorectal tumors	[6]
Detection of AI of chromosome 18q	AI of 18q is associated with vascular invasion in colorectal carcinomas	[5]
Detection of AI of chromosomes 8p and 18q	AI of 8p and 18q is a better predictor of prognosis than histopathological stage in colorectal cancer patients without metastasis	[19]
Detection of AI using 8 SNP markers with high frequency of allelic loss in ovarian cancer	AI can be detected with high specificity and sensitivity in plasma DNA samples from patients with ovarian cancer	[31]
Detection of AI using 7 SNP markers in ovarian, colorectal and pancreatic cancers	Detection of AI can be a useful adjunct for the detection of cancer in ascitic fluid	[32]
Detection of *BAT26* alterations in fecal DNA	Presence of *BAT26* mutations in fecal DNA provides a promising marker for colorectal cancer screening	[34]
Quantitative detection of APC gene expression	Small changes in expression of APC gene affect predisposition to familial polyposis coli	[35]

AI: Allelic imbalance; APC: Adenomatous polyposis coli; LOH: Loss of heterozygosity; SNP: Single nucleotide polymorphism.

used directly on the PCR products in the same wells, are particularly well suited for this purpose [2]. Currently, molecular beacons are extensively used to detect the PCR products in digital PCR assays [3]. For mutational analysis, a pair of molecular beacons is designed with one hybridizing to the wild type sequence that harbors the mutation and the other hybridizing to the neighboring sequence (FIGURE 1). Therefore, the mutational status of a specific allele in a well is determined by the ratio of fluorescence intensity of the two beacons in that particular well. As multiple wells are counted, digital PCR can be used to detect mutations present at relatively low levels in the samples to be analyzed. The sensitivity of mutation detection depends on the number of wells that are included for analysis and the intrinsic mutation rate of the polymerase used for amplification. For assessing allelic imbalance, single nucleotide polymorphisms (SNPs) are used to represent the paternal or maternal alleles. A pair of PCR primers and a pair of molecular beacons are designed for each SNP (FIGURE 2). Digital PCR is performed using a SNP marker for which the patient is heterozygous. The resultant PCR products are then analyzed using molecular beacon probes to determine allelic representation. The mechanism of how molecular beacons discriminate between maternal and paternal alleles is briefly summarized. Molecular beacons are single-stranded oligonucleotides which contain a fluorescent dye and a quencher on their 5′ and 3′ ends, respectively (FIGURE 1). Both beacons are identical except for the nucleotide corresponding to the SNP and the fluorescent label (green or red). Molecular beacons include a hairpin structure, which brings the fluorophore closer to the quencher, and do not emit fluorescence when not hybridized to a PCR product [4]. Upon hybridization to their complimentary nucleotide sequences, the quencher is distanced from the fluorophore, resulting in increased fluorescence. Therefore, the ratio of fluorescence intensity of two allele-specific beacons with either green or red fluorescence is calculated to determine the allele type in one PCR reaction (well). With hundreds or thousands of wells (reactions) counted, the percentage of mutant alleles or the ratio of maternal and paternal alleles can be determined. For allelic status, a rigorous statistical method is then used to conclude whether allelic imbalance is present in the background of normal DNA [5,6].

Applications of digital PCR

Mutational analysis

For a variety of basic research and clinical applications, the identification of rare mutations is very important. Analysis of the early effects in tumorigenesis often depends on the ability to detect small populations of mutant cells [7,8]. Reliable technology to demonstrate the presence of mutations in clinical specimens holds great promise for cancer detection, as mutations represent a molecular genetic hallmark of neoplastic diseases.

To address whether digital PCR is useful for mutation detection in cancer, Vogelstein and Kinzler have analyzed the DNA from stool specimens in patients with colorectal cancer [11]. Their study focused on the *KRAS* gene mutation, which is a frequent molecular genetic event in colorectal cancer [9,10]. As the stool DNA is pool DNA released from a mixed-cell population including both normal and tumor cells, approximately 1–10% of the *KRAS* genes purified from stool contained mutant alleles [11]. Therefore, digital PCR appears a well-suited technique to assess the presence of mutated *KRAS* gene in stool. A 384-well digital PCR experiment was established to include positive controls (48 of the wells contained 25-genome equivalents of DNA from normal cells) and negative controls (48 wells without DNA template). The other 288 wells contained an appropriate dilution of stool DNA. In this study, molecular beacon red fluorescence indicated that 102 of these 288 experimental wells contained PCR products, whereas the other 186 wells did not. The red/green ratios of the 102 positive wells suggested that five contained mutant *KRAS* alleles. To determine the nature of the mutant *KRAS* genes from stool in the five positive wells, the PCR products were sequenced directly to reveal Gly12Ala mutations (GGT to GCT at codon 12) in four of them, whereas the sequence of the other indicated a silent C>T transition at the third position of codon 13. This transition presumably resulted from a PCR error during the first productive cycle of amplification from a wild type template. Thus, approximately 4% (4/102) of the *KRAS* alleles present in this stool sample contained a Gly12Ala mutation. The mutant alleles in the stool presumably arose from the colorectal cancer of the patient, as direct sequencing of PCR products generated from DNA of the cancer identified the identical Gly12Ala mutation [11].

In another study, digital PCR has been used to identify *KRAS* mutations in paraffin tissues of appendiceal mucinous adenomas in identical twins [12]. One of the twins suffered from a rare disease called pseudomyxoma peritonei (PMP), which produces an overwhelming amount of mucin in the intra-abdominal cavity as a result of the rupture of the appendiceal mucinous tumor. As the mucinous adenoma is a single layer of neoplastic cells embedded in abundant stromal cells and mucin, traditional methods, such as direct nucleotide sequencing, may not be sensitive enough to detect *KRAS* mutations, even when laser capture microdissection is employed to enrich the tumor cell population. In this study, the tumor tissue on paraffin sections was dissected under an inverted microscope and genomic DNA was purified and subjected to digital PCR. The study demonstrated that identical *KRAS* mutations were detected in the appendiceal adenoma and peritoneal tumor from the twin with PMP, whereas the adenoma from the other twin harbored a different mutation. The *KRAS* mutational analysis supported the view of the authors that PMP is clonally derived from the associated appendiceal mucinous adenoma. The different types of mutations in *KRAS* in the tumors from both siblings suggested that mutation in *KRAS* occurs somatically in adenomas and is independent of the identical genetic background of the twins.

Assessing allelic imbalance in tissue

Genetic instability is a molecular signature of most human cancers [13] and at the molecular level is characterized by allelic imbalance (AI), representing losses or gains of defined chromosomal regions. Analysis of AI is important in elucidating the molecular basis in the development of cancer. There are, however, at least two major problems associated with the current methods for assessing AI in tissue sections using microsatellite markers. First, DNA purified from microdissected tissues is always a mixture of neoplastic and non-neoplastic DNA and the latter, released from non-neoplastic cells, can mask AI because it is difficult to quantify the allelic ratio using microsatellite markers. Second, such DNA is often degraded to a variable extent, producing artifactual enrichment of smaller alleles when microsatellite markers are used for analysis [14]. Thus, digital PCR promises to overcome these technical difficulties associated with the molecular genetic analysis of AI in which the paternal or maternal alleles within a plasma DNA sample are individually counted, thereby allowing a quantitative measure of such imbalance in the presence of normal DNA. Statistical methods are used to evaluate the strength of the evidence for loss of heterozygosity in each tumor sample. Currently, the sequential probability ratio test (SPRT) is used to

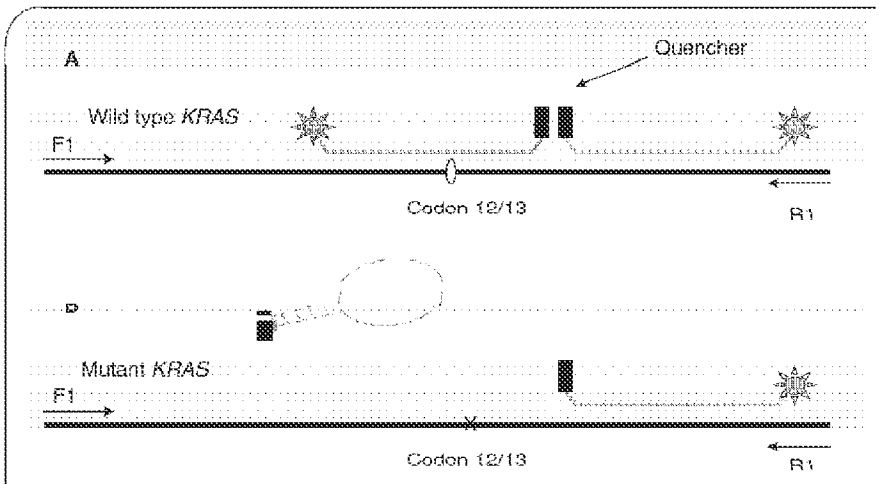

Figure 1. Design of molecular beacons and PCR primers for digital PCR analysis to detect *KRAS* mutations. Forward (F1) and reverse (R1) primers amplify exon 1 of the *KRAS* gene containing the relevant codons 12 and 13. Asymmetrical amplification generates single-stranded DNA complementary to the molecular beacons by using excessive R1 primer. The green beacon recognizes the common sequence in both wild type and mutant, while the red beacon only recognizes wild type sequence containing codons 12 and 13. Therefore, both red and green fluorescence is detected in wild type DNA but predominant green fluorescence is detected in PCR products with mutations at and around codons 12 and 13 as a result of mismatched hybridization.

conclude the presence of AI in tumor tissues [15]. SPRT allows two probabilistic hypotheses to be compared as data accumulate and, on average, guarantees a smaller amount of testing for a given level of confidence than any other method. Hypothesis one is that a sample has no loss of heterozygosity (LOH), that is, the tumor cells have the same proportion of alleles as normal cells. This corresponds to p = 50%, where p is the proportion of either allele in the overall sample. Hypothesis two is that the same one of the two alleles is absent in every tumor cell. This does not correspond to an allelic proportion of 100% in the tested sample because isolation of pure tumor cell populations from human tumors is almost impossible for routine samples. Given the conservative assumption that at least 50% of the DNA from the microdissected samples originated from neoplastic rather than non-neoplastic cells, the hypothesis of LOH corresponds to the probabilistic hypothesis that the observed proportion would be at least 66.7%. A SPRT is therefore constructed to choose between the hypotheses p = 50% and p = 66.7%, with a threshold likelihood ratio of 8. Generally, for each case, the number of alleles studied in each sample is plotted on the abscissa and the ratio of wells containing the allele with the higher counts to total number of wells containing either allele is plotted on the ordinate. Samples represented by points above curve one are interpreted to have allelic loss, meaning that the likelihood ratio for p = 66.7% versus p = 50% exceeds 8. The samples below the bottom curve are categorized as having no LOH. Indeed, it has been shown that AI can be demonstrated in a much higher percentage of colorectal carcinomas using digital SNP analysis than the traditional method using microsatellite markers [5,16].

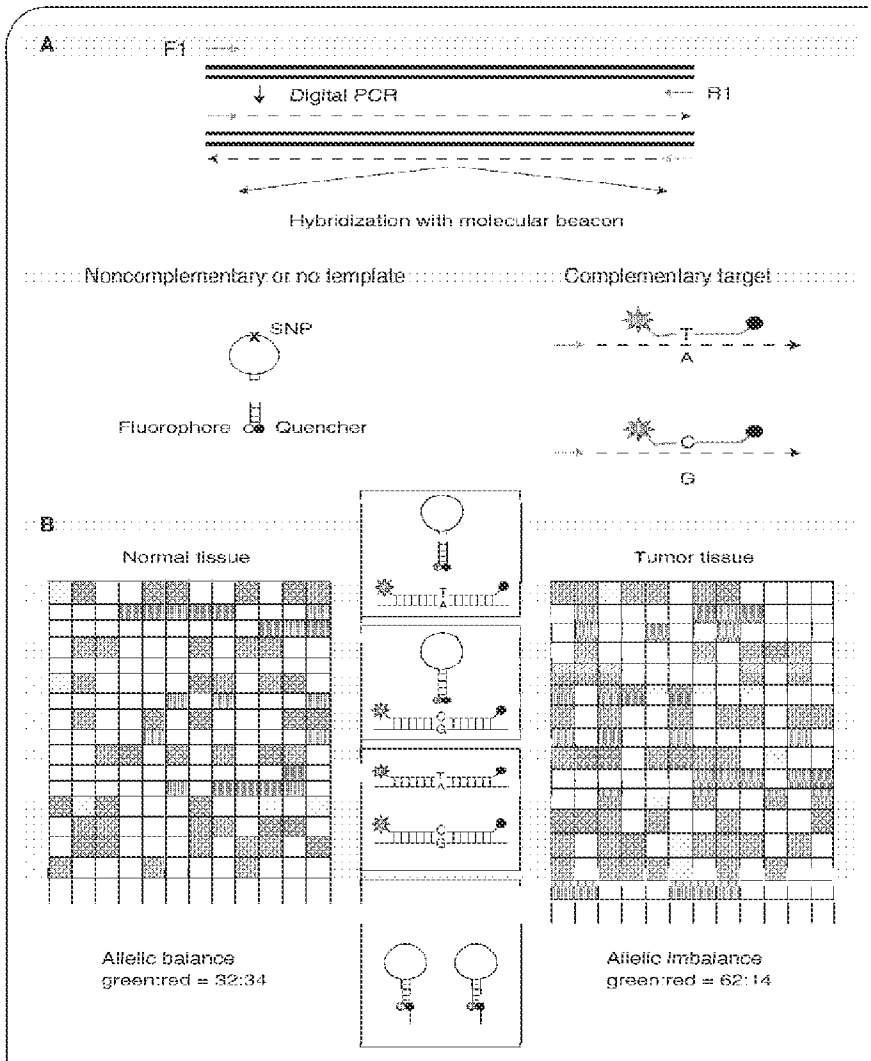

Figure 2. Digital PCR analysis to assess allelic imbalance. (A) Molecular beacon design. A pair of primers are designed to amplify approximately 100 bp of PCR product that contains a single nucleotide polymorphism (SNP) marker in the center region. The two beacons used for analysis of a specific SNP are identical except for the base pair corresponding to the SNP and the fluorescence label. Green and red represent fluorescein and hex labels, respectively. The molecular beacons do not emit fluorescence when not hybridized to a PCR product, as the 3'-dabcyl group (open circle) quenches the signals. Upon hybridization to their complimentary sequences, the quencher is distanced from the fluorophore, resulting in increased fluorescence. (B) Schematic illustration of a digital PCR analysis of the previous format. DNA from samples is distributed to a 384-well PCR plate. After completion of the PCR, molecular beacons are added to each reaction to determine allele status. Modified protocol has been used by combining digital PCR and allelic determination of molecular beacons in a single step [17].

Digital PCR has been used to characterize AI in small colorectal adenomas [6]. The investigators analyzed the allelic status in a total of 32 adenomas with an average size of 2 mm (range 1 to 3 mm). AI of chromosome 5q markers occurred in 55% of tumors analyzed, consistent with a gatekeeping role of the adenomatous polyposis coli (*APC*) tumor suppressor gene located at chromosomal position 5q21. AI was also detected in each of the other four chromosomes tested. The fraction of adenomas with AI of chromosomes 1p, 8p, 15q and 18q was 10, 19, 28 and 28%, respectively. Over 90% of the tumors exhibited AI of at least one chromosome and 67% had AI of a chromosome other than 5q. These findings demonstrate that AI is a common event, even in very small tumors, and led the authors to conclude that chromosomal instability occurs very early during colorectal neoplasia [6].

In another study, Singer and coworkers applied digital PCR to assess the pattern of AI during tumor progression in ovarian cancer [17]. This study demonstrated that a progressive increase in the degree of AI of chromosomes 1p, 5q, 8p, 18q, 22q and Xp was observed comparing serous

borderline tumors to noninvasive and invasive micropapillary serous carcinomas (low-grade serous carcinomas). In contrast, high-grade (conventional serous carcinoma) tumors had a high frequency of AI, even in small (early) primary tumors, similar to that found in advanced-stage tumors. Based on these findings, together with mutational analysis of *BRAF* and *KRAS* genes [17,18], Singer and coworkers proposed a dualistic model for ovarian serous carcinogenesis. One pathway involves a stepwise progression from serous borderline tumor to noninvasive and then invasive micropapillary (low-grade) serous carcinoma. The other pathway is characterized by rapid progression from the ovarian surface epithelium or inclusion cysts to a conventional (high-grade) serous carcinoma.

Zhou and coworkers have applied digital PCR to count alleles and use the presence of AI-specific chromosomal regions to predict recurrence of early-stage colorectal cancer [19]. They studied 180 colorectal cancer patients with no evidence of lymph node metastases or distant metastases at the time of surgery, and looked for the presence of AI on chromosome 8p and 18q in these tissue samples. They divided tumors into three groups: L tumors (n = 93) had AIs of chromosomes 8p and 18q, L/R tumors (n = 60) had AIs of either chromosome 8p or 18q but not both, and R tumors (n = 27) retained allelic balance for both chromosomes. Five-year disease-free survival was 100% for patients with R tumors, 74% for patients with L/R tumors and 58% for those with L tumors. These differences were significant and independent of other variables. The authors concluded that in patients without metastasis, AI was found to be a better predictor of prognosis than histopathologic staging.

Cancer detection in body fluid DNA

It is well recognized that tumors release a significant amount of genomic DNA into the systemic circulation, probably through cellular necrosis and apoptosis [20–22]. This tumor-derived DNA can be detected as a result of specific genetic and epigenetic alterations in the tumors, such as microsatellite alterations, translocations, mutations and aberrant methylation. As previously described, genetic instability is a defining molecular signature of most human cancers [13,23] and at the molecular level, it is characterized by AI, representing losses or gains of defined chromosomal regions. Thus, analysis of AI may also provide a molecular basis for cancer detection. Using microsatellite markers, AI has been demonstrated in the serum or plasma obtained from patients with lung [24], breast [25,26], renal [27] and ovarian cancers [28] and melanoma [29]. Some of these were small, early-stage neoplasms at the time of diagnosis, suggesting that detection of AI in plasma is a promising method for population-based screening [30]. Although these studies provide encouraging results, as with assessing AI in tissues, there are the two major problems of plasma DNA being a mixture of neoplastic and non-neoplastic DNA (so AI may be masked as it is difficult to quantify the allelic ratio using microsatellite markers) and DNA is degraded to a variable extent (producing artifactual enrichment of smaller alleles when microsatellite markers are used for analysis [14]). Therefore, detection of AI using digital PCR analysis may overcome these technical difficulties.

Chang and coworkers have performed digital PCR analysis to determine allelic status in plasma DNA and to evaluate the potential of this new technology for cancer detection using plasma samples [31]. This study first analyzed DNA concentration in plasma samples from 330 patients, including 122 patients with various cancers, 164 control patients with non-neoplastic disease and 44 individuals without apparent diseases. The area under the receiver-operating characteristic (ROC) curve for plasma DNA concentration was 0.90 for neoplastic versus healthy patients and 0.74 for neoplastic versus non-neoplastic patients. Given 100% specificity, the highest sensitivity achieved was 57%. Of the 330 patients, digital PCR analysis was performed on 54 ovarian cancer patients and 31 non-neoplastic disease controls. AI of at least one SNP in plasma DNA was found in 87% (95% confidence interval [CI]: 60–98%) of Stage I/II and 95% (95% CI: 83–99%) of Stage III/IV patients and none of 31 patients without neoplastic disease (specificity 100%, CI: 89–100%). For the 63 patients with serum CA125 data, DNA plasma concentration added information to serum CA125 levels by increasing the area under the ROC curve from 0.78 to 0.84. CA125 is the most commonly used tumor marker in ovarian cancer patients. Thus, measurement of plasma DNA levels may not be sensitive or specific enough for use as a cancer screening or diagnostic tool, even in conjunction with CA125. However, detection of AI in plasma DNA using the digital SNP analysis holds great promise for the detection of cancer.

Besides plasma DNA, Chang and coworkers also applied digital PCR analysis to AI in ascites fluid to assess the feasibility of this new technology in detecting malignant ascites [32]. Cytological examination of ascitic fluid is critical for clinical management in patients with peritoneal or pelvic diseases. Such morphologic examination can only achieve a sensitivity less than 62% and thus a molecular test that is able to distinguish benign versus malignant ascites could be clinically useful [33]. With digital PCR analysis, AI in at least one SNP marker was found in 19 of 20 (95%) ascitic fluid DNA samples obtained from patients with cytologically proven carcinomas in ascitic fluid. In contrast, AI was detected in only one of 20 patients with negative cytology. This latter patient with AI in her ascites had known Stage III ovarian carcinoma at the time of cytology sampling. The ascitic specimen of this patient demonstrated presence of carcinoma cells in culture with an identical AI pattern found in the ascitic supernatant and surgical specimen. These findings suggest that detection of AI using digital SNP analysis can be a useful adjunct for the detection of ovarian and other types of cancer in ascitic fluid.

Cancer detection in stool DNA

In addition to *KRAS* mutations, Traverso and coworkers have applied digital PCR to examine the alteration of a microsatellite marker, *BAT26*, in stool DNA from patients with proximal

cancers (located at the right colon) to determine the feasibility, sensitivity and specificity of this new approach [34]. Their study focused on patients with proximal cancers, reasoning that such cancers are difficult to detect since they are located most distally from the anus when colonoscopy is performed. Stool DNA was purified and subjected to digital PCR. The PCR products were sequenced to determine the status of *BAT26* (alterations/mutations vs. wild type). They found that 18 of 46 cancers had microsatellite alterations in *BAT26* and that identical mutations could be identified in the fecal DNA of 17 of these 18 cases. Among the cancer patients with proximal lesions, the clinical sensitivity of the *BAT26* fecal DNA test was 37%. In contrast, there were no positives among 69 individuals with normal colonoscopy findings or among 19 patients with adenomas. The specificity was therefore 100%. This study provides a promising new molecular diagnostic technique for colorectal cancer screening.

Quantification of gene expression of specific alleles

Yan and coworkers have recently applied digital PCR analysis to quantitatively measure gene expression of specific alleles (based on SNP) using cDNA as templates [35]. The principle is similar to that for genomic DNA. The study showed that constitutional 50% decreases in expression of one *APC* gene allele can lead to the development of familial adenomatous polyposis. Similarly, Pohl and coworkers have used the digital PCR assay to quantify the ratio of wild type and mutant *BRAF* gene expression in ovarian cancer [POHL G, UNPUBLISHED RESULTS]. This approach can provide a powerful and useful technique for assessing the success of (epi)genetic knockout of specific alleles (wild type or mutant), such as somatic knockout and siRNA.

Expert opinion

Digital PCR represents an example of the power of PCR and provides new opportunities for genetic analysis. This technique is especially powerful in experiments requiring the quantitative investigation of individual alleles in DNA samples isolated from a mixed-cell population.

There are several advantages of digital PCR compared with other types of PCR-based molecular genetic analyses. First, as compared with microsatellite markers, the PCR products derived from the two SNP alleles at every locus are the same size and therefore their analysis is not biased by the preferential DNA degradation of larger alleles. Second, the digital PCR approach, which amplifies single-allele templates in the PCR reaction, can precisely determine the number of alleles examined in each experiment. Accordingly, SNP genotyping is digital, involving the detection of the presence or absence of a specific allele, rather than analog, as microsatellite genotyping is, which measures the length of microsatellites [1]. Third, a statistical method such as SPRT can be employed to conclude whether AI is present in the background DNA.

Five-year view

Although the sensitivity of digital PCR analysis in the current 384-well format is usually satisfactory, higher sensitivity would be desirable. Sensitivity could be improved by analyzing more wells in the assay, although such an approach may not turn out to be cost effective. New technologies are being developed to perform digital PCR without using multiwell formats. For example, an innovative technology called the BEAMing (beads, emulsion, amplification and magnetics) method has been introduced [36]. In this method, each DNA molecule in a sample is converted into a single magnetic particle to which thousands of copies of DNA with the same sequence to the original are bound. This population of beads then corresponds to a one-to-one representation of the original DNA molecules. Variation within the primary population of DNA molecules can then be simply assessed by counting fluorescently labeled particles using flow cytometry. Therefore, millions of individual DNA molecules can be assessed at the same time. BEAMing can be used for the identification and quantification of rare mutations, as well as to study variations in gene sequences or transcripts in specific populations or tissues. Such innovative techniques for digital PCR are expected to emerge in the next few years, and they may provide another wave of new technologies to facilitate researchers in both basic and clinical science.

Key issues

- The study of DNA sequence variation is important for many areas of research. Current PCR format does not allow the identification and quantification of rare molecular genetic changes because conventional PCR amplifies a pool of DNA templates from the starting material.
- Digital PCR is used to amplify a single DNA template from minimally diluted samples, therefore transforming the exponential, analog signals from conventional PCR to linear, digital signals, allowing statistical analysis of the PCR products.
- Digital PCR has been applied in the quantification of mutant alleles and detection of allelic imbalance in clinical specimens, providing a promising molecular diagnostic tool for cancer detection.

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36 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. USA* 100(15), 8817–8822 (2003).

* **A new innovative technology to perform digital PCR in a powerful and cost-effective way.**

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Affiliations

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B Vogelstein, KW Kinzler - Proceedings of the National ..., 1999 - National Acad Sciences
Abstract The identification of predefined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. Here, we describe an approach for transforming the exponential, analog nature of the ...
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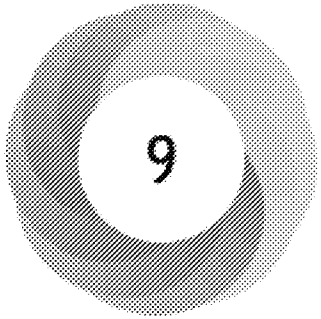

Digital PCR.

The Altmetric score is one measure of the quality and quantity of online attention that this article has received. You can read about how Altmetric scores are calculated here.

This article scored 8.96

The context below was calculated when this article was last mentioned on 8th April 2014

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In the 71%ile

So far Altmetric has tracked 22,318 articles from this journal. They typically receive a lot more attention than average, with a mean score of 13.5 vs the global average of 4.6. This article **has gotten more attention than average**, scoring higher than 71% of its peers.

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Older articles will score higher simply because they've had more time to accumulate mentions. To account for age we can compare this score to the 1,888,361 tracked articles that were published within six weeks on either side of this one in any journal. This article has done well, scoring **higher than 88% of its contemporaries**.

Other articles of a similar age in Proceedings of the National Academy of Sciences of the United States of America

In the 71%ile

We're also able to compare this article to 22,295 articles from the same journal and published within six weeks on either side of this one. This article **has gotten more attention than average**, scoring higher than 71% of its contemporaries.

All articles

In the 88%ile

More generally, Altmetric has tracked 2,255,664 articles across all journals so far. Compared to these this article has done well and is in the

Exhibit 5

Digital PCR Conference: Technologies and Tools for Precision Diagnostics - Day 1

Register by September 5 & SAVE up to $400!

Digital PCR's ability to precisely quantitate, identify mutations and copy number variations, and to perform gene expression analysis is creating waves across the diagnostics landscape. It is well suited for single-cell analysis and shows promise to become a superior tool in the clinic due to its capacity to work with small amounts of sample. The Third Annual Digital PCR event will bring together industry visionaries and early adopters to discuss digital PCR's capabilities, limitations, and future applications. Researchers will examine applications in cancer biomarker and rare mutation detection, non-invasive fetal DNA analysis, and infectious disease quantification, particularly in HIV. Novel digital PCR devices from startups and academic labs will be showcased. Other topics to be addressed include digital PCR integration with existing technologies, solutions for processing difficult samples as well as increasing sample throughput, and guidelines and best practices for digital detection.

Day 1 | Day 2 | Day 3 | Download Brochure

MONDAY, OCTOBER 6

8:00 am Short Course Registration and Morning Coffee

PRE-CONFERENCE SHORT COURSE

9:00 am - 12:00 pm

Digital PCR Experiment Design and Primer

Instructor: Jim Huggett, B.Sc. (Hons), Ph.D., Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC

This course will introduce the concept of digital PCR (dPCR) and explain how it compares to other molecular methodologies, citing both advantages and disadvantages. Included in the discussion will be the application of dPCR to perform minority target detection, absolute quantification and measurement of copy number variation as well as the analysis of RNA using reverse transcriptase dPCR. The discussion will also include some of the specific challenges associated with performing this technique.

* Separate registration required

12:00 pm Main Conference Registration

1:25 Chairperson's Opening Remarks
Stephen Bustin, BA(Mod), Ph.D., FSB, Anglia Ruskin University

» 1:30 KEYNOTE PRESENTATION:

Advancing Diagnostics with Digital Detection: From Quantifying Ultra-Rare Mitochondrial DNA Mutations to Profiling Tumor-Infiltrating Lymphocytes
Jason H. Bielas, Ph.D., Associate Member, Translational Research Program, Fred Hutchinson Cancer Research Center (FHCRC); Affiliate Assistant Professorship, Department of Pathology, University of Washington
In addition to precise quantification of nucleic acids, there are a number of advantages that digital PCR affords over other detection methods. I will discuss these advantages and review a number of novel digital PCR-dependent assays, which we have developed in our laboratory.

PCR MEASUREMENT AND ANALYSIS

2:00 Reverse Transcription Digital PCR
Stephen Bustin, BA(Mod), Ph.D., FSB, Professor, Molecular Medicine, Postgraduate Medical Institute, Faculty of Medical Sciences, Anglia Ruskin University
Digital PCR has potential to be useful for measuring RNA in cellular gene expression studies as well as in RNA diagnostic applications. We have assessed the reliability and linearity of the RT step and conclude that there are enzyme, target and concentration-dependent issues that require urgent attention.

2:30 Digital PCR Standards
Ross Haynes, Biological Science Technician, Biochemical Science Division, National Institute of Standards and Technology (NIST)

Digital PCR has many benefits, including lack of reliance on reference materials. IRMM was the first to certify a CRM with digital PCR. AD413 was certified to have a 1:1 ratio of HMG and MON810 genes on a plasmid construct. NIST later certified SRM 2366 for concentration using digital PCR. Standards and rational behind using them will be discussed.

3:00 Refreshment Break with Exhibit and Poster Viewing

3:45 Defining Reference Standards for Digital PCR and Demonstrating Their Application in Circulating Tumor Assay Development

Jonathan Frampton, Ph.D., Global Product Manager, Horizon Diagnostics

Horizon Discovery's patented gene editing technology (GENESIS™) has enabled the manufacture of genetically defined genomic DNA and FFPE reference standards. The reference standards used in this study include clinically relevant biomarkers (e.g. B-Raf, EGFR, K-Ras & N-Ras, etc.) and can be accurately manufactured to a specific allelic frequency (e.g. 0.01%, 0.1%, 1%, etc.). Here, we demonstrate the use of these reference standards to assess the performance of Digital PCR to detect rare mutations and copy number variants as well as their application in circulating tumor assay development.

4:00 Constellation - A High-Throughput Digital PCR Solution

Doug Roberts, Ph.D., Digital PCR Applications Scientist, Formulatrix Inc.

The benefits of digital PCR are well established; however barriers to widespread adoption remain including ease of use, high cost and low sample/assay throughput. We describe Constellation, a 96-well microplate based high-throughput digital PCR platform and performance data generated for a variety of applications.

4:15 Analysis and Visualization of Digital PCR Experiments – Applications of the dpcR Framework

Stefan Rödiger, Ph.D., Group Leader, InnoProfile, "Image-Based Assays", Brandenburg University of Technology

Recently, we started a unified cross-platform software frame-work, designated "dpcR." Our target user base is deliberately broad, including end users in clinics, academics, developers and educators. dpcR provides a means to understand how digital PCR works, to design and analyze experiments, and to spot potential troubles. Our framework is suitable for teaching and includes references for an elaborated methods set for dPCR statistics. The presentation will showcase customized applications of the dpcR package including a remote browser application and a standalone desktop application.

4:45 A Robust Framework for Digital PCR Data Analysis

Jo Vandesompele, Ph.D., CEO, Biogazelle; Professor, Ghent University

Digital PCR technology is maturing, but data analysis is still in its infancy. Here, we present our efforts to develop a robust mathematical framework for objective processing of this type of analog-digital data with inherent uncertainty. As proof-of-concept, we apply the framework for gene copy number analysis of circulating nucleic acids.

5:15 Welcome Reception with Exhibit and Poster Viewing

Day 1 | Day 2 | Day 3 | Download Brochure

Exhibit 6

Integrating Digital PCR Conference (April 11-12, 2013 - Boston, MA)

Conference Proceeding CD Now Available

Order Now

Single Copy - $350.00 Site License - $995.00

- Speaker Presentations
- Poster Abstracts
- and More!

Attendee Profile: Don't miss networking opportunities with thought leaders in the emerging dPCR space.

Advances in microfluidics and nanofabrication have led to the manufacture of technologies capable of utilizing hundreds to millions of small-scale partitions. Digital enumeration, whether it is done through digital PCR, microfluidics, or next-generation sequencing, is finding growing utility in both basic research and clinical applications. By allowing for detection of nucleic acids at higher resolution and lower target levels, digital detection technologies have the ability to identify diseases earlier in progression, providing an advantage for diagnostics and preventative medicine. Cambridge Healthtech Institute's Inaugural Integrating Digital PCR conference will cover digital PCR, microfluidics, and single-cell analysis and how these technologies are best used in a clinical setting. The evolution of qPCR to a more digital format will also be discussed. Special focus will be given to clinical translation and future trends driving the field.

Featured Presentations:

Use of Digital PCR in Oncology: Changing the Paradigm for Systemic Therapy Administration

Ben Ho Park, M.D., Ph.D., The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Digital PCR in Oncology

PODCAST

Extreme PCR: Efficient Amplification in Less than One Minute

Carl Wittwer, M.D., Ph.D., Professor, Pathology, University of Utah

The Evolution of PCR: Amplification in Under a Minute

PODCAST

Cambridge Healthtech Institute's Integrating Digital PCR will cover:

- Validating digital PCR for clinical use
- Novel digital PCR technologies and techniques
- Moving digital PCR to the clinic
- Single cell analysis methods
- Future trends driving the field

Dinner Short Course:

DIGITAL PCR: A TECHNOLOGY PRIMER

7:00 Dinner Buffet

7:15 Clinical Uses of Digital PCR and Measurement Uncertainty

[illegible]

Digital PCR can be used to detect dynamic changes in molecular load during treatment, identify resistant clones, and more. The presentation will begin with a brief introduction to the principles of digital PCR, followed by a discussion of the clinical applications that digital PCR is enabling, and the challenges that must be overcome to realize these applications.

8:15 Advanced Applications in Digital PCR

[illegible]

BIO-RAD

[illegible]

9:00 Close of Course

Separate registration required

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Digital PCR Conference: Technologies and Tools for Precision Diagnostics

October 7-9, 2013, San Diego CA

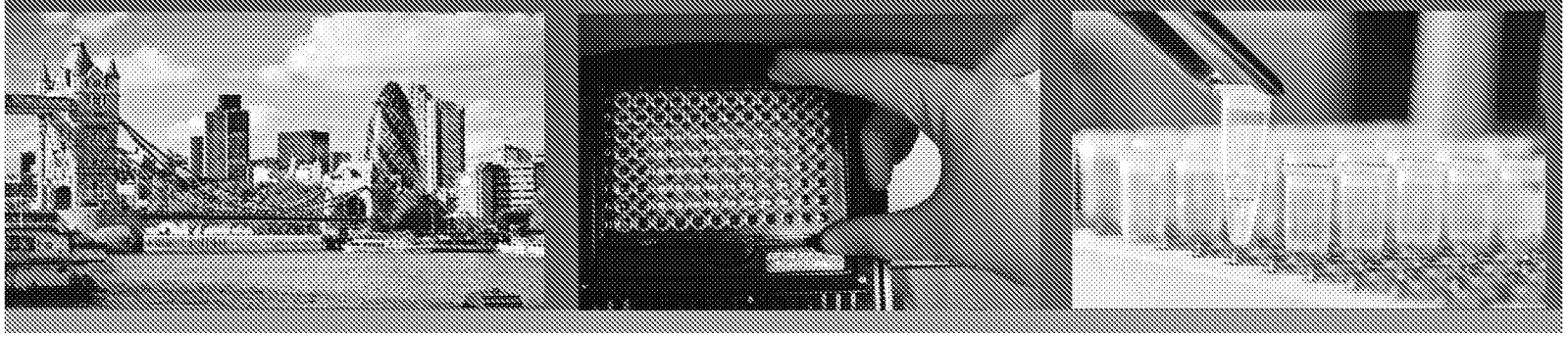
Digital PCR, at its core, is simply a single molecule counting method that quantitatively measures absolute DNA and eliminates the need for standard curves. However, it has already shown potential to be a disruptive technology in many areas of diagnostics. The Second Annual **Digital PCR** event will bring together industry visionaries and early adopters to discuss digital PCR's capabilities, limitations, and future applications. Researchers will examine applications in cancer biomarker and rare mutation detection, non-invasive fetal DNA analysis, and infectious disease quantification, particularly in HIV. Novel digital PCR devices from startups and academic labs will be showcased. Other topics to be addressed include digital PCR integration with existing technologies, solutions for processing difficult samples as well as increasing sample throughput, and guidelines and best practices for digital detection.

Scientific Advisory Board
Kerry R. Emslie, Ph.D., Bioanalysis Group Manager, National Measurement Institute, Australia
Jim Huggett, B.Sc. (Hons), Ph.D., Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC
Ross Haynes, Biological Science Technician, Biochemical Science Division, National Institute of Standards and Technology

» KEYNOTE PRESENTATION

1:30 Digital PCR: A Paradigm Shift in Molecular Measurement
Jim Huggett, B.Sc. (Hons), Ph.D., Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC
Digital PCR (dPCR) offers a unique ability to perform sensitive molecular measurement without the need for a standard curve. By converting the real-time PCR analogue signal to a binary output, measurement is simplified considerably. This also results in improved precision, facilitating the measurement of smaller differences, and the format leads to more sensitive detection of minority mutations. This presentation will discuss the benefits as well as disadvantages of dPCR, discuss the digital MIQE guidelines and offer a prediction of how dPCR may impact molecular measurement in the future.

www.globalengage.co.uk/qpcr.html



2nd qPCR and Digital PCR Congress

DEVELOPMENTS & POTENTIAL OF qPCR & dPCR AS A TOOL FOR PROGRESSING MOLECULAR BIOLOGY RESEARCH

Global Engage is pleased to announce the 2nd qPCR & Digital PCR Congress, which will be held on October 20-21 in London, United Kingdom and co-located with our inaugural Synthetic Biology Congress.

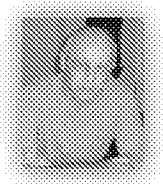
Bringing together over 200 industry & academic experts working in areas such as molecular biology/diagnostics, gene expression, genomics, biomarkers, pathogen detection, GMO, miRNA, NGS, bioinformatics and data management, the congress will examine the latest developments, opportunities and applications of both dPCR and qPCR through case studies across diverse areas such as oncology, infectious diseases, vaccines, prenatal diagnosis, diagnostic & clinical applications, microbiology, food microbiology, plant/ecology genomics and other novel applications.

With increasing numbers of real-time PCR users purchasing digital PCR due to its reduction in cost, absolute quantification, improved sensitivity, precision and greater robustness; and with the gene amplification market predicted to grow to $1.9 billion by 2015, this conference provides a timely opportunity to learn first-hand about dPCR whilst also keeping up to date with latest developments and strategies in qPCR.

The conference will provide an interactive networking forum to both further develop and answer your queries through a vibrant exhibition room full of technology providers showcasing their technologies and other solutions, poster presentation session, expert led case study presentations and interactive Q&A panel discussions from a 40 strong speaker faculty examining topics on four separate tracks covering:

Confirmed Speakers Include:

Stephen Bustin
Professor of Allied Health and Medicine, Anglia Ruskin University, UK



Emir Hodzic
Director, Real-Time PCR Research & Diagnostic Core Facility, University of California, Davis, USA

Lawrence Jennings
Assistant Professor, Pathology & Director, Molecular Pathology, Northwestern University & Robert H. Lurie Children's Hospital of Chicago, USA

Conference Synopsis

Day 1 – Stream One

Digital PCR: Possibilities & Opportunities

- Introduction, benefits, future development of dPCR
- Single cell analysis
- Converting to dPCR from qPCR
- Oncology (Mutant detection, diagnosis, monitoring therapy response, early relapse detection etc)Competition and collaboration committee
- Panel discussion: Future technologies for next generation dPCR/qPCR instruments
- Verifying accuracy of dPCR data
- Complimenting digital PCR with other technologies
  - o Microfluidic tools to compliment dPCR
  - o NGS v dPCR – Advantages over NGS
- Mutation analysis

Day 1 – Stream Two

qPCR: Strategies & Developments

- MIQE guidelines & standardisation
- qPCR assay design, optimisation & validation
- Sample preparation & quality control
- Amplification curve analysis/automated analysis of qPCR data/challenges & new solutions for data analysis

Day 2 – Stream One

qPCR & Digital PCR Case Studies

- Clinical/Diagnostic applications
- dPCR & its effect on patient care
- qPCR/dPCR in diagnosis of neurological disorders
- Prenatal Diagnosis
- Micro RNA/ncRNA/siRNA applications
- Infectious diseases
- Vaccines
- Molecular diagnostics

Day 2 – Stream Two

Plant, Food & Environmental Case Studies

- qPCR in Plant Research
- Plant/Ecology Genomics
- Detection And Identification Of Plant Pathogens
- Gene expression analysis
- qPCR in Food Research
- Genetically modified organism quantification in food

Confirmed Speakers

Stephen Austin
Professor of Allied Health and Medicine, Anglia Ruskin University, UK

Anders Ståhlberg
Senior Scientist, Department of Pathology, University of Gothenburg, Sweden

Bram Magnusson
Senior Scientist, Cellular Reagents and Assay Development, AstraZeneca, Sweden

Marios Ioannides
Researcher at the Translational Genomics Team, The Cyprus Institute of Neurology and Genetics, Cyprus

Angelika Beaver
Senior Scientist, BMGeo Wiesbaden Fertility Center, Germany

Moisés Egea Gutiérrez-Cortines
Professor of Genetics, ETSIA, Spain

Florian Schreiber
Leader Research Field, Innovative Detection Technologies and Assay Design, Fraunhofer IME, Germany

Joshua Yuan
Associate Professor and Director, Texas A&M Agrilife Synthetic and Systems Biology Innovation Hub, Texas A&M University, USA

Stefan Rödiger
Group Leader, BTU Cottbus – Senftenberg, Germany

Charlotte Guldberg Hyveld
Associate Professor, HemoDiagnostic Laboratory, Aarhus University Hospital, Denmark

Jan Koster
Principle Investigator, Department of Anatomy, Embryology & Physiology, Academic Medical Centre, Amsterdam University, The Netherlands

Valerie Taly
Group Leader/CNRS researcher, CNRS/Université Paris-Descartes, France

Deborah Morgan
Reader, School of Biosciences, Cardiff University, UK

Mario Capasso
Assistant Professor, University of Naples Federico II, Italy

Victor Turcanu
Senior Lecturer in Allergy, Kings College London, UK

Lawrence Jennings
Assistant Professor of Pathology ; Director, Molecular Pathology, Northwestern University/Ann and Robert H. Lurie Children's Hospital of Chicago, USA

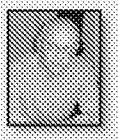
Pawel Bazan
Team Leader Microfluidics Team, KIST Europe, Germany

Martin Dufva
Associate Professor, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark

Mikhail Bogoi
Team Leader - Analytical Microbiology & Molecular Biology, Danone, France

Richard Oliver
Professor of Agriculture, University of Curtin, Australia

Jim Huggett
Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC, UK



Emir Hodzic
Director, Real-Time PCR Research & Diagnostic Core Facility, University of California, Davis, USA

Andrew deMello
Professor of Biochemical Engineering, Department of Chemistry and Applied Biosciences, ETH Zurich and Professor of Bioengineering, MRC Clinical Sciences Centre, Imperial College London

Jan Hellemans
Postdoctoral Fellow, University of Ghent, Belgium

Tony Godfrey
Associate Chair, Surgical Research, Associate Professor of Surgery, Boston University, USA; INSERM, France

Florent Mouliere
Postdoctoral Research Associate, Cancer Research UK Cambridge Institute, University of Cambridge, UK

Julie Pascale Young
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Peter Molloy
Senior Principal Research Scientist, Preventative Health Flagship, Commonwealth Scientific and Industrial Research Organisation, Australia

Justin O'Grady
Lecturer in Medical Microbiology, Norwich Medical School, University of East Anglia, UK

Alexander Pastmak
Researcher, Laboratory of Experimental Virology, Amsterdam University Medical Center, The Netherlands

Also:

Adam S. Corner, Senior Field Application Scientist, PathSense Technologies

David Rodríguez-Lázaro, Assistant Professor of Microbiology, University of Burgos, Spain

Yin-Lian Fang, Division Vice President, R&D, Abbott Molecular, USA

Aurita Menezes, qPCR Product Manager, IDT Technologies

Eduardo Gonzalez-Couto, Chief Strategy Officer, Integromics

Senior Representative, Life Technologies

Brian Lockhart, Director, Molecular Pharmacology & Pathophysiology, Servier, France

Anna Wilbrey, Senior Scientist, Pfizer, UK

Andrej-Nikolai Spiess, Group Leader, University Hospital Hamburg-Eppendorf, Germany

Claudia Paret, TRON - Translational Oncology at the Johannes Gutenberg University of Mainz, Germany

Steve Millington: New Business Development, OptiGene

Nicky Bauher, Product Development Manager, Arcogen

Tigst Demeke, Research Scientist, Canadian Grain Commission, Canada

Dev Mani Pandey, Associate Professor of Biotechnology, Birla Institute of Technology, Mesra, India

Jim White, Senior Field Application Scientist, NanoString Technologies

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08.00-08.30	Registration & Coffee
08.30-08.35	Global Engage Welcome Address & Chairperson's Opening Remarks Chairperson: TBC

08.00-08.35 Keynote Address:

Reverse Transcription Revisited

Reverse transcription (RT) continues to be a key procedure in the detection, quantification and sequencing of RNA. A detailed analysis of reverse transcriptase: (RTase:) published ten years ago identified the RT step as a major source of variability, with cDNA yields highly RTase- and target-dependent. This has serious implications for many commonly used techniques, including RT-qPCR, RT-dPCR, microarray analysis and whole transcriptome shotgun sequencing. Since there has been a huge expansion in the number as well as the quality of RTases and their associated buffers, we have taken a fresh look at the performance of a large number of commercially available RTases. We report our comparative findings with respect to their sensitivity, linearity and the variability associated with their use.

Confirmed:

Stephen Bustin, Professor of Allied Health and Medicine, Anglia Ruskin University, UK

	Digital PCR Congress	qPCR Congress
08.35-08.45	Stream Chair: TBC	Stream Chair: Mark Stevens, Head of Research and Development, PCR

08.45-10.10 Solution Provider Presentation:

How to Take Advantage of 10 Million Droplets Using Single Molecule Counting on the RainDrop™ Digital PCR System

RainDance Technologies' RainDrop™ digital PCR system is a highly sensitive tool for precise quantification of nucleic acids using standard probe-based reagents.

The RainDrop offers unique analytical advantages, including:

- Exceptionally high sensitivity without the need for standard curves
- Multiplex capability up to a 10 plex capacity
- Extensive application portfolio including DNA and RNA sample analysis, rare variant detection, viral DNA & RNA analysis and copy number determination

Platinum Sponsor

RainDance Technologies

Confirmed:

Adam S. Corner, Senior Field Application Scientist, RainDance Technologies

Solution Provider Presentation:

AccuCAL, A Novel Reagent for Absolute Quantitation of qPCR Products

- Accugen Pty Ltd have developed a novel method for quantifying nucleic acids for gene expression studies using qPCR without the need to normalize data to housekeeping gene(s) or to use a synthetic standard specific for each gene of interest.
- Our patented methodology is based on a universal reference calibrator, AccuCAL, to generate a calibration curve from which the absolute level of amplified target nucleic acid can be determined. This novel method eliminates any bias potentially introduced by the change in levels of housekeeping genes under different experimental conditions and also overcomes pipetting and dilution errors of standards prior to amplification. AccuCAL is not amplified during the reaction and is not part of the original experiment and is therefore not subject to the traditional problems and biases.
- AccuCAL can be used to accurately quantitate one or more amplicon(s) of 60 – 200bp in the same qPCR run. Furthermore, the setup of the reference calibrator is extremely simple, is easily implemented into existing user protocols, and we have customised software to make the analysis easy.

ACCUGEN

Confirmed:

Nicky Boulter, Product Development Manager, Accugen

10.10-10.35 **Detection of Circulating, Cell Free DNA in Plasma from Oesophageal Adenocarcinoma Patients using ThunderBolts™ DNA Sequencing and RainDrop™ Digital PCR.**

Detection and quantification of mutated, circulating tumour DNA in plasma or serum of cancer patients has great potential as a biomarker for cancer detection, treatment selection and measuring response to therapy. We have identified mutations in oesophageal adenocarcinoma specimens using ThunderBolts™ targeted DNA sequencing followed by sensitive detection of patient-specific mutations in serum/plasma using digital PCR on the Raindrop platform. Preliminary data will be presented to evaluate this approach for monitoring response to neoadjuvant therapy in patients with oesophageal adenocarcinoma.

Confirmed:

Tony Godfrey, Associate Chair, Surgical Research, Associate Professor of Surgery, Boston University, USA

Biomarkers and qPCR
Title To Be Confirmed

Confirmed:

Brian Lockhart, Director Biotechnology: Biomarker Research, Servier, France

10.35-11.45	Morning Refreshments and Poster Presentation Session Speed-to-Date Networking Meetings

11.55-12.10

Picoliter Droplet Digital PCR for Cancer Patient Follow-up

- Picoliter droplet digital PCR is a powerfull tool for highly sensitive and quantitative cancer biomarker detection.
- Multiplex procedures can be developed in picoliter droplet digital PCR.
- Picoliter droplet digital PCR based strategies could be designed to perform efficient detection of circulating tumor derived DNA in plasma of patients with cancer as well as to detect low frequency subclones in patient tumor tissues.
- Potential applications of such strategies will be presented and illustrated.

Confirmed:
Valerie Taly, Group Leader/CNRS Researcher, CNRS/Université Paris-Descartes, France

Title To Be Confirmed

Confirmed:
Yiu-Lian Fong, Division Vice President, R&D, Abbott Molecular, USA

12.10-12.35

Tumor Heterogeneity at the Single-cell Level

- We will demonstrate how multi-analyte analysis in single-cells can add value to our understanding of tumor heterogeneity.
- We will discuss the potentials and drawbacks of single-cell analyses in understanding tumor heterogeneity.
- We will show how subpopulations in myxoid liposarcoma and breast cancer can be identified and how these data can add value to our understanding of these tumour entities.

Confirmed:
Anders Ståhlberg, Senior Scientist, Department of Pathology, University of Gothenburg, Sweden

Single Cell Quantitative Real-Time PCR: An Insight into Directed Neural Differentiation

Differentiation of embryonic stem cells down a specific lineage can often produce mixed populations of cells that are difficult to dissect using whole population analyses. Here, we report single cell qPCR following a timecourse of embryonic stem cells differentiated towards anterior neuroectoderm. This differentiation protocol uses small molecule inhibitors and animal-free components to make it more clinically relevant. This data shows reduced expression of the pluripotency markers NANOG and POU5F1 during the first 2 days, followed by upregulation of the neuroectoderm marker PAX6, as well as brain and eye field markers. Single cell analysis allows us demonstrate that the heterogeneity of the population increases with time and to dissect sub-populations that would not be apparent by whole population qPCR analysis.

Confirmed:
Anna Wilbrey, Senior Scientist, Pfizer, UK

12.35-13.00

Tools n' Source Code for qPCR and dPCR Experiments

The precise quantification of nucleic acids is an essential element of bioanalytics. Methods and strategies based on DNA microbeadarray, isothermal amplification, PCR and related technologies are dominantly used. In particular, combined array-like formats are used as more rapid, cost-effective, sensitive and multiplexed methods for precision medicine. We developed the highly versatile fluorescence microscope-based "VideoScan platform" and novel probe system for the analysis of several genes of interest. My talk will show the developer's perspective on new multiplex microbead-based quantitative PCR and digital PCR technologies and the importance of open computer programs. I will describe how a single platform can make the transition from pure endpoint quantification to highly multiplex quantitative PCR and chamber-based droplet digital PCR.

Confirmed:
Stefan Rödiger, Group Leader, BTU Cottbus – Senftenberg, Germany

Phenotypic Screening on Human Pre-adipocytes with Quantitative Real-time PCR

In the pharmaceutical industry, the use of reporter gene assays in transformed cell lines have been extensively used. However, the utilization of such "artificial" models have been questioned, and screening assays with higher biological relevance has been desired. In this talk, I will present a phenotypic screen on human pre-adipocytes, where transcription of a gene-of-interest is analysed with quantitative real-time PCR (qPCR). The workflow does not involve any RNA purification, and will be further described in the talk. Assay development and data analysis will also be briefly discussed. In addition to the primary qPCR assay, we have developed an orthogonal protein-based assay, as well as a functional assay. The level of agreement between these readouts will be discussed. Finally, findings from the screening activities will be presented and put in their biological context.

Confirmed:
Björn Magnusson, Senior Scientist, Cellular Reagents and Assay Development, AstraZeneca, Sweden

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13.00-13.30

Solution Provider Presentation:

NanoString Technologies - the bridge to the Clinic for DNA and RNA Biomarkers

The nCounter System from NanoString Technologies, uses direct single molecule imaging with molecular barcodes to multiplex and detect up to 800 targets in a single reaction direct from total RNA, cell lysates, FFPE, LCM or single cell samples. The assay technology captures and labels target nucleic acid biomarkers, through a multiplex hybridization process, providing a simple workflow, with minimal pipetting, enhancing the precision and reproducibility of peer-to-peer analysis. The output is digital, one molecule one count, with a typical five-log dynamic range with a 50ng input of total RNA. The system supports applications for DNA copy number analysis, ChIP-String-epigenetic analysis, gene expression analysis and miRNA profiling. The nCounter System has proven capability with FFPE samples, as a tool to profile gene signatures in clinically relevant samples. With the potential to accelerate the development of companion diagnostics for disease profiling with increased statistical power.

nanoString TECHNOLOGIES

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Senior Representative, NanoString Technologies

Solution Provider Presentation:

Title To Be Confirmed

Agilent Technologies

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Confirmed:
Senior Representative, Agilent Technologies

13:30-14:30	Lunch	
14:30-14:55	**Why We Need MIQE Guidelines for Digital PCR** • Present the rationale behind the dMIQE guideline; • Detail some of the advantages dPCR has over other molecular methods • Discuss (with examples) some of the sources of error when performing dPCR • Offer a prediction of how dPCR may impact on molecular measurement in the future Confirmed: **Jim Huggett, Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC, UK**	**Fluidigm-Based Expression Analysis for the Identification of Relevant Transcripts for Immunotherapy** Confirmed: **Claudia Paret, TRON - Translational Oncology at the Johannes Gutenberg University of Mainz, Germany**
14:55-15:20	**Detection of Genetic Alterations by Digital PCR and TAm-seq on Circulating Tumour DNA** • Circulating tumour DNA (ctDNA) is a non-invasive source of tumour material and it is released in large proportions in the bloodstream of cancer patients. ctDNA exhibits some genetic alterations from its tumour of origin, and can be used as a "liquid biopsy" for monitoring tumour progression. • Digital PCR (dPCR) and its derivatives open the way to highly sensitive detection of point mutations on ctDNA, which are essential for the detection of early stage patients and for the follow-up of therapeutic efficiency. • Plasma ctDNA levels of somatic genetic alteration are assessed with dPCR and tagged-amplicon deep sequencing. Rare mutations driven to therapeutic resistance are identified by TAm-seq and the quantification and the follow-up of the ctDNA concentration exhibiting this mutation will be done by dPCR. Confirmed: **Florent Mouliere, Postdoctoral Research Associate, Cancer Research UK Cambridge Institute, University of Cambridge, UK**	**Amplification Curve Analysis and qPCR Reaction Quality Control** A recent comparison of qPCR data analysis methods showed that some of the amplification curve analysis methods perform better than the classic standard curve approach on indicators like variability and sensitivity. In this comparison the ability of amplification curve analysis to analyse characteristic of the amplification curve, and thus do assessment of curve quality remained under-exposed. We compared several datasets with varying concentrations of cDNA and primers, various master mixes, different amplicons and PCR cycling programs. Gel electrophoresis and melting curve analysis were used to check the PCR product and thus validate the PCR reaction. Thereafter, we used LinRegPCR to determine the following characteristics of the amplification curves: baseline, plateau and PCR efficiency as well as the (relative) target quantity. The resulting data were analysed to identify the relation between input parameters and the quality of the amplification curves. The results of this analysis shows that such amplification curve analysis can be serve to objectively assign quality marks. This will help in the decision making during assay development and optimization, as well as during data analysis. Confirmed: **Jan Ruijter, Principle Investigator, Department of Anatomy, Embryology & Physiology, Academic Medical Centre, Amsterdam University, Netherlands**
15:20-15:45	**Twenty Years of Microfluidic PCR: An Academic Curiosity of Indispensable Tool?** My talk will assess the impact that microfluidic technology has had on the Polymerase Chain Reaction. I will describe how microfluidics tools have been used to improve the efficiency, speed, throughput and application of the reaction in molecular biology. In addition, I will introduce the concept of flow segmentation and describe how this method has been successfully used to perform digital and single copy PCR in ultra-high throughput Confirmed: **Andrew DeMello, Professor of Biochemical Engineering, Department of Chemistry and Applied Biosciences, ETH Zürich and Professor of Bioengineering, MRC Clinical Sciences Centre, Imperial College London**	**Surprising Structural Features in the Baseline Region of qPCR Curves** In recent years, many methods have been developed for reliable quantification of qPCR experiments. With the exception of some newly developed that are derived from recursive algorithms of PCR mechanistics, the majority of methods are based on quantification of parameters obtained at the exponential phase of PCR. Although it is known that the baseline region of the first cycles of qPCR has substantial impact on the accuracy of quantification, it is widely considered and generally termed as "noise". We interrogated four published and six self-created datasets with a large number of technical replicates and specifically analysed the first 10 cycles or the same cycle over all samples in respect to hidden structural features. To our astonishment, significant structural features pertaining to the hardware or chemistry could be isolated from this region using classical algorithms applied in signal analysis. We conclude that the baseline region should be viewed as an own entity harboring defined system-specific characteristics that potentially have an impact on qPCR analysis. Confirmed: **Andrej-Nikolai Spiess, Group Leader, University Hospital Hamburg-Eppendorf, Germany**
15:45-16:15	Solution Provider Presentation: **Title To Be Confirmed** life technologies A ThermoFisher Scientific Brand Platinum Sponsor Confirmed: **Senior Representative, Life Technologies**	Solution Provider Presentation: **Title To Be Confirmed** IDT INTEGRATED DNA TECHNOLOGIES Platinum Sponsor Confirmed: **Anita Menezes, qPCR Product Manager, IDT Technologies**
16:15-17:15	Afternoon Refreshments and Poster Presentation Session One-to-One Networking Meetings	

17.05-17.30	**Smart Phone Sized Real-Time PCR** Here we demonstrate a handheld, smartphone-sized device for real-time polymerase chain reaction (PCR). It is powered by a small battery capable of concurrently analysing four samples with volumes of 100 nl in less than 15 minutes. It fulfils the requirement of a handheld portable PCR system for point-of-care and field diagnostics. The system is programmed by a USB interface and the results are displayed on a LCD display. All components are enclosed in a computer numerical control (CNC) made housing resulting in size of a smartphone. The PCR is capable of performing four reactions a time consisting of a positive control, negative control and two actual samples. Confirmed: **Pavel Neužil, Team Leader Microfluidics Team, KIST Europe, Germany**	**qPCR Applications for Biomarker Discovery and Diagnostic Assays in Allergy** Allergies increased over the last few decades in the developed countries. As more countries adopt a 'westernized' lifestyle, their prevalence of allergic diseases also rises. Current allergy diagnostic tests are not always able to differentiate between allergic sensitisation and fully developed allergic diseases. Therefore there is an unmet need for identifying better diagnostic biomarkers. Since allergic diseases are mechanistically underlied by T helper cell, which can transfer the disease, several Th subset cells were purified and their gene expression was analysed using microarrays. Potential biomarkers were further monitored using qPCR in mononuclear blood cells. Confirmed: **Victor Turcanu, Senior Lecturer in Allergy, Kings College London, UK**
17.30-18.00	Panel Discussion: **Experiment Design - Future Technologies for the Next Generation dPCR/qPCR Instruments** Confirmed: **Jim Huggett, Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC, UK** Invite to: **Senior Representatives x4**	
18.00-18.15	**Small Company Showcase** For opportunities please contact Steve Hambrook at steve@globalengage.co.uk	**Small Company Showcase** For opportunities please contact Steve Hambrook at steve@globalengage.co.uk
18.15-18.35	**An Alternative Fluidic Array Platform for Digital Assays** Digital assays methodology has large impact on the detection limit, dynamic range and ability to probe a sample deep for e.g. mutations. Here we present a simple to work with platform as alternatives to microfluidic droplet or valved arrays. The microfluidic chip creates millions of half droplets on a 2D surface in a few seconds using a simple pipetting step. The method is compatible with allele specific hybridization, sandwich immunoassays, in vitro transcription and translation reactions, cell growth and is currently explored for thermo cycling reactions. The chip can alternate solid phase reactions and solution phase reactions as needed. We expect that the platform will enable more labs to employ digital methods due to its flexibility and simple handling. Confirmed: **Martin Dufva, Associate Professor, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark**	**Title To Be Confirmed** Reserved: **Suzanne Drury, Translational R&D Scientist, Great Ormond Street Hospital for Children NHS Trust, UK**
18.35	Chairman's Closing Remarks	
18.35-19.35	Drinks Reception	

Co-Located with the Inaugural Synthetic Biology Congress

With a focus on Synthetic Biology in the healthcare/drug discovery and agricultural sector, this interactive meeting will provide the opportunity to take home cutting edge research, strategies, case study examples and methods to allow you to keep up to date with the latest advancements, novel methods and applications of Synthetic Biology within your field.

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For more information please contact Steve Hambrook, Conference Director, Global Engage Ltd.

08.30-08.40	Coffee & Networking Meetings
08.35-08.40	Stream Chair Welcome Address

08.40-09.10 Keynote Address:

Optimizing Droplet Digital PCR for Minimal Residual Disease Testing

The viability of 'personalized healthcare' relies heavily on the accurate quantification of minimal residual disease (MRD). For the detection of MRD, we have previously shown droplet digital PCR to have clear advantages as compared to conventional real-time PCR with regards to accuracy, simplicity and lower limit of detection and quantification. We are extending this work by addressing challenges we have encountered with other targets including background noise and the lack of traceable calibrators.

Confirmed:

Lawrence Jennings, Assistant Professor of Pathology; Director, Molecular Pathology, Northwestern University/Ann and Robert H. Lurie Children's Hospital of Chicago, USA

qPCR & Digital PCR Case Studies	Plant, Food & Environmental Case Studies
09.10-09.15 Stream Chair: TBC	Stream Chair: Dev Mani Pandey, Associate Professor of Biotechnology, Birla Institute of Technology, Mesra, India

09.15-09.45 Solution Provider Presentation:

New Frontiers and Versatility in Droplet Digital PCR

1. Bio-Rad's Automated Droplet Generator for QX200 Droplet Digital™ PCR system

Bio-Rad now offers an Automated Droplet Generator that works with both hydrolysis probes and EvaGreen fluorescence detection chemistries. Bio-Rad's Automated Droplet Generator simplifies the Droplet Digital™ PCR workflow, making digital PCR both scalable and practical. The AutoDG™ system prepares droplets for up to 96 samples at a time with minimal hands-on time required.

2. Direct Quantification of residual host cell DNA using Bio-Rad's ddPCR Supermix for Residual DNA

Many therapeutic protein and vaccines are manufactured using bacterial and mammalian host cells. Manufacturing processes are prone to leaving biological impurities derived from these cells, such as host cell DNA (HCD). The presence of HCD in drug substances poses safety concerns and HCD must be removed to ensure product quality and safety. Real-time quantitative PCR (qPCR) is currently the most commonly used technique to monitor residual HCD. Bio-Rad now introduces a highly precise and sensitive method for residual HCD quantification, without the need for DNA extraction, using Bio-Rad's QX200™ Droplet Digital™ PCR (ddPCR™) System.

3. Single Color Multiplexing Using Bio-Rad's EvaGreen-Based ddPCR

When performing intercalating dye reactions in qPCR, the quantification of multiple amplification products from one reaction mixture is inconceivable as only a single fluorescent measurement is made in each detection channel. However, with ddPCR™, variations in the fluorescence signal intensity of EvaGreen due to the mass of DNA present in each droplet can actually be utilized for multiplexed detection.

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Confirmed:

Pallavi Shah, Senior Scientist, Digital Biology Centre, Bio Rad Laboratories

Solution Provider Presentation

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09.45-10.10 **Comparison of RNA Sequencing with validated RT-qPCR Assays for All Human Genes**

The MIQE guidelines describe the assay parameters to be evaluated in the lab before giving green light on a qPCR assay for use in nucleic acid quantification studies. Wet lab validation of a qPCR assay appears to be a challenge or extra burden for many users. Here, we describe the procedures by which we have successfully completed the full wet-lab validation of RT-qPCR assays for all protein coding genes of the human and mouse transcriptome. One of the key improvements in our validation approach is the use of massively parallel amplicon sequencing for an unprecedented stringency on assay specificity. Following extensive validation, we have used all human assays (n=18,841) on the four MicroArray Quality Control (MAQC) RNA samples to obtain the first RT-qPCR based human transcriptome expression profile. The data reveal an impressive dynamic range with 90% of protein coding genes detected over a 20 million-fold range. In addition, this study enabled a thorough comparison of various quantification performance aspects of qPCR with those of microarrays and RNA sequencing obtained in the MAQC and SEQC studies, respectively. We will show how expression levels correlate between the different platforms and how the platform choice affects the achievable sensitivity.

Confirmed:

Jan Hellemans, Postdoctoral Fellow, University of Ghent, Belgium

Pooling of Fecal and Environmental Samples for the Detection of Salmonella spp. by qPCR

Several studies have evaluated the use of qPCR for the detection of Salmonella spp. in fecal and environmental samples. The purpose of this study was to evaluate the pooling of feces and environmental samples following a selective enrichment culture step for the detection of Salmonella spp. by qPCR. Equine fecal and environmental samples were collected at veterinary hospitals, inoculated into selenite broth and incubated overnight, then processed for DNA purification. Salmonella spp. qPCR assay targeting the invasion A gene was performed. The pooling strategy of collected samples incubated in selective enrichment broth was able to detect all culture and individual PCR positive samples. This strategy appears to be cost- and time-effective in a hospital environment with a low prevalence for Salmonella spp.

Confirmed:

Emir Hodzic, Director, Real-Time PCR Research & Diagnostic Core Facility, University of California, Davis, USA

10.10-11.00	Morning Refreshments and Poster Presentation Sessions One-to-One Networking Meetings

11.08-11.38 Solution Provider Presentation:
Title To Be Confirmed

Silver Sponsor

Confirmed:
Eduardo Gonzalez-Couto, Chief Strategy Officer, Integromics

Solution Provider Presentation:
Plant Pathogens Do Not Respect Borders: The Development of a Rapid 'In field' Solution

OptiGene Limited was formed in 2008. The goal was to harness the inherent advantages of isothermal amplification of DNA and RNA to enable testing at point-of-application. We have brought to market a number of innovative products that support sensitive and specific detection of bacteria and viruses for use in the fields of plant health, food safety, veterinary medicine, environmental monitoring and healthcare. Our expertise in both instrument design and enzymology has allowed us to develop a sophisticated open platform that will support all isothermal amplification methods. State-of-the art instrumentation is supported by reagents that offer very fast reaction rates. These products support rapid detection from crude samples allowing very simple preparation. Ultra-sensitive molecular detection that has been constrained to laboratory use by highly-qualified personnel and taking hours to complete can now be deployed to point-of-application and run with very little training, producing results in single minutes.

Gold Sponsor

Confirmed:
Steve Millington: New Business Development, OptiGene

11.38-11.55 **Using RTqPCR to Reveal Disease Mechanisms in Arthritis**

Osteoarthritis is a common, age-related disease that causes pain and disability and cannot be treated. Abnormal loading of joints causes osteoarthritis, but underlying disease mechanisms are unknown. We have used RTqPCR in various in vitro and in vivo models to relate gene expression to mechanical strain. We found that glutamatergic signals that respond to load also regulate pain, inflammation and degeneration in arthritis, providing a direct link between musculoskeletal biomechanics, disease symptoms and underlying mechanisms. Our current studies investigating mechanical regulation of miRNA expression in individual patients are revealing the limitations of what we can detect in real life.

Confirmed:
Deborah Mason, Reader, School of Biosciences, Cardiff University, UK

Molecular Approach to Food Microbiology Diagnostics: New Solutions for Old Challenges

Confirmed:
David Rodriguez-Lázaro, Assistant Professor of Microbiology, University of Burgos, Spain

11.55-12.20 **Comparison of Droplet Digital PCR and Seminested Real-Time PCR for Quantification of Cell-Associated HIV-1 RNA**

Cell-associated (CA) HIV-1 RNA is a sensitive marker for assessment of viral reservoir dynamics and antiretroviral therapy (ART) response in HIV-infected patients. We compared droplet digital PCR (ddPCR) and the seminested qPCR for quantification of CA HIV-1 RNA. Seminested qPCR showed better quantitative linearity, accuracy and sensitivity in the quantification of synthetic standards than ddPCR, especially in the lower quantification ranges. Both methods demonstrated equally high detection rate of unspliced HIV-1 RNA in patient samples on and off ART. No-template controls were consistently negative in the seminested qPCR, but yielded a positive ddPCR signal for some wells. Quantitative assays for CA HIV RNA have the potential to improve monitoring of patients on ART and to be used in clinical studies aimed at HIV eradication.

Confirmed:
Alexander Pasternak, Researcher, Laboratory of Experimental Virology, Amsterdam University Medical Center, The Netherlands

Advancing Analytical Microbiology with qPCR: Opportunities for Dairy Industry

- In dairy industry, microbes, such as fermenting microbes or probiotics, are used for their technological and/or for their health beneficial assets and are thus major components of the product
- A key requirement in dairy industry is that lactic acid bacteria (LAB) and probiotics used in yoghurt and fermented milks are alive through all the shelf life of the product. Some analytical standardized methods are available to quantify these beneficial bacteria but generally rely on culture-dependent methods that show poor discriminatory power to enumerate specifically each LAB and or probiotics present simultaneously in more and more fermented dairy foods
- This challenge can be faced with new alternative methods based on molecular biology (PCR) that can offer new analytical solutions to dairy microbiology.

Confirmed:
Mickaël Boyer, Team Leader - Analytical Microbiology & Molecular Biology, Danone, France

12.20-12.50 Solution Provider Presentation:
Title To Be Confirmed

Platinum Sponsor

Confirmed:
Senior Representative, Life Technologies

Solution Provider Presentation

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12.50-13.50 Lunch

13.55-14.15 **Finding the Needle in the Haystack – Pathogen DNA Enrichment Strategies for the Molecular Diagnosis of Sepsis**

- The importance of sepsis and challenges associated with diagnosis
- Trends in new molecular diagnostics tests and the role of pathogen DNA enrichment
- Comparison of pathogen DNA enrichment strategies and conclusions

Confirmed:
Justin O'Grady, Lecturer in Medical Microbiology, Norwich Medical School, University of East Anglia, UK

qPCR for Food Quality and Identification of Bioactive Components in Foods

qPCR may be used for researching both food quality and potential bioactive components in foods:

- Suppression of boar taint in meat has become an important issue as many European countries are banning castration of male pigs. qPCR was used to identify regulation of detoxification enzymes in the liver of importance for degradation of skatole, which is involved in the development of boar taint.
- Using qPCR, an up-regulation of the angiopoietin like 4 gene (ANGPTL4) was identified upon exposure of milk to a human colon cell line with possible implication for weight regulation. Further identification of the fraction and specific components in the milk responsible for this regulation was carried out using the qPCR technique in both cell and intestinal tissue from pigs.

co-authors: Søren Drud Nielsen, Trine Kastrup Dalsgaard, Marlo Krøyer Rasmusen and Bo Ekstrand

Confirmed:
Jette Feveile Young, Associate Professor, Aarhus University, Denmark

14.15-14.40 **Helper-Dependent Chain Reaction (HDCR) for Sensitive and Specific Detection of Cancer-Derived DNA**

- HDCR is an amplification technology that that uses a pair of oligonucleotides at an end of a target amplicon. The combined action of the oligonucleotides ensures selection for sequences within the target amplicon in every amplification cycle, providing enhanced specificity for detection of minimal amounts of target DNA
- We have combined HDCR with the use of methylation-dependent restriction enzymes (eg. GlaI) to develop assays for genes commonly methylated in cancers. Because these assays provide a positive amplification of methylated DNA without the requirement for bisulphite treatment they have significant advantages in clinical application to biopsy samples or for detection of circulating tumour-derived DNA.
- HDCR technology can also be applied for selective amplification of target mutations, such as in KRAS or BRAF genes.

Confirmed:
Peter Molloy, Senior Principal Research Scientist, Preventative Health Flagship, Commonwealth Scientific and Industrial Research Organisation, Australia

Analysing Zygosity by qPCR: An Innovative Approach for Plant Breeding and Generation of New Transgenic Crops

For the generation and propagation of new plant lines zygosity is an important criteria. High-yielding plant hybrids can only be obtained by crossbreeding of homozygous parental lines. But identifying the zygosity state of germplasms during plant propagation is crucial for the offspring generation of marketable hybrid seeds. Existing methods to determine zygosity in plants are rare, time consuming and error-prone frequently resulting in losses of up to 30% during seed production. Recently, we developed a novel qPCR based approach to facilitate routine zygosity determination in crop plants. Utilizing the novel and innovative qPCR approach we are now able to screen hundreds of individual plant samples in high-throughput and reliably identify the zygosity status of specific incorporated transgenes and/or specific conventional genomic trait loci.

Confirmed:
Florian Schröper, Leader Research Field: Innovative Detection Technologies and Assay Designs, Fraunhofer IME, Germany

14.40-15.05 **qPCR Methods to Improve Prediction of Cancer Prognosis**

Cancer is a leading cause of death worldwide and the prognostic evaluation of cancer patients is of great importance in medical care. qPCR methods have emerged as a highly sensitive method to identify appropriate genetic markers of cancer progression. In this talk, I will show different applications of this analytic method to predict the prognosis of different tumors. I will present i) our sensitive and fast qPCR technology to detect erbB2 copy number variations in the free DNA from plasma of esophageal carcinoma patients; ii) our cost-effectives qPCR quantification strategy of 18 genes for predicting survival in stage 4 neuroblastoma, easily applicable in routine laboratories, and minimal required amounts of RNA. This work demonstrates the clinical utility of qPCR methods and their potential in improving patient outcome.

Confirmed:
Mario Capasso, Assistant Professor, University of Naples Federico II, Italy

Use of qPCR to Identify Biases in Next-Generation Sequencing of Complex Samples

- Experiments of metabarcoding and metagenomics are based on the amplification and sequencing of complex samples comprising an unknown number of species. The hope is to identify and maybe quantify the biodiversity.
- We found that in metabarcoding experiments of plants the quantities could vary over 2000 fold between fragments for the same DNA sample. We found further evidence for biases caused by the amplification during the HGS sequencing process. We analysed the different steps using qPCR and found that Ct values could predict to some extent the degree of bias in the HGS process.
- Our work indicates that using qPCR including assays and the corresponding mathematical analysis will shed light on NGS projects helping to correct unavoidable biases caused by the amplification procedures

Confirmed:
Marcos Egea Gutiérrez-Cortines, Professor of Genetics, ETSIA, Spain

15.05-15.35 Afternoon Refreshments and Poster Presentation Sessions

15.35-16.00	**Quantification of Minimal Residual Disease in Haematological Malignancies by qPCR** • In haematological malignancies minimal residual disease (MRD) is defined as miniscule amounts of leukaemia or lymphoma cells residing in the patients either during treatment or at remission with no other clinical sign of malignancy • MRD is nowadays acknowledged as an important factor for risk stratification of the patients and numerous studies have shown that the kinetics of the disappearance of the malignant clone greatly influences the prognosis. In addition, an increase in MRD level can predict a forthcoming clinical relapse • High specificity and sensitivity of the qPCR assays used for quantification of MRD are mandatory in order to use this diagnostic approach in a clinical setting. The genetic targets vary from consensus sequences to patient specific sequences, which challenge the design of the qPCR assays. Confirmed: **Charlotte Guldberg Nyvold, Associate Professor, HemeDiagnostic Laboratory, Aarhus University Hospital, Denmark**	**Plant Science and qPCR** **Title To Be Confirmed** Confirmed: **Joshua Yuan, Associate Professor and Director, Texas A&M AgriLife Synthetic and Systems Biology Innovation Hub, Texas A&M University, USA**
16.00-16.25	**New Technologies to Increase the Multiplexing Degree and Reduce Costs of Digital PCR** In digital PCR only one target at a time per reaction compartment is amplified. This aids in multiplexing, since the depletion of reaction components due to competing reactions is less likely. Though some methods for multiplexing in digital PCR are already available, most of them bare disadvantages, e.g. the extensive probe design and associated costs. We aim to minimize the probe design complexity and costs. Two approaches will be presented. 1) an innovative hydrolysis-probe based method which can detect more than one target per color. 2) The mediator probe PCR, which uses label-free primary probes in combination with universal reporter oligonucleotides. Confirmed: **Simon Wadle, Head of Lab-on-a-Chip Nucleic Acid Analysis, Laboratory for MEMS Applications, IMTEK, University of Freiburg, Germany**	**Use of Droplet Digital PCR for Absolute Quantification of Genetically Engineered Traits** RT-PCR is the most widely used method for the detection and quantification of genetically engineered (GE) traits. Use of certified reference materials is essential for RT-PCR to generate standard curves and accurately determine the amount of genetically engineered material in given samples. Certified reference materials are not available for many GE events, and this poses a challenge for quantification with real-time PCR. In addition, accurate quantification of GE materials at low concentration levels is a challenge. In droplet digital PCR (ddPCR), thousands to millions of small droplets are generated from a PCR sample and absolute quantification is achieved by counting the number of positive amplifications. Reference material is not required for absolute quantification of GE material using dPCR. We explored the use of BioRad QuantaDrop™ dPCR for absolute quantification of GE trait in flax, canola and soybean. Improved separation of target and reference droplets was achieved by using sheared DNA. Absolute quantification of GE materials was achieved by combining reference and target primers and probes in a single reaction (duplex ddPCR). Successful quantification was achieved for GE samples spiked at 1%, 0.1% and 0.01% levels. Confirmed: **Yigzi Demeke, Research Scientist, Canadian Grain Commission, Canada**
16.25-16.50	**Assessment of Oocyte Ploidy with Digital PCR** Pregnancy rates after in vitro fertilization are notoriously low due to the high frequency of aneuploid oocytes. We analyze the chromosome and chromatid content of all chromosomes in the two oocyte by-products – polar body one and two – with digital PCR. This allows definition and selection of euploid oocytes for embryo transfer and increase of pregnancy and take home baby rates Confirmed: **Angelika Daser, Senior Scientist, SNGen Wiesbaden Fertility Center, Germany**	**Using NGS efficiently in probe design** A DNA probe to detect particular nucleic acid sequences should have optimized specificity and sensitivity. In a typical PCR detection project, the goal is to find PCR primers that amplify with maximum sensitivity the designed organisms (in-organisms) whilst all other genomes present in the sample (out-organisms) do not amplify or interfere. The flood of data from next-gen sequencing methodologies has created new opportunities to design optimized probes. Optimised sensitivity can be achieved using repetitive sequences from the in-genomes. However NGS methods are poor substrates for assembling repetitive region of genomes. We are developing an algorithm that utilises unassembled NGS reads from both the in and out organisms to detect sequences long enough for PCR or LAMP applications that are (1) unique to the in-organism and (2) multicopy. Progress in applying the technique to designing probes to detect fungal pathogens in leaf samples will be presented Confirmed: **Richard Oliver, Professor of Agriculture, University of Curtin, Australia**

16.58-17.15 **Non-Invasive Prenatal Diagnosis Using Digital PCR**

Non-invasive prenatal diagnosis has been gaining a lot of interest in the last few years. The presence of fetal DNA in the maternal circulation has allowed the development of genetic, genomic and epigenetic non-invasive prenatal diagnosis tests for the detection of aneuploidies and more recently microdeletion and microduplication syndromes. Most of these tests employ NGS in order to detect and quantify the minute differences in copy number resulting from the gain or loss of genetic material in the fetus. We will provide an overview of past and current efforts to employ digital PCR in non-invasive prenatal diagnosis.

Confirmed:

Marios Ioannides, Researcher at the Translational Genomics Team, The Cyprus Institute of Neurology and Genetics, Cyprus

Molecular Beacon Probe-Based Real Time PCR Assay for the Identification of Promoter Motifs in Rice

Differentially expressed genes (DEG) are regulated by interaction of transcription factors (TF) with promoter motifs. In anoxic rice coleoptile promoter motifs GCC box and mutated GCC box (TCC box) were predicted in co-expressed up-regulated and down-regulated genes, respectively. Subsequently, molecular beacon (MB) probe based qRT-PCR was performed to identify the presence of GCC and TCC box in the promoter of DEGs. The presence of GCC box in up-regulated-DEGs (Ubiquitol cytochrome C chaperon gene; *UCCC* and methyltransferase domain containing protein gene; *MTE*) and TCC box in DR-DEGs (the GAP domain containing protein gene; *RoGAP*) were validated. Therefore, MB probe based qRT-PCR enabled to identify the GCC box in UR-DEGs while TCC box in DR-DEGs.

Confirmed:

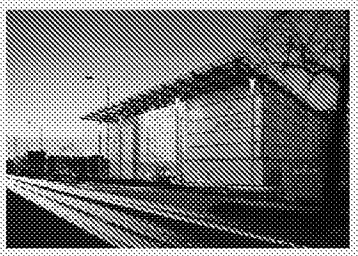
Dev Mani Pandey, Associate Professor of Biotechnology, Birla Institute of Technology, Mesra, India

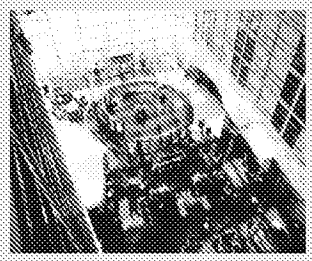
17.15-17.20 Chairperson's Closing Remarks & Conference Close

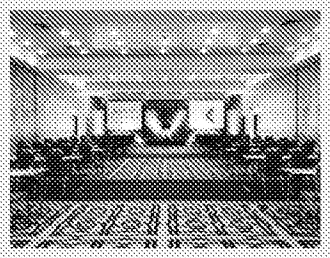
Venue

London Heathrow Marriott Hotel
Bath Road
Hayes, UB3 5AN
United Kingdom

A discounted group rate is available to all attendees. Details of how to book are available on registration. Space is limited and accommodation is available on a first come basis.







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tataabiocenter

2 Day Course: MIQE – How to get Good Quality Control in qPCR

Are you working with qPCR? Do you have control of the quality in all the steps of the analysis procedure? This course will go deep into the MIQE guidelines, describe the important steps in RNA and DNA analysis with qPCR and how you should work to fulfil the guidelines. To follow the guidelines will give you a better control of the quality of your results. The course is theoretical with a few practical computer-based exercises. The course content includes:

- **Background about the MIQE guidelines**
  - What are the MIQE guidelines?
  - Why are they important?
- **What to think about when doing experimental design**
- **How to do nucleic acid extraction and quality control of extracts**
  - Different approaches for nucleic acid extraction
  - How can we check the quality of extracted samples?
  - How do we identify inhibitory samples?
- **The Reverse Transcription reaction**
  - Different priming strategies for reverse transcription
  - Pros and cons for different strategies
- **How to do primer and probe design**
  - How to search for gene sequences
  - Which are the factors that affect design?
  - How do we avoid primer dimer formation?
  - Other important considerations for primer design
  - How to design hydrolysis probes
  - Practical exercises in primer design
- **How to optimize qPCR assays**
  - Which factors affect the PCR?
  - Which factors can be optimized?
- **How to validate qPCR assays, LOD and LOQ**
  - How to determine LOD and LOQ
  - Precision estimation
  - Which controls to use
- **Data analysis**
  - How does qPCR software process the data?
  - How to evaluate curves and set threshold
- **Normalization**
  - Different ways to normalize
  - How to find stable reference genes
- **How to do relative quantification**
  - Quantification methods and equations
  - How to interplate calibration
- **Absolute quantification strategies**
  - What is a suitable standard?
  - How to do absolute quantification

Key Information:

Location: **London Heathrow Marriott Hotel**

Date: **22-23 October 2014**

Cost:

Academia: **£495.00**

Industry: **£595.00**

Course Leader: Kristina Lind, Lead Instructor, TATAA Biocenter

Kristina Lind received her PhD in Molecular Biotechnology from Chalmers University of Technology, Gothenburg, in 2007. Her PhD project included development of the protein quantification technique Immuno-qPCR. She has held courses in TATAAs regime since 2005 and is now responsible for the course program and core facility at TATAA Biocenter. She is also leading a group that is involved in the commissioned services at the company and is project manager for a number of customer projects. As assistant Page 1 of 2 she is deeply involved in the quality control and how to work with qPCR according to the MIQE guidelines.

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The 2nd qPCR and Digital PCR Congress

20th October 2014 at 08:00 - 21st October 2014 at 17:20 (GMT+00:00) London

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Type:	Congress
Venue:	London Heathrow Marriott Hotel
Location:	Bath Road, Hayes, London Heathrow, United Kingdom
Website:	The 2nd qPCR and Digital PCR Congress
Phone number:	44(0)1865849841
Area	Life Sciences
Specialty	Molecular Cell Biology
Subject	Molecular Biology

Some 150 attendees came to the first qPCR & Digital PCR Congress, held in Lyon, France last year.

Building on the success of the first meeting, the 2014 qPCR & Digital PCR Congress will have more speakers and expanded coverage of developments in dPCR while also addressing the key advances in qPCR.

We are delighted that our new speakers include Stephen Bustin, Professor of Allied Health and Medicine, Anglia Ruskin University, UK and Jim Huggett, Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC Genomics, UK. Also returning to address the congress are Anders Ståhlberg, Senior Scientist, Department of Pathology, University of Gothenburg, Sweden and Valerie Taly, Group Leader/CNRS researcher, CNRS/Université Paris-Descartes, France

Introducing the Agenda for London 2014
Day One: 20 October, 2014
Stream 1 – Digital PCR Strategies & Application Case Studies – Clinical Applications
Stream 2 -- qPCR Strategies & Application Case Studies

Day Two: 21 October, 2014
Stream 1 -- qPCR & Digital PCR Case Studies
Stream 2 -- Plant & Food Case St

Deadlines
Abstract Deadline:
1st October 2014
Registration Deadline:
1st October 2014

Speakers

Stephen Bustin, Professor of Allied Health and Medicine, Anglia Ruskin University, UK
Anders Ståhlberg, Senior Scientist, Department of Pathology, University of Gothenburg, Sweden
Jan Ruijter, Principle Investigator, Department of Anatomy, Embryology & Physiology, Academic Medical Centre, Amsterdam University, The Netherlands
Jim Huggett, Science Leader, Nucleic Acid Metrology, Molecular & Cell Biology, LGC, UK
Emir Hodzic, Director, Real-Time PCR Research & Diagnostic Core Facility, University of California, Davis, USA
Andrew deMello, Professor of Biochemical Engineering, Department of Chemistry and Applied Biosciences, ETH Zürich and Professor of Bioengineering, MRC Clinical Sciences Centre, Imperial College London
Alexander Pasternak, Researcher, Laboratory of Experimental Virology, Amsterdam University Medical Center, The Netherlands
Tony Godfrey, Associate Chair, Surgical Research, Associate Professor of Surgery, Boston University, USA
Florent Mouliere, Postdoctoral Research Associate, Cancer Research UK Cambridge

Institute, University of Cambridge, UK
Valerie Taly, Group Leader/CNRS researcher, CNRS/Université Paris-Descartes, France
Björn Magnusson, Senior Scientist, Cellular Reagents and Assay Development, AstraZeneca, Sweden
Marios Ioannides, Researcher at the Translational Genomics Team, The Cyprus Institute of Neurology and Genetics, Cyprus
Adam S. Corner, Senior Field Application Scientist, RainDance Technologies
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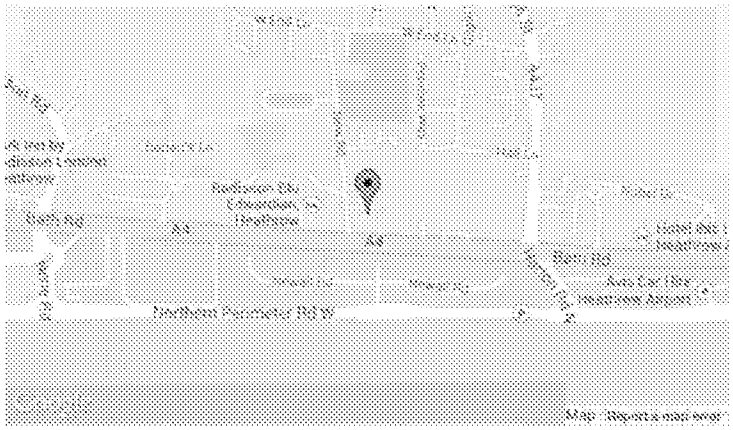

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REVIEW ARTICLE

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Advances in the research of fetal DNA in maternal plasma for noninvasive prenatal diagnostics

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Summary

Molecular analysis of fetal DNA present in the maternal circulation allows noninvasive, early, and precise determination of fetal genetic status in prenatal diagnostics. The most common clinical applications, i.e. prenatal gender determination and fetal RhD genotyping, are possible already in the first trimester using specialized protocols for DNA isolation from plasma and subsequent real-time PCR detection. Recent advances in molecular techniques enable other applications of fetal DNA purified from maternal plasma samples. Chromosomal abnormalities (e.g. trisomy 21) can be diagnosed by digital PCR, which offers higher accuracy in quantifying DNA sequences than standard real-time PCR. Digital PCR, but also MALDI-TOF, are suitable for detecting point mutations, widening the spectrum of applications to monogenic diseases. The ongoing lowering of costs for massively parallel sequencing might lead to replacement of most of the other currently used approaches. Adopting specialized protocols for the purification of fragmented circulating fetal DNA and improving the bioinformatic analysis of raw data can bring us closer to sequencing the fetal genome as the ultimate goal of prenatal DNA diagnostics, with wide-ranging medical applications. The discussion and solution of ethical issues beyond early fetal gender or paternity determination is hanging just behind the rapid technical progress of noninvasive prenatal DNA diagnostics.

key words: **circulating nucleic acids • maternal plasma • genetic digital PCR • next-generation sequencing • molecular prenatal diagnostics**

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Background

The discovery of the presence of cell-free fetal DNA in maternal plasma is on its way to revolutionizing prenatal molecular diagnostics [1]. It is the noninvasive, safe, and easy sampling approach compared with amniocentesis or chorionic villi sampling that makes the analysis of fetal DNA circulating in maternal blood so interesting. Numerous methodological hurdles must, however, be overcome in order to reduce or completely replace currently used invasive procedures in prenatal DNA diagnostics. Recent rapid advances and the development of new molecular techniques have made some of the hurdles disappear quickly.

The origin of fetal DNA in maternal plasma was a matter of debate for years. It is currently generally accepted that cell-free fetal DNA (cff DNA) originates from the placenta, especially from nucleosomes of particular cells [2]. It seems that these nucleosomes are parts of apoptotic bodies of trophoblasts from the placenta [3]. The quantification of fetal DNA concentration in plasma was even suggested for use as a marker of trophoblast status during pregnancy [4]. After delivery, fetal DNA rapidly disappears from the maternal circulation, having an estimated half-life of cca. 16 minutes [5], but termination of the pregnancy in the first trimester is not followed by such rapid clearance [6]. Research on fetal DNA in maternal plasma is progressing quickly. Since the last thorough review on the use of circulating fetal DNA [7], a number of new applications and methodological approaches have been tested and/or proved that should be covered by this review (Figure 1).

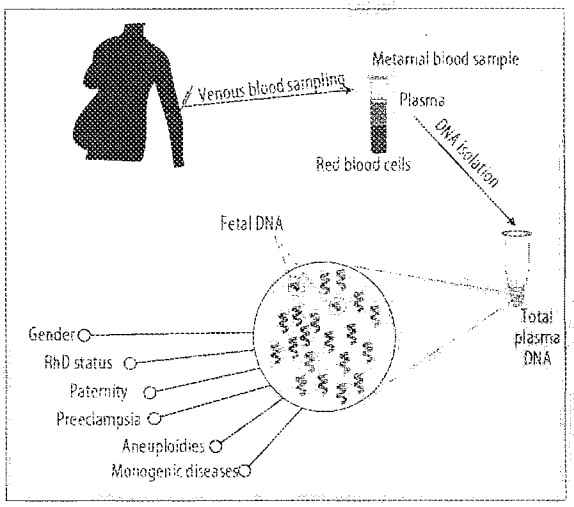

Figure 1. Fetal DNA – Proof of fetal DNA in maternal plasma dates back to 1997 [1], starting the field of noninvasive prenatal diagnostics. In principle, any prenatal genetic diagnostic test can be carried out using DNA from maternal plasma. While gender and RhD status determination are already widely implemented, the greatly desired detection of fetal aneuploidies and monogenic diseases remains challenging.

Applications

The currently used prenatal screening of standard medical care for monitoring different pregnancy-related disorders includes various biochemical blood tests in the first and second trimester [8]. The research of cff DNA in maternal plasma is a true translational science with direct application in clinical diagnostics (Table 1). In recent years, thanks to standardization in multi-center projects such as the European SAFE (Special Noninvasive Advances in Fetal and Neonatal Evaluation) project, the qualitative analysis of circulating fetal DNA is in routine clinical and/or commercial use for gender specification, the detection of some paternally inherited alleles, and RhD (the Rhesus blood group system D antigen) genotyping and has the potential to complement and possibly replace some of the currently used methods [9]. Until now, gender determination has probably been the most widely used application, important particularly in pregnancies at risk of specific inherited sex-linked diseases. The sensitivity and specificity of the PCR assay vary among laboratories, but reaches 95–100% already in the first trimester when the ratio of fetal to maternal DNA in plasma is very low [10,11]. Fetal RhD genotype determination is important for RhD-negative pregnant women in the prevention a hemolytic disorder in the newborn [12]. RhD status testing in high-risk sensitized RhD-negative pregnant women was among the first medical application of fetal DNA isolated from maternal plasma [13]. The determination of the genetic status of the fetus can help to minimize the risk of complications occurring during pregnancy or at birth in case of ABO fetomaternal incompatibility [14]. Early fetal ABO genotype can also be accurately assessed by an analysis of circulating fetal DNA purified from maternal plasma [15]. Recent studies showed that cell-free fetal DNA can be reliably used for the prenatal diagnosis of Huntington's disease [16,17]. In some countries with a high prevalence of thalassemia, the targets of the analysis are disease-causing alleles of the particular globin genes. The usefulness of this approach was proved in several studies [18–20].

One year after the discovery of fetal DNA in the maternal circulation, it was already suggested that the quantity of fetal DNA in maternal plasma might be of diagnostic value in the early detection of some pregnancy-related disorders [21]. One of the applications that rely on quantitative assessment of fetal DNA is the risk assessment of preeclampsia. Higher risk is associated with a higher number of genomic equivalents of cff DNA in maternal plasma. The use of specific paternally inherited markers (beyond the Y chromosome-specific sequences) also makes it possible to implement this monitoring for female fetuses [22]. In preeclampsia, but also in pregnancies with fetal growth restriction, fetal DNA concentrations in maternal plasma rise considerably [23]. Although the variability is high, the plasma of preeclamptic pregnant women contains cca. 5 times more fetal DNA than physiological pregnancies [24]. Another study showed that there is a weak but significant association between fetal DNA concentration and the severity of symptoms of preeclampsia [25].

Methodological Advances

Isolation

DNA isolation is usually taught in the first course for laboratory technicians. However, the isolation of circulatory nucleic acids is far from being that so simple. The high activity of nucleases, the fragmentation of cff DNA (most of the fetal DNA fragments are less than 300 bp in length), and

Table 1. Short overview of some currently applied methods in noninvasive prenatal diagnostics.

Method	Advantage	Disadvantage	Application
Real-time PCR	High sensitivity and specificity; clinical applications	Qualitative analysis of only paternally inherited sequences	Gender determination and RhD genotyping [13]
MALDI-TOF	Automated fast and accurate method, high sensitivity	Analysis only by mass (mass has to be known)	RhD genotyping [33]; point mutation detection [31,32]
Digital PCR	Highly accurate quantification of fetal sequences	Expensive microfluidic PCR device	Point mutation detection [50]
Massively throughput sequencing	Analyzes the whole sequence of fetal DNA; parallel analysis of hundreds of samples	High cost per sample	Trisomy 21 diagnostics [51]
Methylation-specific PCR	Identification of fetal sequences according to differences in methylation – universal fetal-specific marker	Conversion of unmethylated bases is unspecific	Diagnosis of aneuploidies [57,58]

especially the overall low concentration of nucleic acids in plasma make it a difficult task to solve. Column-based kits for viral nucleic acid isolation have shown the best performance and have thus been the most widely used [26]. A new commercially available kit directly offered for the isolation of circulating nucleic acids is now on the market. Whether the promised higher yields outweigh the complicated protocol and longer hands-on time will be seen. Universal robotic pipetting or specific DNA isolation systems enable high-throughput preparation of samples and subsequent analysis. This is of special interest for large laboratories analyzing a large number of samples in parallel [27].

Due to the fact that fetal DNA represents only a minor part of the whole DNA isolated from maternal plasma, numerous attempts have been made to increase the concentration of fetal DNA in the template. Simple approaches include those using the smaller molecular weight of fetal DNA fragments. Isolation of these smaller fragments can be column based, but a simple agarose gel electrophoresis also works well [28,29]. Size fractionation and enrichment for size around 200 bp improves further analysis, such as the highly sensitive automated MALDI-TOF mass spectrometry for point mutations [30]. PCR of the sequence of interest with a subsequent base extension reaction is an option for MALDI-TOF mass spectrometry pre-treatment [31,32]. Mass spectrometry has also been applied for RhD genotyping with results similar to real-time PCR [33].

Besides size fractionation, fetal DNA can also be enriched by sequence specificity, as shown recently using magnetic beads for RhD exon fragments [34]. Specific PCR pre-amplification of fetal targets such as the specific locus on the Y chromosome can greatly increase the sensitivity of the analysis, but prevents quantitative applications [35]. Other laboratories use multi-copy targets instead, which showed a higher correct fetal gender prediction rate [36]. A combination of more than one Y-specific loci as the target obviously increases the accuracy of gender determination [37].

In a much-discussed study published in Lancet, Dhallan et al. showed the ability to diagnose trisomies 21 and 13 with quantitative real-time PCR and circulating fetal DNA as the template [38]. Interestingly, although published in a high-profile clinical journal, one of the discussed topics was the addition of formaldehyde to blood to prevent cell lysis and so to increase the fraction of fetal DNA previously already reported by the same laboratory [39]. The reported enrichment, however, was not reproduced in other laboratories [40,41]. The issue of using formaldehyde during the pre-analytic phase remains controversial. It seems that the enrichment effect can only be seen if the samples are processed later than one day after blood collection, which is not the case in routine practice [42].

Applications related simply to the presence of a specific paternally inherited allele, such as standard real-time PCR detection of the Y chromosome, the RhD genotype, or specific microarray detection of disease-causing alleles, can be applied without any fetal DNA enrichment [43,44]. Other approaches include PCR followed by an allele-discriminating ligase reaction and capillary electrophoresis, as shown for estrogen receptor gene polymorphisms [45]. A rarely used but interesting method is the clamp of maternal wild-type alleles by peptic nucleic acids enriching the potentially mutant fetal alleles for further microchip analysis [44].

Digital PCR

The so-called digital PCR is based on the premise that the average number of template molecules per single reaction is <1. A high number of parallel reactions enable quantification of the positive PCR reactions and thus the number/concentration of the template (Figure 2). As each individual reaction is qualitative, digital PCR outperforms real-time PCR in precision, which is needed especially in the screening and detection of aneuploidy [46]. Digital PCR has been proven as an effective approach in noninvasive prenatal diagnostics of trisomy 21 [47]. By using this more accurate method it has been shown that the actual fractional concentration of fetal DNA in the maternal plasma in all three trimesters is two times higher than the previously reported amount determined by real-time PCR. Expensive microfluidic PCR devices are, however, needed for this advanced approach [48].

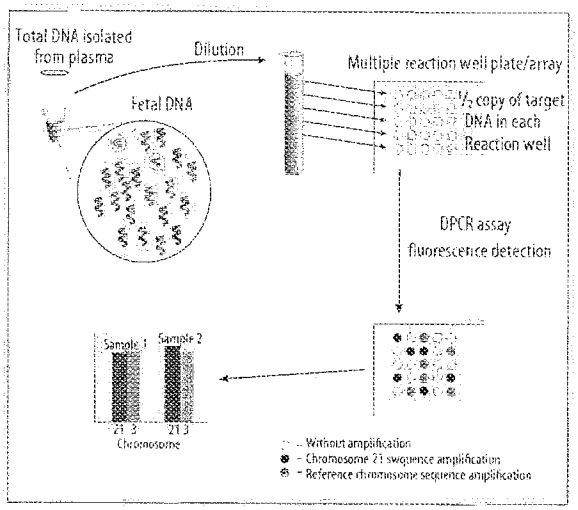

Figure 2. Digital PCR – For digital PCR (DPCR) the sample is diluted and distributed into a multi-reaction array (e.g. 384-well plate format) so that each reaction contains on average virtually ½ of a target molecule, or only every second reaction contains a target template. Subtle ratio differences, as in fetal aneuploidies, can be determined by counting positive PCRs of the target and reference sequence.

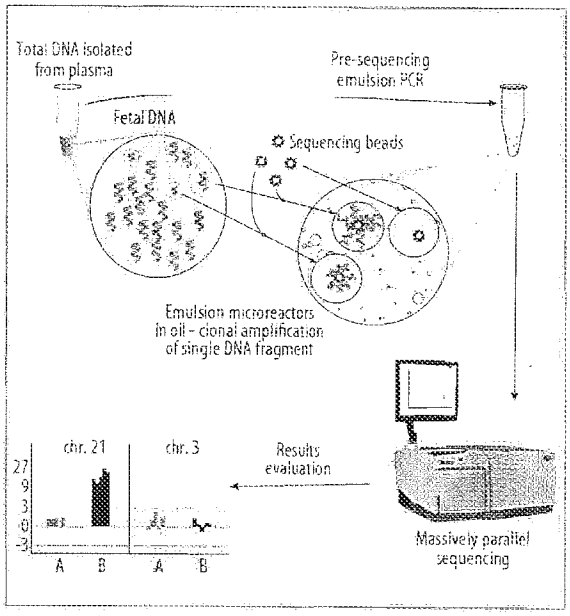

Figure 3. Massively parallel sequencing – Massively parallel (MP) sequencing technology is extremely well suited for overcoming the problem of low relative content of fetal DNA in maternal plasma. In principle, a single template molecule gets amplified in each "microreactor" of emulsion PCR attached to a special carrier. In massively parallel sequencing, from millions to many billions (according to technology implementation) of amplified single templates are sequenced. In principle, sequencing data of millions to billions of single templates should allow the most straightforward detection of subtle ratio changes, which is the key challenge of detecting fetal aneuploidies in maternal plasma.

A review on this method and its application has been published recently [49]. Current developments of digital PCR and the preparation of template DNA by so-called digital nucleic acid size selection (NASS) widens the spectrum of application of digital PCR also to tests for monogenic diseases. The advantage lies in the digital relative mutation dosage approach. Effective quantification of allele frequency by digital PCR makes possible the precise evaluation of balance/imbalance between mutant and wild-type alleles [50].

Massively parallel sequencing

Obviously, the ultimate goal of prenatal diagnostics is to obtain and analyze the complete sequence of the fetal genome. The recent boom of the second and third generations of systems for massively parallel sequencing brings us closer to this goal (Figure 3). Although it still is a problem to distinguish between maternal and fetal sequences, a parallel analysis of paternal DNA helps to identify the haplotypes passed to the fetus. Bioinformatic analysis including quantification of the presence and length of the reads might further help to elucidate which sequences are maternal and which are of fetal origin. The ability to diagnose trisomy 21 in the first trimester of pregnancy by genomic sequencing of cff DNA from maternal plasma samples was shown by the lab of the discoverer of circulating fetal DNA [51]. In addition to the diagnostic value of massively parallel sequencing, the results of such analyses showed the overrepresentation of nucleosome sequences indicating the source of cff DNA in maternal plasma [52].

Although the costs for massively parallel sequencing are still relatively high, the possibility to analyze tens or hundreds of samples in a single run using tags or other improvements could make this a suitable and cost-effective method if a large number of samples is analyzed in parallel. A more sensitive approach is the sequencing of DNA from fetal cells present in the maternal blood [53]. Although the principal techniques for the isolation of these rare cells exist, the procedure is yet to be standardized. Interestingly, fetal cells can be isolated much more easily in preeclamptic pregnancies or in pregnancies with RhD incompatibility than in physiological pregnancies [54]. Looking at the developments in other related fields, it is, nevertheless, more probable that the crude sequencing approach with subsequent bioinformatic analysis will be widely used in the near future [55].

Other methods

Differences in methylation pattern between maternal cells and fetal cells from the placenta can be used to track and identify fetal sequences using PCR on bisulfite-treated DNA. Hypermethylated fetal sequences can be used as a universal marker of fetal DNA, enabling its quantification and thus preventing false-negative results due to the low concentration of fetal DNA [56]. In addition, these differences enable the diagnosis of aneuploidies (such as trisomies 21 and 18) by bisulphate sequencing or by methylation-sensitive primer extension [57,58].

Denaturing high-performance liquid chromatography (dHPLC) is a widely used technique for the screening of rare genotypes, for example gene mutations, by temperature separating DNA duplexes that differ in the identity of one

or more base pairs. Although it is a unique study, to evaluate its potential utility, dHPLC has been applied and shown to be sensitive enough for the detection of paternally inherited mutated CRB1 gene in maternal plasma and thus for the prenatal diagnosis of Leber's congenital amaurosis [59]. Another interesting method is the commercially available multiplex genotyping SNaPshot technology, a so-called mini-sequencing reaction of several PCR products of variable length amplified during the primer extension-based reaction. Subsequent capillary electrophoresis can then uncover the genotypes of several SNPs (single nucleotide polymorphisms) or point mutations. In a single study, this method was successfully used for the prenatal molecular diagnostics of cystic fibrosis [60].

Sex Determination and Ethical Concerns

It is crucial for every diagnostic method to be submitted to an analysis of sensitivity and specificity in large-scale studies before application in clinical diagnostics. This is the main principle of evidence-based medicine, which is now considered the best available. From this view it can be only welcomed that such large studies with more than 1000 pregnant women participating have been performed with the diagnostic use of cff DNA in maternal plasma at least for RhD genotyping [61]. Similar results were obtained in a Belgian retrospective study with more than 500 pregnancies [62] and in an earlier meta-analysis combining the results of more than 3000 tests [63]. Although the results are in general convincing, further studies are needed directly comparing standard management and the use of a cff DNA-based diagnostic approach in the prevention of hemolytic disorders of the newborn, as pointed out in a recent systematic review [64]. However, proof of accuracy is the first requirement for a diagnostic method to be ethically correct.

Studies concentrating on the first trimester have shown that fetal gender determination is principally possible in 5th–6th week of pregnancy with a high accuracy and much sooner than the routinely used ultrasound detection [65]. This early fetal sex determination is useful in clinical diagnostics in pregnancies at risk of inherited sex-linked diseases, but can be also applied in non-clinical applications, without any specific medical need. The sensitivity of the assays is astonishing given the limited volume of starting material, the fragmentation of the target sequences, and the concentrations of fetal DNA, with less than 10 genome equivalents per ml of plasma. Multiplex PCR using fluorescently labeled primers targeting highly variable short tandem repeats used as a bar code for the identification of individuals in forensic medicine has already been adapted for identification of fetal DNA and thus can be used for prenatal paternity testing [66,67]. Although multiplex PCR requires further optimization, already standard commercially available identification kits can be potentially used for prenatal paternity testing [68]. Although the legislation varies considerably among countries, the possibility of such an early detection of fetal gender or even paternity may potentially lead to selective abortion. Whether reporting the test results after the 12th week, when such a procedure is no longer legal in several countries, really solves the ethical issue is questionable.

Another potential scientific breakthrough and also an ethical issue is the use of other biological fluids for the analysis of fetal DNA. Besides in the maternal plasma, fetal DNA has been identified in the cerebrospinal fluid [69]. This is hardly an alternative to blood sampling. However, other fluids can easily be obtained. Especially urine and, potentially, also saliva may contain fetal DNA suitable for genetic analysis [70]. Attempts to use so-called transrenal DNA were successful in some studies [71]; in other papers the authors report too low fetal DNA concentrations and thus low sensitivity of subsequent analyses [72,73]. These issues may be only a temporal obstacle. Given the easy sampling and the available fetal gender determination kits, it is highly probable that this novel technology might be misused in some countries with many cases of socially driven sex-selective abortion. A governmental ban on plasma/blood sample-based tests may in this way be circumvented.

Conclusions

Genomics was the first branch of system biology to enter noninvasive prenatal diagnostics, although additional evaluation is needed to determine how effective and precise the test applications are. In the near future, transcriptomics and proteomics are expected to bring new insight into the physiology and pathophysiology of pregnancy as well as new opportunities for noninvasive prenatal molecular diagnostics [74]. Further advances in this field can be expected in fetal DNA selection from the maternal DNA background, the use of other biological fluids, as well as the application of easier low-cost procedures such as the LAMP (loop-mediated isothermal amplification of DNA) method and the cycling reaction, in which a set of four different primers is employed for sensitive amplification of specific DNA sequences under isothermal conditions [75]. Despite several technological and ethical issues, rapid progress in molecular biology and biomedicine is completely changing prenatal DNA diagnostics and opening the door for early prenatal gene therapy in the future.

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Digital PCR for the molecular detection of fetal chromosomal aneuploidy

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Trisomy 21 is the most common reason that women opt for prenatal diagnosis. Conventional prenatal diagnostic methods involve the sampling of fetal materials by invasive procedures such as amniocentesis. Screening by ultrasonography and biochemical markers have been used to risk-stratify pregnant women before definitive invasive diagnostic procedures. However, these screening methods generally target epiphenomena, such as nuchal translucency, associated with trisomy 21. It would be ideal if noninvasive genetic methods were available for the direct detection of the core pathology of trisomy 21. Here we outline an approach using digital PCR for the noninvasive detection of fetal trisomy 21 by analysis of fetal nucleic acids in maternal plasma. First, we demonstrate the use of digital PCR to determine the allelic imbalance of a SNP on *PLAC4* mRNA, a placenta-expressed transcript on chromosome 21, in the maternal plasma of women bearing trisomy 21 fetuses. We named this the digital RNA SNP strategy. Second, we developed a nonpolymorphism-based method for the noninvasive prenatal detection of trisomy 21. We named this the digital relative chromosome dosage (RCD) method. Digital RCD involves the direct assessment of whether the total copy number of chromosome 21 in a sample containing fetal DNA is overrepresented with respect to a reference chromosome. Even without elaborate instrumentation, digital RCD allows the detection of trisomy 21 in samples containing 25% fetal DNA. We applied the sequential probability ratio test to interpret the digital PCR data. Computer simulation and empirical validation confirmed the high accuracy of the disease classification algorithm.

circulating fetal nucleic acids | noninvasive prenatal diagnosis | sequential probability ratio test | trisomy 21 | RNA SNP

The detection of fetal trisomy 21 (T21) is an important indication for prenatal diagnosis. The sampling of fetal materials by amniocentesis and chorionic villus sampling are invasive, with a finite risk of fetal loss (1). A variety of screening methods, such as ultrasound, have been investigated (2). However, these screening methods typically target T21-related epiphenomena instead of the core chromosomal abnormality and thus have suboptimal diagnostic accuracy and disadvantages, such as being highly influenced by gestational age.

The discovery of cell-free fetal DNA in maternal plasma in 1997 offered new possibilities for noninvasive prenatal diagnosis (3, 4). This method has been readily applied to sex-linked (5) and certain single-gene (6, 7) disorders, but its use for fetal chromosomal aneuploidies has been a challenge (4). First, fetal nucleic acids coexist in maternal plasma with a high background of maternal nucleic acids that can often interfere with analysis (8). Second, fetal nucleic acids circulate in maternal plasma in a cell-free form, making it difficult to derive chromosome dosage information. Significant developments have recently been made (9–11). One approach focuses on the detection of nucleic acid species that are fetal-specific, including DNA fragments with a placenta-specific DNA methylation pattern (10, 12) and RNA molecules expressed by the placenta (9). Because circulating fetal nucleic acids are mainly derived from the placenta, the problem of maternal background interference can be overcome by targeting such molecules in maternal plasma (4). Dosage of chromosome 21 (chr21) is then inferred from the ratios of polymorphic alleles in the placenta-derived DNA/RNA molecules. However, the dependence on genetic polymorphisms limits the use of these approaches to heterozygous fetuses.

It would be ideal if a noninvasive test for fetal T21 detection based on circulating fetal nucleic acid analysis were not dependent on the use of genetic polymorphisms. Theoretically, even with the small fractional concentration of fetal DNA (8), a T21 fetus would contribute an additional dose of chr21 sequences per genome equivalent (GE) of fetal DNA released into maternal plasma. For example, a maternal plasma sample from a euploid pregnancy containing 50 GEs per milliliter of total DNA with 5 GEs per milliliter of DNA contributed by the fetus (i.e., 10% fetal DNA) should contain a total of 100 copies (90 maternal copies plus 10 fetal copies) of chr21 sequences per milliliter of maternal plasma. For a T21 pregnancy, each fetal GE would contribute three copies of chr21, resulting in a total of 105 copies (90 maternal copies plus 15 fetal copies) of chr21 sequences per milliliter of maternal plasma. At 10% fetal DNA concentration, the amount of chr21-derived sequences in the maternal plasma of a T21 pregnancy would therefore be 1.05 times that of a euploid case. If an analytical approach could be developed to determine this small degree of quantitative difference, a polymorphism-independent test for noninvasive prenatal diagnosis of fetal T21 would be achieved.

Gene dosage assessment requiring 2-fold discrimination power can readily be attained with quantitative PCR (13). Through DNA quantification of a chr21 locus and a reference locus in amniocyte cultures, Zimmermann *et al.* (14) were able to detect the 1.5-fold increase in chr21 DNA sequences in T21 fetuses. Because a 2-fold difference in DNA template concentration constitutes a difference of only one threshold cycle (Ct), the discrimination of a 1.5-fold difference has been the limit of conventional real-time PCR. To achieve finer degrees of quantitative discrimination, alternative strategies are needed. Here, we explore the use of digital PCR (15) for this purpose.

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Conflict of interest statement: Y.M.D.L., F.M.F.L., K.C.A.C., N.B.Y.T., K.C.C., B.C.Y.Z., C.R.C., and R.W.K.C. have filed patent applications on aspects of noninvasive prenatal diagnostics. Y.M.D.L. has equity in Plasmagene Biosciences Limited. C.R.C. has equity in Sequenom, Inc., and is the Chief Scientific Officer of Sequenom, Inc.

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Abbreviations: T21, trisomy 21; chr, chromosome; RCD, relative chromosome dosage; SPRT, sequential probability ratio test.

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Digital PCR involves multiple PCR analyses on extremely dilute nucleic acids such that most positive amplifications reflect the signal from a single template molecule (15), permitting the counting of individual template molecules. The proportion of positive amplifications among the total number of PCRs analyzed allows an estimation of the template concentration in the original nondiluted sample. This technique has been proposed to allow the detection of a variety of genetic phenomena (15), including the detection of loss of heterozygosity (LOH) in tumor samples (16) and plasma of cancer patients (17). Because template molecule quantification by digital PCR does not rely on dose–response relationships between reporter dyes and nucleic acid concentrations, its analytical precision should theoretically be superior to that of real-time PCR. To test whether this approach is precise enough to detect fetal chromosomal aneuploidies in maternal plasma, we first assessed whether digital PCR could measure the allelic ratio of *PLAC4* mRNA in maternal plasma (9), thereby distinguishing T21 from euploid fetuses. This is referred to as the digital RNA SNP method. We then evaluated whether the increased precision of digital PCR would allow the detection of fetal chromosomal aneuploidies without depending on genetic polymorphisms. We call this digital relative chromosome dosage (RCD) analysis.

Results

Principles of Digital PCR. The first step in digital PCR is the dilution of the extracted nucleic acids to a concentration such that, on average, one template molecule is present per reaction well. PCR is then set up so that a multitude of such single-molecule PCRs is analyzed per sample. We used 96-well and 384-well reaction plates and distributed each diluted nucleic acid sample to the reaction wells of one or more plates. Under these conditions, the actual number of template molecules distributed to each reaction well followed the Poisson distribution. Thus, an individual reaction well could contain zero, one, or more template molecules. The expected proportion of wells with no template is given by e^{-m}, where m is the average concentration of template molecules per well. For example, at an average concentration of one template molecule per well, the expected proportion of wells with no template molecule is given by e^{-1}, i.e., 0.37 (37%). The remaining 63% of wells will contain one or more template molecules. Typically, the number of positive and informative wells in a digital PCR run would then be counted. The definition of informative wells and the manner by which the digital PCR data are interpreted depend on the application (15) and are described below.

Principles of Digital RNA SNP. Digital RNA SNP is a digital version of our previously reported approach (9) for T21 detection by determining an imbalance in the ratio of polymorphic alleles of an A/G SNP, rs8130833, located on *PLAC4*. For a heterozygous euploid fetus, the A and G alleles should be equally represented in the fetal genome (1:1), whereas, in T21, an additional copy of one of the SNP alleles would give a 2:1 ratio. Digital RNA SNP analysis aims to determine whether the amounts of the two *PLAC4* alleles in the sample are equal or otherwise. Thus, both the A and G *PLAC4* alleles are the target templates. The analytical steps are schematically shown in Fig. 1.

After digital real-time PCR analysis of the *PLAC4* SNP alleles in 384-well plates, the number of informative wells was counted. An informative well is defined as one that was only positive for the A or G allele but not both (Fig. 1). For a euploid case, we expect an equal number of A-positive and G-positive wells (Fig. 1). However, when template molecules from a T21 fetus are analyzed, the number of wells containing just one allele should be higher than the number containing just the other allele (Fig. 1). In short, allelic imbalance is expected for T21. The same degree of imbalance would be expected if this approach were applied to the analysis of placental DNA, placental RNA, and maternal plasma RNA [*PLAC4* mRNA in maternal plasma being completely fetal in origin (9)].

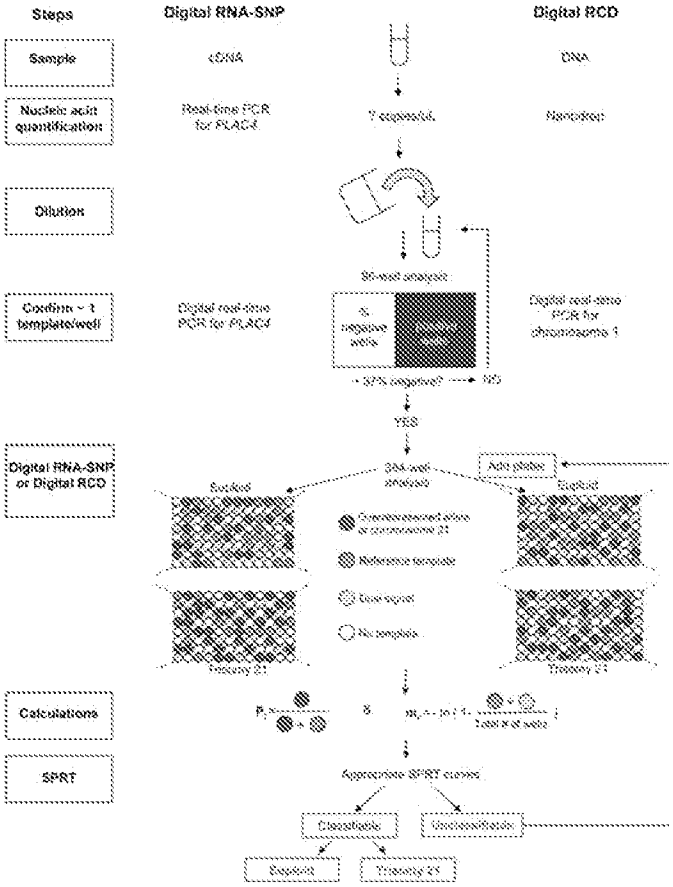

Fig. 1. Illustration of the analytical steps in digital RNA SNP and digital RCD analyses for T21 detection. Only a representative 96-well subset of the 384-well data is shown for one euploid and one T21 case for each of digital RNA SNP and digital RCD analyses, respectively. The T21 data depicted in the digital RNA SNP experiment represent a case where the G allele is overrepresented, i.e., a fetal genotype of AGG.

The allele with the higher number of counts is referred to as the overrepresented allele, and its proportion among all informative wells, P_t, was calculated (Fig. 1). The sequential probability ratio test (SPRT) (16, 18) (see below) was applied to determine whether the P_t indicated the degree of allelic imbalance that would be expected for a T21 sample. Alternatively, the SPRT analysis may indicate that the available data are not yet adequate for disease classification. When classification was not achieved, additional 384-well plates were analyzed until the aggregated data became classifiable by SPRT.

Principles of Digital RCD. We determined chromosome dosage by digital PCR analysis of a nonpolymorphic chr21 locus relative to one located on a reference chromosome, chr1. We aimed to differentiate a change in the ratio of chr21 to chr1 from 2:2 in the genome of a euploid fetus to 3:2 in a T21 fetus (Fig. 1). Here, an informative well is defined as one that is positive for either the chr21 or chr1 locus but not both. For a euploid fetus, the number of informative wells positive for either locus should be approximately equal (Fig. 1). For a T21 fetus, there should be an overrepresentation of wells positive for chr21 (Fig. 1). The degree of overrepresentation would depend on the fractional fetal DNA concentration in the sample. For example, when placental DNA is analyzed, the theoretical RCD ratio in the fetal genome should be 3:2, i.e., a 1.5-fold difference. However, as described earlier, the theoretical

RCD ratio would decrease to 1.05 when analyzing a maternal plasma sample containing 10% fetal DNA. The P_r was calculated by dividing the number of wells positive only for the chr21 locus by the total number of informative wells (Fig. 1). The P_r was subjected to SPRT analysis (16, 18) for disease classification. If the data were unclassifiable, one or more additional 384-well plates were analyzed.

Assessment of Allelic or Chromosomal Imbalance by Digital PCR. To determine whether the analyzed sample is from a T21 case, the observed RNA SNP or RCD ratio would be compared with that expected for a T21 case. The theoretical RNA SNP ratio is 2:1, and the RCD ratio is 3:2 for a pure T21 sample. However, due to the Poisson distribution, the exact ratios are not the same as those in the fetal genome. Furthermore, template concentration is a key variable in the Poisson equation. Thus, the exact ratios are dependent on the template concentration used in a particular experiment. Because the total number of template molecules for a given volume of sample from a T21 subject would be greater than that for a euploid case, we standardize our definition of the level of diluted template concentration as the average number of reference template molecules per reaction well, m_r. For digital RNA SNP analysis, the reference template would be the allele that was not overrepresented, whereas the reference template for digital RCD analysis would be the chr1 locus. Thus, the dilution of one target template molecule of any type per well for the digital PCR analysis of a euploid case equates to an m_r of 0.5.

The basis for the difference between the theoretical and expected degree of allelic or chromosomal imbalance and the calculations to determine the latter for a range of m_r values are shown in supporting information (SI) Tables 3 and 4. In digital RNA SNP analysis of a T21 sample, when the m_r value was 0.5, the digital RNA SNP ratio (namely, the ratio of wells containing just the overrepresented allele with respect to wells containing just the reference allele) was 2.65 (SI Table 3). In digital RCD analysis of a specimen composed of 100% fetal DNA, when the m_r value was 0.5, the digital RCD ratio (namely, the ratio of wells positive solely for the chr21 locus with respect to those positive solely for the chr1 locus) was 1.7 (SI Table 4). As the fractional fetal DNA concentration decreases, the digital RCD ratio decreases for the same m_r (SI Table 4). As shown in SI Tables 3 and 4, the extent of allelic or chromosomal overrepresentation increases with m_r. However, the percentage of informative wells approaches its maximum near an m_r value of 0.5 and decreases gradually with further increase in m_r. In practice, the decline in the proportion of informative wells could be compensated by increasing the total number of wells analyzed if the amount of specimen template molecules is not limiting, with an associated increase in reagent costs. Hence, optimal digital PCR performance is a tradeoff between the template concentration and total number of wells tested per sample.

SPRT Analysis. To determine whether an observed degree of overrepresentation of a *PLAC4* allele in digital RNA SNP, or the chr21 locus in digital RCD, is statistically significant, a SPRT-based approach was used (16, 18). SPRT is a method that allows testing of a hypothesis as data accumulate. SPRT has been used to interpret digital PCR data for loss of heterozygosity (LOH) in tumor samples (16, 18). In T21 detection, the null hypothesis is that there is no allelic or chromosomal imbalance (i.e., T21 is not detected). The alternative hypothesis is that allelic or chromosomal imbalance exists (i.e., T21 is detected). Operationally, SPRT can be performed with a pair of SPRT curves that are constructed to define the probabilistic boundaries for accepting or rejecting the null hypothesis (Fig. 2*A* and *SI Materials and Methods*). These curves show the required proportion of informative wells positive for the overrepresented allele or chr21, P_r (*y* axis, Fig. 2*A*), for a given total number of informative wells (*x* axis, Fig. 2*A*) needed for classification. Samples with data points that are above the top curve

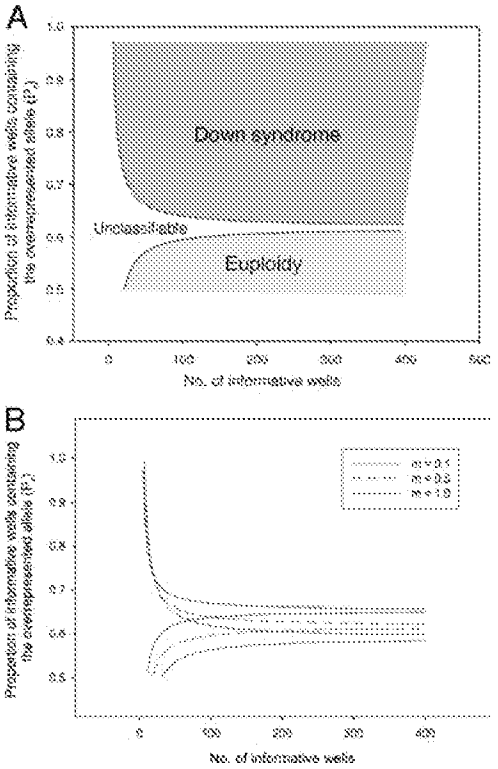

Fig. 2. SPRT analysis. (*A*) A pair of SPRT curves delimits the decision boundaries for accepting or rejecting the hypotheses that the sample belonged to a euploid or aneuploid fetus. (*B*) The decision boundaries of the SPRT curves would vary according to the template concentration. Curves applicable to digital RNA SNP analysis are shown.

are classified as trisomic (Fig. 2*A*). Samples with data points that are below the bottom curve are classified as euploid. Samples with data points in between the two curves are unclassifiable and would require an increased total number of informative counts before classification. SPRT thus offers the advantage that a smaller amount of testing is required for a given level of confidence than other statistical methods. This feature is of particular relevance to the analysis of plasma nucleic acids in which the number of available template molecules is limited.

As discussed above, the exact degree of allelic or chromosomal imbalance depends on the actual template concentration per experiment. We therefore constructed a series of SPRT curves for a range of m_r values (*SI Materials and Methods*). Each set of digital PCR data should be interpreted with the curves relevant to the m_r of that particular run. Thus, in practice, after digital RNA SNP or digital RCD analysis, m_r and P_r are calculated (Fig. 1). m_r is calculated by using the Poisson equation and the proportion of wells negative for the reference template (*SI Materials and Methods*). P_r is the proportion of informative wells positive just for the overrepresented template. The experimentally derived P_r is interpreted with the relevant SPRT curves selected by the corresponding m_r. This is in contrast to the previously reported use of SPRT for molecular detection of loss of heterozygosity (LOH) by digital PCR, where a fixed set of curves was used (16). Because the expected degrees of allelic or chromosomal imbalance for the digital RNA SNP and RCD approaches are different (2:1 for the former and 3:2 for the latter), different series of SPRT curves are needed. Fig. 2*B* illustrates the degree of differences in the SPRT curves for m_r values of 0.1, 0.5, and 1.0 for digital RNA SNP analysis. Compared with the use of a fixed set of SPRT curves in previous studies (*SI Materials and Methods*) (16, 18), the proportion of

Table 1. Digital RNA SNP analysis in placental tissues of euploid and T21 pregnancies

Sample	Genotype	No. of wells positive for individual alleles				m_r	SPRT result		
		A only	G only	AG	All negative		P_t	Unclassifiable region	Classification
Placental DNA									
N677	AG	85	83	126	90	0.79	0.51	0.63–0.65	Euploid
N710	AG	102	83	73	126	0.52	0.55	0.61–0.63	Euploid
N435	AGG	49	157	130	48	0.63	0.76	0.62–0.64	T21
N981	AAG	135	69	82	98	0.50	0.66	0.61–0.63	T21
Placental RNA									
V533	AG	103	93	71	117	0.56	0.53	0.61–0.63	Euploid
V943	AG	89	100	74	121	0.55	0.53	0.61–0.63	Euploid
N435	AGG	52	138	95	99	0.48	0.73	0.61–0.63	T21
T215	AAG	146	58	138	42	0.71	0.72	0.62–0.64	T21

The no. of wells for all samples was 384. Genotypes were determined by mass spectrometric assay. The m_r value indicates the average no. of reference molecules per reaction well. The P_t values were calculated by using the following equation: no. of wells positive for the overrepresented allele/(no. of wells positive for A only + no. of wells positive for G only). The unclassifiable region for the corresponding m_r is shown. "Euploid" was assigned when the P_t was below the unclassifiable region; "T21" was assigned when the P_t was above the unclassifiable region.

unclassifiable data is much lower with our approach (SI Tables 5 and 6). For example, when using our approach, at an m_r value of 0.5, 14% and 0% of T21 samples would be unclassifiable for 96-well and 384-well digital RNA SNP analyses, respectively, but 62% and 10%, respectively, would be unclassifiable when using fixed curves (SI Tables 5 and 6).

Computer Simulation of Classification Accuracies of Digital PCR Detection of T21. Computer simulation was performed to estimate the accuracy of diagnosing T21 by using the SPRT approach. Separate simulations were performed for different values of three parameters, namely, reference template concentration (m_r), number of informative counts, and projected degree of allelic or chromosomal imbalance (P_t). For digital RNA SNP, simulations of a 384-well experiment with m_r values of 0.1–2.0 were performed. At each m_r value, we simulated the scenario whereby 5,000 euploid and 5,000 T21 fetuses were tested (*SI Materials and Methods*). The SPRT curves appropriate for the given m_r were used to classify the 10,000 fetuses. The percentages of fetuses correctly and incorrectly classified as euploid or aneuploid and those unclassifiable for the given informative counts were determined (SI Table 7). The accuracies for diagnosing euploid and aneuploid cases are both 100%, for m_r values between 0.5 and 2.0. When the m_r value was 0.1, only 57% and 88% of euploid and T21 fetuses could be accurately classified by using 384 wells. Simulation results, using an illustrative repetition number of 300 times, are shown in SI Fig. 4.

Computer simulations for digital RCD analysis for a pure (100%) fetal DNA sample were similarly performed (SI Table 8 and SI Fig. 5). The extent of chr21 overrepresentation in digital RCD analysis depends on the fractional concentration of fetal DNA in the tested specimen. Because the fractional fetal DNA concentration becomes lower, the degree of chr21 overrepresentation diminishes, and thus a larger number of informative wells for accurate disease classification is required. Hence, simulations were further performed for fetal DNA concentrations of 50%, 25%, and 10% for a total well number ranging from 384 to 7,680 wells at an m_r value of 0.5 (SI Table 9). The performance of digital RCD is better for cases with a higher fetal DNA fractional concentration. At a fetal DNA concentration of 25% and with a total number of 7,680 PCR analyses, 97% of both euploid and aneuploid cases would be classifiable with no incorrect classification. The remaining 3% of cases require further analyses until classification can be achieved.

Validation of T21 Detection When Using Digital RNA SNP for *PLAC4*. The practical feasibility of digital RNA SNP was demonstrated by using the rs8130833 SNP on the *PLAC4* gene (*SI Materials and Methods*) (9). Placental DNA and RNA samples from two euploid and two T21 heterozygous placentas were analyzed. The placental DNA samples were analyzed with the omission of the reverse transcription step, thus essentially converting the procedure to digital DNA SNP analysis. We diluted the samples, aiming for approximately one allele of any type per well, and confirmed this

Table 2. Digital RNA SNP analysis of maternal plasma from euploid and T21 pregnancies

Sample	Genotype	No. of wells positive for individual alleles				m_r	SPRT result		
		A only	G only	AG	All negative		P_t	Unclassifiable region	Classification
M2390P	AG	90	100	97	97	0.67	0.526	0.62–0.64	Euploid
M2391P	AG	97	105	65	117	0.55	0.520	0.61–0.63	Euploid
M2473P	AG	66	92	34	192	0.30	0.582	0.59–0.62	Euploid
M2524P	AG	29	28	3	324	0.08	0.509	0.54–0.64	Euploid
M2528P	AG	112	85	44	143	0.41	0.569	0.60–0.62	Euploid
M2601P	AG	90	101	72	121	0.55	0.529	0.61–0.63	Euploid
M2607P	AG	73	91	57	163	0.41	0.555	0.60–0.63	Euploid
M2638P	AG	66	90	52	176	0.37	0.577	0.59–0.62	Euploid
M2639P	AG	71	56	17	240	0.21	0.559	0.58–0.62	Euploid
M2525P	AAG	110	53	21	200	0.21	0.675	0.58–0.61	T21
M2272P	AAG	246	127	112	283	0.37	0.660	0.60–0.61	T21
M2718P	AGG	66	114	66	138	0.42	0.633	0.60–0.62	T21
M1519P	AGG	58	130	54	142	0.34	0.691	0.59–0.62	T21

The number of wells for all samples except M2272P was 384. The number of wells for sample M2272P was 768. Genotypes were determined by mass spectrometric assay. The m_r value indicates the average no. of reference molecules per reaction well. The P_t values were calculated by using the following equation: no. of wells positive for the overrepresented allele/(no. of wells positive for A only + no. of wells positive for G only). The unclassifiable region for the corresponding m_r is shown. "Euploid" was assigned when the P_t was below the unclassifiable region; "T21" was assigned when the P_t was above the unclassifiable region.

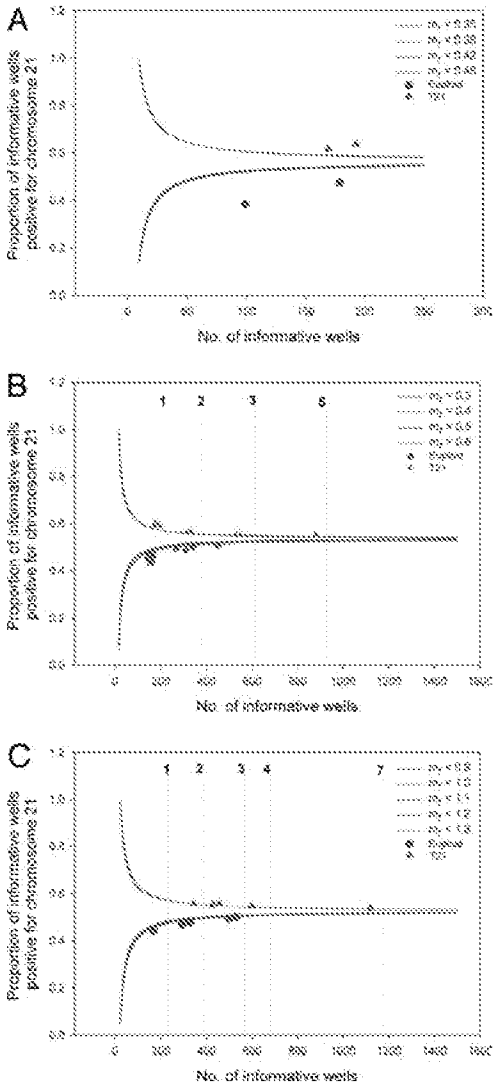

Fig. 3. SPRT interpretation of digital RCD analyses. (*A*) Placental DNA samples. (*B*) DNA mixtures of 50% placenta/maternal buffy coat. (*C*) DNA mixtures of 25% placenta/maternal buffy coat. Numbers at the top of *B* and *C* indicate the number of 384-well plates required before the data set was classifiable for the cases delimited by the dotted lines surrounding each number.

by a 96-well digital PCR analysis (Fig. 1). This was followed by a 384-well digital RNA SNP experiment. P_t and m_t were calculated, and the SPRT curve for this m_t value was used for data interpretation. The results are shown in Table 1. Each of these DNA and RNA samples was correctly classified with one 384-well experiment. We further tested plasma RNA samples from nine women carrying euploid fetuses and four women carrying T21 fetuses. All cases were correctly classified (Table 2). Initial results for one T21 case (M2272P) fell within the unclassifiable region between the SPRT curves after one 384-well experiment. Thus, we performed an additional 384-well run. New m_t and P_t values were calculated from the aggregated data of 768 wells, and the classification was performed by using a new set of SPRT curves selected based on this m_t value. The case was then scored correctly as aneuploid.

Validation of T21 Detection When Using Digital RCD. Placental DNA samples from two euploid and two T21 placentas were diluted to approximately one target template for either chromosome per well and confirmed by a 96-well digital PCR analysis (*SI Materials and Methods*). Each confirmed sample was analyzed by a 384-well digital RCD experiment, and the P_t and m_t values were calculated. For digital RCD, the chr1 paralog (19) was the reference template. This m_t value was used to select a corresponding set of SPRT curves for data interpretation. All of the placental samples were correctly classified (Fig. 3*A*). To demonstrate that digital RCD is applicable to nonpure fetal DNA samples (e.g., fetal DNA in maternal plasma), mixtures containing 50% and 25% of T21 placental DNA in a background of euploid maternal blood cell DNA were analyzed. Placental DNA from 10 T21 and 10 euploid cases was mixed with an equal amount of euploid maternal blood cell DNA, thus producing 20 DNA mixtures of 50%. Similarly, placental DNA from five T21 and five euploid cases was each mixed with a 3-fold excess of euploid maternal blood cell DNA, thus producing 10 DNA mixtures of 25%. All of the euploid and aneuploid DNA mixtures were correctly classified (Fig. 3 *B* and *C*). Each sample reached the point of being classifiable after a number of 384-well digital PCR analyses (Fig. 3 *B* and *C*). For the 50% DNA mixtures, the number of 384-well plates required ranged from one to five. For the 25% DNA mixtures, the number of 384-well plates required ranged from one to seven. The cumulative proportion of cases correctly classified increased progressively with the addition of each 384-well digital PCR analysis, as predicted in SI Table 9.

Discussion

In this study we have outlined and demonstrated the principle of digital PCR-based detection of chromosomal aneuploidy, using T21 as an example. As the statistical tool, we used SPRT, previously used for digital PCR-based detection of loss of heterozygosity (LOH) in samples with 50% tumor-derived DNA. In this setting, 50% of target DNA is contributed by normal cells with two copies of target chromosomes, and the other 50% is contributed by cancer cells where one target chromosome is lost (16, 18). We realized that a fetal trisomic cell is analogous to the combination of one noncancer cell and one cancer cell. The degree of allelic imbalance in a cancer sample containing 50% tumor-derived DNA is the same as that in a clinical sample containing pure fetal DNA (e.g., amniotic fluid) or RNA [e.g., *PLAC4* mRNA in maternal plasma (9)] from a pregnancy involving a T21 fetus. In both the cancer and the prenatal diagnosis scenarios, the ratio of the more abundant allele to the less abundant allele is 2:1. We further refined the SPRT analysis by constructing specific SPRT curves appropriate for the exact template concentration for any given digital PCR run and extended this strategy for the polymorphism-independent digital RCD approach. Alternative statistical methods, such as that based on the false discovery rate (20), could be further evaluated in future studies.

Our experimental and simulation data show that digital RNA SNP is an effective and accurate method for T21 detection. Because *PLAC4* mRNA in maternal plasma is derived purely from the fetus, for 12 of the 13 maternal plasma samples tested, only one 384-well digital PCR experiment was required for correct classification. This homogenous, real-time PCR-based approach thus offers an alternative to the previously described mass spectrometry-based approach for RNA SNP analysis (9). Apart from placental-specific mRNA transcripts, other types of fetal-specific nucleic acid species in maternal plasma could be used. One example is fetal epigenetic markers (12, 21) which have recently been used for the noninvasive prenatal detection of trisomy 18 via the epigenetic allelic ratio (EAR) approach (10). Thus, we predict that digital EAR would be a possible analytical technique.

Digital RCD was developed to overcome the requirement of heterozygosity for a polymorphism-based approach such as digital RNA SNP. Digital RCD could readily discriminate T21 and euploid placental DNA samples, thus supporting its applications to samples containing virtually pure fetal DNA, e.g., amniotic fluid and chorionic villus samples.

The application of digital RCD to DNA extracted from maternal plasma is complicated by the fact that fetal DNA constitutes only

a minor fraction of maternal plasma DNA, with a mean fractional concentration of some 3% between weeks 11 and 17 of gestation (8). Nevertheless, we have shown that digital RCD allows aneuploidy detection even when the fetal fraction is a minor population. With a decreasing fractional concentration of fetal DNA, e.g., during early gestation, a larger number of informative counts is needed for digital RCD. The significance of the present work, as summarized in SI Table 9, is that we have provided a set of benchmark parameters, e.g., fractional fetal DNA and total template molecules required, toward which future research can work. In our opinion, 7,680 reactions for a fractional fetal DNA concentration of 25% should be achievable (SI Table 9) and allows correct disease classification 97% of the time.

To achieve a fractional fetal DNA concentration of 25%, methods are needed to allow the selective enrichment of fetal DNA (22) or the suppression of the maternal DNA background (11, 23) in maternal plasma. For example, although the effect of formaldehyde has not been universally observed by all groups (24, 25), Dhallan *et al.* (11) reported that 85% (51 of 60) of their formaldehyde-treated plasma samples had fractional fetal DNA concentrations ≥25% and Benachi *et al.* (26) reported a mean fetal DNA concentration of 36.8% in their formaldehyde-treated plasma samples. Besides physical methods for fetal DNA enrichment and maternal DNA suppression, molecular enrichment strategies, such as targeting fetal DNA molecules that exhibit a particular DNA methylation pattern (12, 21, 27), may be possible. In this regard, placenta-specific DNA methylation markers from chr21 have recently been identified (S. S. C. Chim, S. Jin, T. Y. H. Lee, F.M.F.L., W. S. Lee, L. Y. S. Chan, Y. Jin, N. Yang, Y. K. Tong, T. Y. Leung, *et al.*, unpublished work).

The number of plasma DNA molecules that are present per unit volume of maternal plasma is limited (8). For example, in early pregnancy, the median maternal plasma concentration of an autosomal locus, the β-globin gene, has been shown to be 986 copies per milliliter, with contributions from both the fetus and mother (8). To capture 7,680 molecules, DNA extracted from some 8 ml of maternal plasma would be needed. This volume of plasma, obtainable from ≈15 ml of maternal blood, is at the limit of routine practice. However, we envision that multiple sets of chr21 and reference chromosome targets can be combined for digital RCD analysis. For five pairs of chr21 and reference chromosome targets, just 1.6 ml of maternal plasma would be needed to provide the number of template molecules needed for analysis. Multiplex single-molecule PCR would thus be needed. The robustness of such multiplex single-molecule analysis has been demonstrated previously for single-molecule haplotyping (28). Thus, the SPRT approach outlined here could be modified for the analysis of multiple target loci by methods like mass spectrometry (28).

The implementation of digital PCR, as illustrated in this proof-of-principle study, is rather labor-intensive, requiring one or more 384-well PCR plates to be set up per case. However, alternative approaches for conducting digital PCR, such as using microfluidic digital PCR chips (29, 30), emulsion PCR (31), and massively parallel genomic sequencing (32), are now available. These latter methods would greatly enhance the clinical applicability of the methods proposed here for noninvasive prenatal diagnosis and for other applications in which allelic or chromosome imbalance is seen.

Materials and Methods

Digital RNA SNP Analysis. A real-time PCR assay was designed to amplify *PLAC4* mRNA, with the two SNP alleles being discriminated by TaqMan probes. *PLAC4* mRNA concentrations were quantified in extracted RNA samples followed by dilutions to approximately one target template molecule of either type (i.e., either allele) per well. We distributed the diluted sample to 96 wells for real-time PCR analysis to confirm that a usable dilution has been achieved. When ≈37% (i.e., e^{-1}) of the wells were shown to be negative for any amplification, we proceeded to the digital RNA SNP analysis using the same diluted sample for 384-well analyses. Details are given in the *SI Materials and Methods*.

Digital RCD Analysis. Extracted DNA was quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE) and diluted to a concentration of approximately one target template from either chr21 or chr1 per well. A real-time PCR assay was designed to amplify a paralogous sequence (19) present on both chromosomes, distinguishable by a pair of TaqMan probes. The diluted DNA sample was first analyzed by the assay using the chr1 probe only in a 96-well format to confirm whether ≈37% of the wells were negative; then we proceeded to digital RCD analysis using both TaqMan probes in 384-well plates. Details are given in the *SI Materials and Methods*.

Computer Simulation of Classification Accuracy. The computer simulation was performed with Microsoft Office Excel 2003 software (Microsoft, Redmond, WA) and SAS 9.1 for Windows software (SAS Institute, Cary, NC). Details are given in the *SI Materials and Methods*.

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

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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

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[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

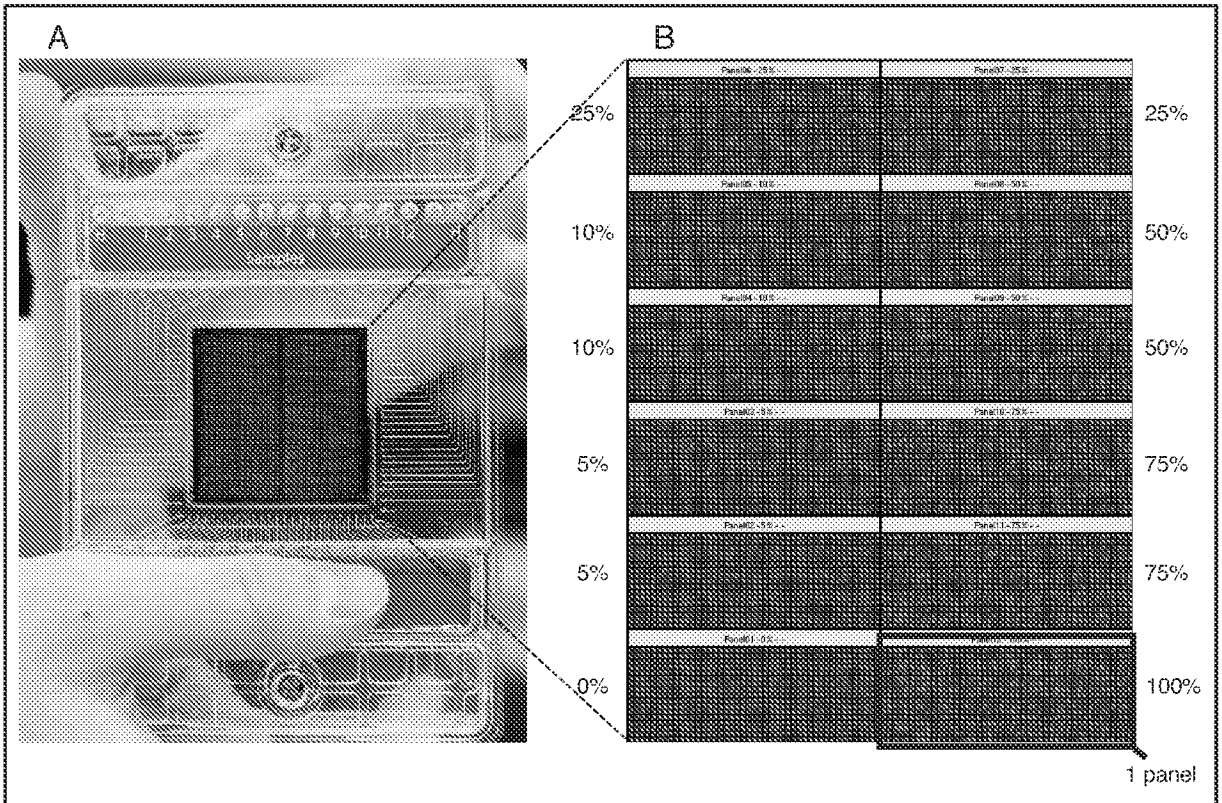

Fig. 1. Application of microfluidics chip for digital PCR analysis.

(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 non-digital *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

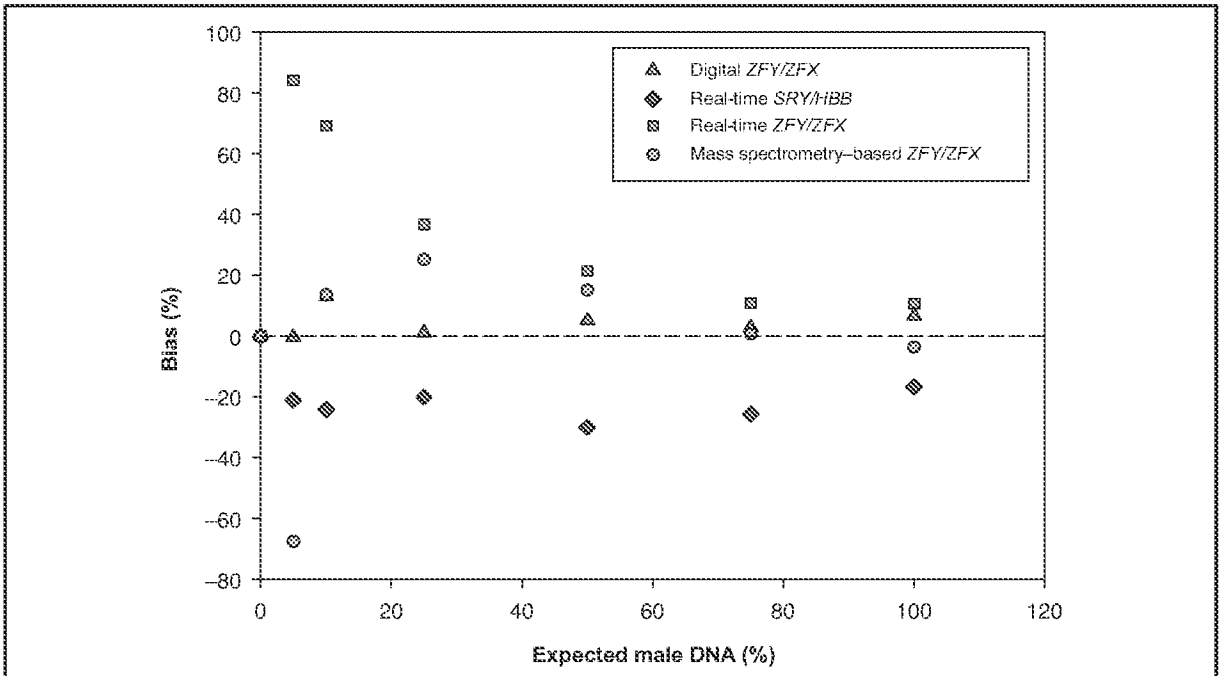

Fig. 2. Difference plot.

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

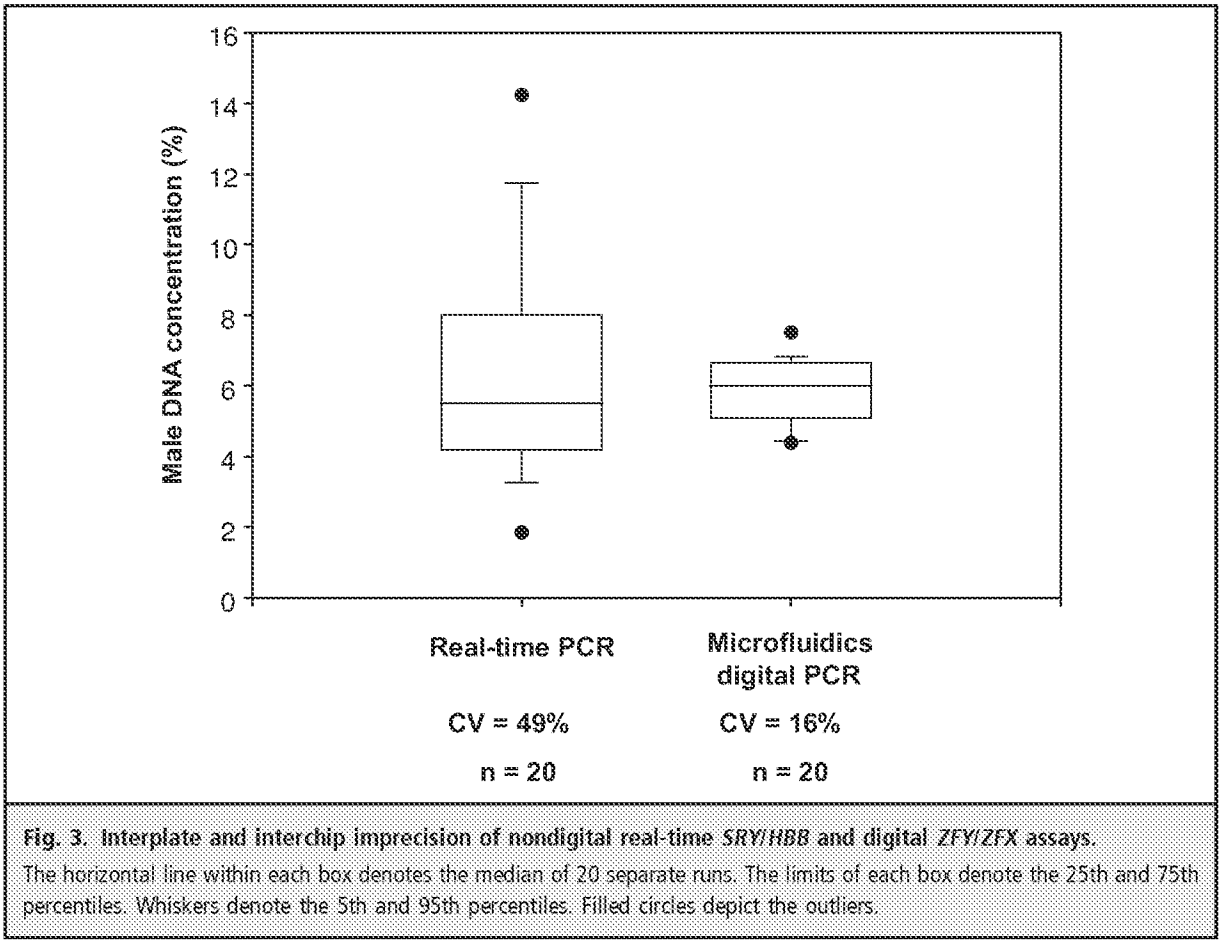

Fig. 3. Interplate and interchip imprecision of nondigital real-time *SRY/HBB* and digital *ZFY/ZFX* assays.
The horizontal line within each box denotes the median of 20 separate runs. The limits of each box denote the 25th and 75th percentiles. Whiskers denote the 5th and 95th percentiles. Filled circles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

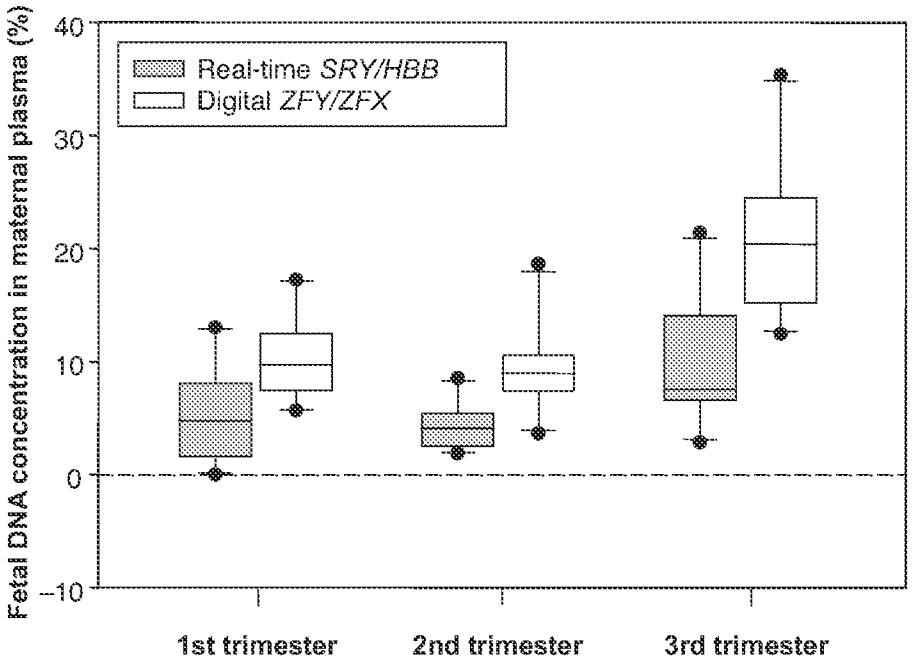

Fig. 4. Box plots of male fetal-DNA percentages measured with the nondigital real-time *SRY/HBB* assay and the digital *ZFY/ZFX* assay in first-, second- and third-trimester maternal plasma.

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.

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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.

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Review Article

Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine

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ABSTRACT

The efficient delivery of personalized medicine is a key goal of healthcare over the next decade. It is likely that PCR strategies will play an important role in the delivery of this goal. Digital PCR has certain advantages over more traditional PCR protocols. In this article we will discuss the current status of digital PCR, highlighting its advantages and focusing on how it can be utilized in biomarker development and analysis, including the use of individualized biomarkers. We will explore recent developments in this field including examples of how digital PCR may integrate with next generation sequencing to deliver truly personalized medicine.

1. Introduction

There is currently a great emphasis in both academia and the biotechnology industry on the development of meaningful molecular biomarkers to assist in the appropriate clinical management of patients. Such biomarkers will assist the rational matching of patients to effective therapies that should benefit them, and will facilitate the use of molecular stratification to inform prognosis and clinical decision-making.

There are significant biological, clinical, logistical and economic complexities in the delivery of high quality personalized medicine. However, it is clear that defining and validating molecular biomarkers is central to the process. Although many biological substrates can be used to derive biomarkers much work to date has concentrated on detecting and quantitating nucleic acids – RNA and DNA. In this review we focus on the potential of digital PCR as a platform to analyse nucleic acid biomarkers. Digital PCR is both conceptually simple and extremely robust in terms of assay performance; it has a number of specific attributes that may make it particularly applicable to biomarker assay in clinical scenarios.

2. Principles of digital PCR

The term "digital PCR" was coined by Kinzler and Vogelstein in 1999 [1], although the conceptual framework of limiting dilution of DNA and single molecule detection was laid out in prior reports [2,3]. The origins and principles of digital PCR have been extensively reviewed [1,4–6], but the concept remains relatively poorly understood and we will briefly review the principles here.

Digital PCR depends on the ability of PCR to detect a single molecule of a target locus. The sample is greatly diluted and divided into a large number of aliquots, so that some aliquots receive at least one molecule of the target ("positive" aliquots), whilst others do not. The number of positive aliquots, as determined by PCR, then reflects the abundance of the target locus in the sample Fig. 1.

If the sample is sufficiently dilute, only a few of the aliquots will be positive, and each of these positive aliquots can be assumed to have contained only a single target molecule. In this case, the process equates to a direct and simple counting of molecules – the "digital" in "digital PCR".

More often, though, the sample is not diluted quite so far. Then many (but not all) of the aliquots will be found to be positive, and some of these positive aliquots will probably (and unbeknownst to the experimenter) have contained two, three or more target molecules. Therefore, simply counting positive aliquots will underestimate the true number of molecules. This can be corrected by using the Poisson equation, a simple statistical tool which calculates the *average* number of molecules per aliquot from the observed proportion of positive aliquots. (The equation is $A = -\log_e(1-P)$, where A is the average number of molecules per aliquot, and P is the proportion of positive aliquots.)

In this way, it is easy to calculate the absolute abundance of the target sequence in the sample [1,3]. More commonly, though, the abundance of the target sequence is compared to that of a reference sequence analysed in the same way, to determine the target's

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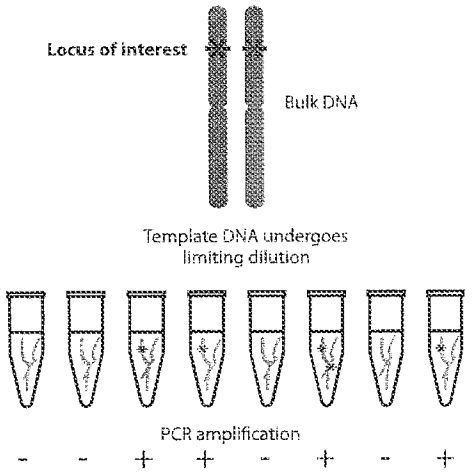

Fig. 1. Limiting dilution PCR. The principles underpinning digital PCR are very simple. DNA undergoes limiting dilution. PCR is then used to probe each aliquot for the presence (+) or absence (−) of a locus of interest.

relative abundance [1,7]. The reference sequence is usually chosen to be one whose abundance is known – for example, one which is present in two copies per diploid cell.

Besides being able to accurately quantify target sequences, digital PCR can also be used to identify rare variants, such as mutations present in only a small minority of the cells from which the DNA is isolated. Digital PCR experiments can be designed so that each positive aliquot is the result of a single or a few template molecules being amplified. In those aliquots in which a rare variant is present, it's detection is not swamped by the more common variant, as it would be if bulk DNA were amplified [8,9]. In practice, rare variant detection requires well-designed experimental protocols with marker validation and non-template controls, but here the point is to emphasize the potential of digital PCR in terms of sensitivity and quantitation of rare variants.

The precision (reproducibility) of digital PCR-based quantitation and its capacity to detect very rare variants depends on the total number of aliquots that are interrogated – the precision and sensitivity increase as more aliquots are analysed.

The degree of dilution of sample is also important: if it is too dilute, then very few aliquots will be positive, and the data will be unreliable. If it is not dilute enough, then all the aliquots will be positive, and no quantitative information can be obtained. A number of authors have investigated how linear the response is to DNA concentration using various platforms [10–12]. In a recent study using a droplet digital PCR system (ddPCR) and interrogating 20,000 partitioned reactions (microdroplets), a linear response to DNA concentration was obtained in droplet saturation in the interval 0.16–99.6% [11]. However, the relative uncertainty in DNA concentration varied across this dynamic range – in particular at the lower end of this range the impact of stochastic events on the estimated copy-number increased. Similar observations were made in a study using a microfluidic based approach [12].

The uniformity of partition volume is also a critical determinant of the accuracy of copy-number estimation and becomes particularly important when the number of partitions exceeds one thousand [11].

Finally, in digital PCR as opposed to QPCR, the efficiency of reactions only has to reach a threshold at which a product will be detected if present. Therefore, it may be unimportant if one reaction is more efficient than another as long as both are sufficiently efficient to amplify a molecule if present. This will potentially reduce the number of primer design 'failures' when biomarker assays are designed.

There are a number of clinical circumstances in which the accuracy and precision of quantitation of potential biomarkers that can be delivered by digital PCR may be very attractive.

3. Digital PCR – attributes

3.1. Rare variant detection

There is a move towards using molecular biomarkers obtained from peripheral blood sampling to detect specific mutations and monitor disease progression, recurrence and stability [13–15]. The assay needs to be able to detect a low proportion of mutant alleles in a huge excess of wild type alleles. Digital PCR can readily achieve this aim. There is also increasing evidence that each individual's cancer may have diverse subclonal populations [16,17]. The clinical relevance of this is that subclones may harbor specific mutations that confer resistance to currently available cancer therapeutics. Examples of this are discussed in more detail in Section 5.

3.2. Estimating copy-number variation

The clinical implications of very precise estimates of germ-line or somatic copy number variants (CNVs) are unclear and will vary depending on the clinical scenario. However, CNVs do alter gene expression [18] and therefore may well be of clinical importance.

The attributes of digital PCR discussed above facilitate the accurate and precise discrimination of the number of copies of specific loci. Assuming reference diploid loci have been validated and therefore have a relative copy-number of two, it is possible to distinguish between one (indicating allelic loss) and two copies, and also between higher integers, for example, five and six copies [9,19,20]. Digital PCR performs better in this regard than other currently available methods including QPCR [9,10,21].

3.3. Minimal template requirements

A key advantage of the digital PCR strategies is that template requirements are generally low. This is of particular importance in some clinical scenarios when tissue samples may be limited in size and/or heterogeneous, or when extracted nucleic acids are degraded as a result of processing [22]. In many genomic analyses (array CGH, next generation sequencing) of limited clinical material a pre-amplification step has been used with the intention of increasing the abundance of all sequences of interest, without altering their *relative* abundances. In practice, however, unbiased pre-amplification is very difficult to achieve, and has been shown to introduce bias in digital PCR [10] and other genomic platforms [23,24]. The importance of this bias will depend on the specific application.

With respect to digital PCR, the low template requirements mean pre-amplification should generally be unnecessary – therefore the data generated will not be subject to pre-amplification bias.

3.4. Ease of analysis

The digital nature of the results means that data handling is relatively straightforward. Some platforms have automated thermocycling, data capture and analysis meaning that the generation of results can be streamlined. A basic analysis of the results for relative quantitation that would probably be sufficient for most readers purposes is relatively straightforward, requiring only the

application of the Poisson equation (to convert the proportion of positive aliquots into an abundance), and the normalization of the abundance of the query sequence to that of one or more reference loci. Those readers interested in more detailed discussions on the analysis of digital PCR data and the potential sources of error in quantitation of loci are referred to the following publications and the references therein [10–12,21].

3.5. Integration with next generation sequencing protocols

Arguably the greatest biotechnology achievements of the last few years have been in the domain of sequencing. The potential for clinical benefit is huge although much more work is required to define the best use of next generation sequencing (NGS) in the clinic and the balance between whole genome and more targeted approaches. Digital PCR may be a very useful complementary technology. Examples of this have already emerged, notably in the use of digital PCR to detect individualized biomarkers in patients whose tumours have undergone paired-end NGS analysis [14,25]; but also in the quantitation and preparation of NGS libraries [26]. It remains unclear what corroboration of NGS results will be deemed clinically necessary – at present anomalies detected by next generation screens are often confirmed using standard Sanger sequencing [27], so that there may be a role for PCR-based confirmation of NGS mutations.

4. Digital PCR – other issues

4.1. Familiarity

The profile of digital PCR remains low, although there are signs that this is changing, with the entry of more biotechnology companies and new platforms into the market.

4.2. Contamination

In any PCR protocol, appropriate systems and controls are critical to ensure that contamination does not occur. This is particularly true for digital PCR, as the assay is sensitive to even a single contaminant molecule. The systems necessary to avoid contamination include a reliable supply of clean reagents, a dedicated PCR suite and controls on the concentration of template DNA permitted in the laboratory.

4.3. Standardization/sources of bias

A huge effort has been made to produce the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines for the standardization of experimental design, analysis and reporting in quantitative PCR (QPCR) [28]. Many of the remaining issues for this technique concern the pre-analytical handling of samples. Such efforts at standardization afford confidence to laboratories considering using biomarkers validated using QPCR.

The reproducibility of digital PCR has recently been shown to be superior to QPCR [10]. However digital PCR is a relatively young technology and there are potential sources of bias that need to be identified and minimized. As with QPCR, DNA fragment size can significantly impact upon digital PCR results [10,12]. Similarly, results can be affected by sustained template exposure to high temperatures and variation in partition volumes [12,29].

When the sources of variation in a digital PCR experiment are better understood, an international effort to develop digital PCR guidelines similar to the MIQE guidelines would be warranted.

4.4. Multiplexing/throughput

It will be clinically important to measure a number of separate clinical biomarkers on individual patient samples. There are two issues which affect the ability to achieve this – first, whether the experimental platform lends itself to multiplex analysis, the second the quality and quantity of nucleic acids that are available from the clinical specimen. The choice of platform will be discussed in Section 6.

In many clinical scenarios, tissue (blood) and therefore DNA/RNA is plentiful and does not limit analysis. However, in many other cases diagnostic tissue samples are heterogeneous, limited in size and fixed in formalin to preserve histological integrity. Therefore, a key goal will be to deliver a system that facilitates the routine analysis of multiple clinically relevant biomarkers using limited template. This is an aim that we have been particularly focused on in the past [22,30,31], albeit using a relatively low throughput platform. The challenge will be to deliver, on an automated high-throughput platform, a truly multiplexed digital PCR system capable of the parallel analysis of sequence mutations and copy-number variations (CNVs) using limited quantities of template derived from diagnostic specimens.

As well as the number of specific biomarkers that can be interrogated on a given sample the throughput potential and demands on laboratory staff with respect to "hands-on" time are critical issues for any clinical biomarker assay.

5. Application of digital PCR to biomarker detection

Digital PCR strategies have already been successfully applied to measure biomarkers in a range of clinical scenarios. We will review a number of these examples, in an attempt to illustrate the potential for broad application of this technology. Although we emphasize the potential application in oncology, the principles discussed may be applied to many aspects of clinical medicine.

5.1. Mutation/rare variant detection

Since the successful development of imatinib for the treatment of chronic myeloid leukemia with the pathological bcr–abl fusion [32] there has been intense interest in developing biological therapies that target specific gene-products. For example, it is increasingly common for patients to be stratified to targeted therapies on the basis of the presence or absence of specific mutations. Solid organ tumours are now routinely screened for mutations in oncogenes such as *EGFR*, *PIK3CA* and *KRAS* since they predict response to specific and traditional chemotherapeutic regimes [33,34]. Digital PCR strategies have been used to detect EGFR mutations directly in tumour samples, and importantly, the frequency of EGFR mutant alleles can also be accurately estimated using this approach [35]. The importance of the mutant allele frequency has been touched on before [36,37], but is not assessed in current practice. Indeed, it could be argued that the use of very sensitive screens for specific mutations that do not inform the user of the mutant allele frequency may be misleading, and result in the targeted treatment of what is, in effect, a minority subclone (Fig. 2).

It is therefore plausible that two important parameters – (1) the percentage of cancer cells that carry a specific druggable mutation; and (2) whether a specific allele is amplified as well as mutated – may have a major impact on the response to specific therapies. Clearly, this mandates the analysis of as pure population of cancer cells as is feasible with negligible contamination from surrounding stroma cells. This is less challenging than it may previously have been with newer semi-automated laser capture microdissection.

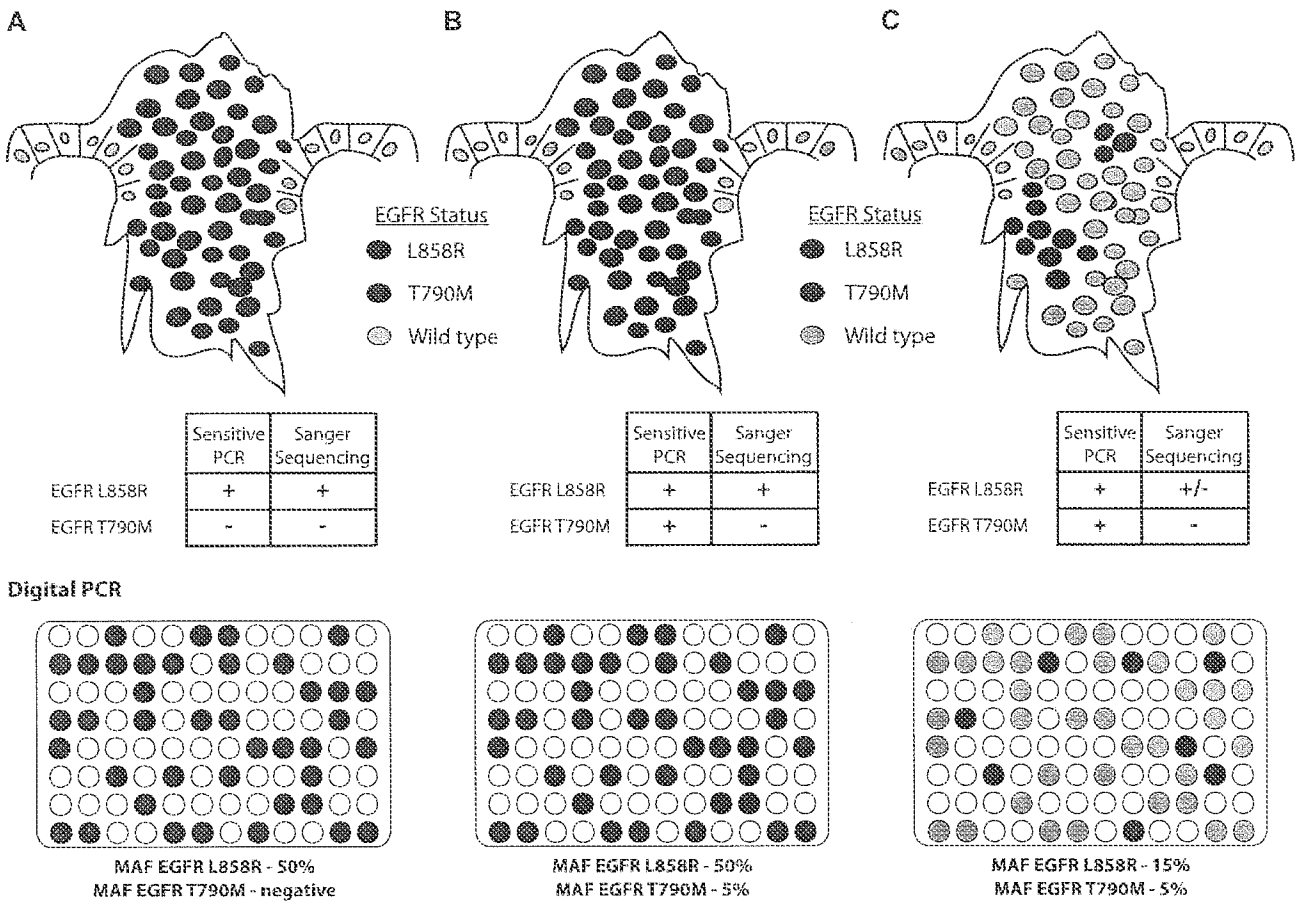

Fig. 2. Digital PCR, mutant allele frequency (MAF) and test sensitivity. The issue of sensitivity and mutant allele frequency in biopsy material has rarely been addressed but may have a significant impact on the interpretation of molecular biomarkers and the delivery of personalized cancer medicine. Currently available detection strategies (probe-based, COLD-PCR) report a sensitivity of approximately 1% for mutations. However the mutant allele frequency is not estimated. In this figure there are cartoons depicting three potential genetic scenarios in lung tumours that are morphologically identical. In each case the tumours are tested for the common mutations in the epidermal growth factor receptor (EGFR) including the activating mutation L858R that generally confers sensitivity to specific tyrosine kinase inhibitors (TKIs) and the T790M mutation (which confers resistance to the same TKIs). In scenario A, all cancer cells harbor the L858R mutation but none have T790M. Traditional (Sanger) sequencing on "bulk DNA" can detect variants to a lower MAF threshold of 20–30%. It would therefore capture this mutation, as would the more sensitive protocols available now. Recent work (Section 5.1) has shown that there is often a subclone of cells that carry the T790M mutation as is suggested in B. This rare mutation would be detected by sensitive protocols but not traditional sequencing. A further possible scenario is C in which three subclones exist, but the dominant clone does not have any mutation in EGFR. Currently, all three tumours would be treated in the same way, but are likely to have very different responses to EGFR TKIs. The potential advantages of digital PCR are that it can detect rare variants but also estimate the absolute frequency of each mutant allele. Therefore, tumours B and C that would now be genotyped as equivalent by sensitive sequencing, could be recognized from the outset to be significantly different from a biological perspective. We could then start to tease out the impact of MAF on outcome or response to therapy.

To emphasize this point, it has recently been confirmed that cancers often consist of multiple subclones [17,38]. This presents multiple challenges to successful targeted therapy, especially if a pre-existent subclone harbors a mutation that confers resistance to a drug under consideration [16,39]. Such drug resistant subclones can exist at the start of treatment and their pretreatment detection may be useful to identify patients who are likely to relapse early after therapy [40] or those who may benefit from combination targeted therapy. This proven tumour heterogeneity presents a direct challenge to the notion of personalized medicine.

A recent study has illustrated the potential importance of digital PCR in evaluating the presence of rare variants/subclonal populations. In this NGS study of hepatocellular carcinoma a mutation in a key tumour suppressor gene was not detected in a whole genome screen with 30× coverage, but was detected in a paired exome resequencing screen with 76× coverage [37]. The same mutation was then sought by traditional capillary sequencing and was difficult to call with certainty. However, a digital PCR analysis both detected the mutation easily, and precisely quantified the mutant allele frequency at 13.2% [37]. Therefore platform sensitivity and ability to accurately call mutant allele frequency may be a major focus of future research.

A potentially very important application of the ability of digital PCR to detect rare variants is the use of routine blood samples for the analysis of nucleic acids originating from solid tumours – either as a result of metastasizing cells or the leakage of DNA from solid tumours into the peripheral circulation [41]. This would facilitate the (repeated) analysis of molecular biomarkers from peripheral blood with a minimum of inconvenience for the patient [42]. One obvious application would be monitoring a patient's response to chemotherapy. For example, digital PCR has been shown to be effective at detecting residual copies of the bcr–abl fusion transcript in patients with chronic myeloid leukemia [25,43]. PCR detection of residual disease in CML has been used clinically for some time. This recent head-to-head comparison of digital and more traditional PCR approaches demonstrated that digital PCR

may offer better sensitivity without the need for a pre-amplification stage [25]. In patients with lung cancer circulating DNA has been successfully analysed for EGFR mutations using digital PCR [42]. Encouragingly, using a microdroplet system the ability to detect a mutant allele at a ratio of 1:100,000 was recently demonstrated [9,20]. Again, the clinical relevance of such low frequency circulating mutant alleles in solid organ tumours has not yet been clarified, although it suggest the lack of a complete response to treatment: this is an issue that needs to be addressed in future studies.

A non-oncological application for the detection of rare variant DNA in peripheral blood has been demonstrated in analysing fetal DNA in maternal peripheral blood samples. The proof of principle for this type of approach has been established in Trisomy 21 [44]; however it may be clinically superseded by the use of NGS platforms to detect fetal trisomy [45,46].

In a related, and very exciting potential application in the field of transplant medicine, digital PCR was recently used to quantify cell-free donor-specific DNA molecules in the peripheral blood of heart transplant recipients [47]. On the premise that the quantity of donor DNA in the circulation would reflect cellular rejection of the graft, the quantity of donor specific loci was used as a molecular biomarker that predicted graft rejection. Again, digital PCR was not used to identify the biomarker (performed by shotgun NGS), but it was the method of choice for quantitation of specific loci in consecutive clinical samples.

5.2. Pharmacogenetics

In addition to the detection of somatic variants that inform the choice of treatment there is evidence that germline variation in certain genes can profoundly affect the individual's response to particular therapies [48,49]. In some cases these are point mutations or SNPs that may affect drug metabolism; in other cases copy-number variants at specific loci may be predictive of how an individual will handle a drug. The added precision of digital PCR may become useful in the future when more data emerges on the role of germline CNVs in pharmacogenetics.

5.3. Gene expression analysis

As well as the analysis of genomic DNA, gene expression analysis using cDNA as template is theoretically and technically straightforward using digital PCR protocols. The dynamic range required to analyse variably expressed genes and reference/housekeeping genes may be more suited to QPCR, particularly when comparing common highly expressed reference loci to rarer transcipts. The dynamic range afforded by newer digital PCR platforms could support this application using well chosen reference loci.

An important potential niche for digital PCR may be the profiling of transcripts from single cells or small numbers of cells. For the reasons discussed earlier, digital PCR is particularly well suited to samples in which material is limited. Single cell transcript analysis has been much discussed in the recent literature and in particular the greater cell-to-cell variability in RNA levels and the potential for bias in a preamplification step prior to QPCR [50]. With respect to dynamic range, most digital PCR platforms (assuming good experimental design and choice of reference transcript) will have sufficient partitioned reactions to deliver precise absolute quantitation of specific transcripts from single cells. However, it remains to be seen whether single cell expression analyses, even if optimized, could be exploited for developing or assaying biomarkers in the clinic.

There has been an explosion of interest in the biology of non-coding RNA (ncRNA) in the last few years. The role of a subgroup of ncRNA–microRNAs – in human pathology has been a subject of particularly high research activity [51,52]. A microfluidic system has been successfully used for miRNA analysis in a RT-PCR protocol with pre-amplification [53]. In principle, microRNA analysis using digital PCR will be feasible, as it has been used to quantitate other transcripts, and has the potential to complement discovery platforms (NGS) in the validation and analysis of ncRNAs.

5.4. Methylation-specific digital PCR

The epigenetic control of gene expression is altered in multiple disease states, in particular cancer. For many years there has been interest in exploiting this to derive molecular biomarkers for both prediction and prognosis. To some extent progress has been disappointing in terms of FDA-approved diagnostics, although there is evidence to support the use of specific methylated loci in some diseases [54]. Methylation-specific PCR has been the cornerstone of these efforts. Digital PCR protocols have also been optimized for the analysis of methylated loci and again, have the potential to afford some advantages over standard techniques [55]. Examples include the use of digital protocols to quantitatively assess methylation at specific loci in colorectal cancer specimens and in plasma obtained from patients with breast cancer [56]. Others have developed digital protocols that allow the single molecule capture of both methylation and histone modifications [57].

6. Choice of platform

There are now numerous available platforms for digital PCR. A significant distinction is whether a platform incorporating a microfluidic chip is chosen [20,58–61], or if the PCR is performed in microdroplets [9], [62,63]. Attributes of the currently available systems are outlined in Table 1. Choice of platform depends on a user's specific experimental/assay requirements and it would be advisable to contact the companies involved as platforms are constantly being refined.

The issues on which to base a decision will be weighed by the degree of precision required (number of partitions, availability of template DNA), the cost of the platform, the cost per assay and the throughput required. Further important considerations will include whether applications other than digital PCR are desired, whether measurement of absolute or relative quantitation is more relevant, whether there is potential to integrate the platform with other technology such as NGS and whether a laboratory would wish to run low volume QPCR experiments on the same platform.

7. Role of digital PCR in the era of next-generation/whole genome sequencing

There has been a huge increase in the use and breadth of applications of next generation sequencing technology in the last three years. NGS has huge experimental capacity and facilitates the parallel analysis of massively multiplexed bar-coded samples. The number of reads per locus affords the potential for data from targeted resequencing protocols to be used to estimate copy-number variation [64]. This means that strong cases need to be made for any proposal to analyse nucleic acids in the future using a non-NGS platform such as digital PCR. The relative advantages of digital PCR remain the accuracy of quantitation, the reproducibility of the data and the ability to analyse very small samples. Furthermore, as discussed in Section 5, there is great potential for the integration of the two protocols in terms of targeted resequencing, the development and use of individualized biomarkers and monitoring the response to chemotherapy in peripheral blood samples.

Table 1
Summary characteristics of the major platforms currently available for digital PCR.

Platform	Description	Number of reactions	Aliquot volume	Analysis	Published sensitivity for rare variants	Integrated thermocycling and analysis	Commercial availability	Published applications
Microdroplets								
ddPCR, BioRad	Microdroplets are generated in an emulsion and transferred to 96 well plates for cycling then to the custom analysis unit (QX100 droplet reader). The reader unpacks the emulsion to single droplets for analysis.	20,000 per 20 μl sample	1 nL	Automated droplet flow cytometer (two colors) with Taqman probes	0.001%	No	Yes QX100 Droplet digital PCR System	Genotyping absolute quantification [9,11]
RainDrop, raindance technologies	Microdroplets, are generated in an emulsion, collected and transferred for thermocycled. The emulsion is then injected onto a microfluidic device and each droplet is analysed	Continuous flow	9 pL	End point analysis with TaqMan probes	0.0005%	No	Yes RainDrop digital PCR system	Genotyping[65] Absolute quantification[63]
BEAMing (beads, emulsion, amplification, magnetics)	Microdroplets containing magnetic beads are generated in an emulsion and transferred to 96 well plates for thermocycling. The emulsion is dispersed and the beads separated. A circularizable probe is hybridized to the sequences on the beads and the changes of interest are labeled with fluorescently labeled dideoxynucleotide terminators	5×10^7 beads	9 μm diameter	Labeled beads are analyzed by flow cytometry	0.01%	No	No	Genotyping Absolute quantification [13]
Microfluidic chambers								
MegaPixel digital PCR	Surface tension based sample partitioning creates aliquots that are thermocycled and analyzed on the device. Fluorescent probes are annealing during thermocycling to enable analysis	1×10^6	10 pL	Microarray scanner	0.001%	Yes	No	Genotyping [20]
Spinning disk platform	Aliquots are generated by passive compartmentalization through centrifugation. These are thermocycled and analysed on the device	1,000	33 nL	CCD camera – end point melting curve analysis	–	Yes	No	Copy number variation and absolute quantification [61]
OpenArray Life technologies/ ABI	Microfluidic reaction chambers are loaded, thermocycled and analysed using the OpenArray system. Chambers may be preloaded with the assay of choice	3,072	33 nL	CCD camera – real time PCR end point melting curve analysis	–	Yes	Yes OpenArray Real-Time PCR platform	
Digital array chip, fluidigm	Microfluidic reaction chambers are loaded, thermocycled and analysed using the BioMark system	9,180 (12 × 765) Prototype 2 × 100,893	6 nL	CCD camera – real time PCR end point melting curve analysis		Yes	Yes BioMark HD system	microRNA expression [53] Single cell gene expression [66] Genotyping [35] Targeted resequencing [67] Copy number variation[19,21,60] Absolute quantification [25]

8. Summary

Despite the important caveats expressed in Section 5.1 about personalized medicine in oncology, it is definitely here to stay. Digital PCR is a quantitative method that combines a robust and well-validated technique (PCR) with unrivalled accuracy and precision of quantitation. It is likely that digital PCR will continue to be a very useful tool for those searching for and validating nucleic acid molecular biomarkers for clinical application.

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Digital PCR hits its stride

Monya Baker

As the less familiar cousin of quantitative PCR moves mainstream, researchers have more options to choose from.

A few years ago, Ramesh Ramakrishnan had to spend so much time explaining what digital PCR was that he had to rush through his explanations of applications when he gave talks at meetings. Now, he says, most audiences are at least familiar with the term, even if they have not performed the technique themselves. "It's no longer an exotic thing," says Ramakrishnan, director of R&D at Fluidigm Corporation.

The strategy for digital PCR (dPCR) has been summarized as 'divide and conquer': a sample is diluted and partitioned into hundreds or even millions of separate reaction chambers so that each contains one or no copies of the sequence of interest. By counting the number of 'positive' partitions (in which the sequence is detected) versus 'negative' partitions (in which it is not), scientists can determine exactly how many copies of a DNA molecule were in the original sample. Among other applications, researchers have used digital PCR to distinguish differential expression of alleles[1], to track which viruses infect individual bacterial cells[2], to quantify cancer genes in patient specimens[3] and to detect fetal DNA in circulating blood[4].

The concept behind digital PCR was first described in 1992 (ref. 5). A few years later, Bert Vogelstein and Ken Kinzler at Johns Hopkins University named the technique and showed that it could be used to quantify disease-associated mutations in stool from patients with colorectal cancer. But although the theory was simple, its implementation was not. Initial demonstrations were performed in commercially available 384-well plates with 5 microliters per partition, requiring volumes of reagents that would daunt most researchers[6].

Advances in nanofabrication and microfluidics have now led to systems that produce hundreds to millions of nanoliter- or even picoliter-scale partitions. Academic technology developers have described several implementations, but so far only a handful of companies have commercialized products or announced plans to do so (Table 1). Fluidigm and Life Technologies create reaction chambers within specially designed chips or plates. Bio-Rad and RainDance sequester reagents into individual droplets.

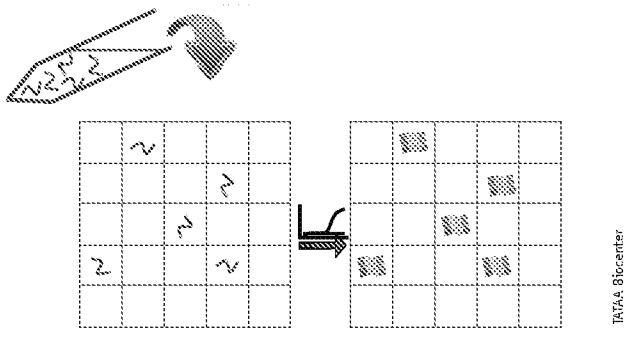
Higher costs, higher precision

The most popular PCR technique to measure the presence and concentration of a DNA sequence is not digital PCR but its more familiar cousin, real-time quantitative PCR (qRT-PCR, or qPCR). In qPCR, DNA is copied until it produces a certain level of signal; the number of amplification cycles needed to reach this point is then used to calculate how many DNA molecules with the particular sequence were originally present relative to other DNA molecules in the sample.

Digital PCR uses the same primers and probes as qPCR but is capable of higher sensitivity and precision. In standard implementations, qPCR cannot distinguish gene expression differences or copy number variants smaller than about twofold. Identifying alleles with frequencies of less than about 1% is difficult because such tests would also detect highly abundant common alleles with similar sequences. In contrast, dPCR can measure a 30% or smaller difference in gene expression, distinguish whether a variant occurs in five versus six copies and identify alleles occurring at a frequency of one in thousands. It can also be used to standardize qPCR assays.

The more partitions, the greater the resolution. "If you want to distinguish between 2 and 3 copies, you need 200 chambers. If you want to distinguish between 10 and 11, you need 8,000," explains Mikael Kubista, CEO of TATAA Biocenter, which provides services and training in both qPCR and digital PCR. In principle, one could also get similar precision by doing 8,000 replicates of qPCR, he says, but such experiments are impractical.

Jim Huggett is the science leader for nucleic acids metrology at LGC, a laboratory services and measurement standards organization in the UK. His team has directly compared the two techniques across several DNA templates and other conditions[7]. Digital PCR offers more accuracy and less ambiguity than qPCR, he says, but qPCR has enticing advantages. It is less expensive and works over a much broader



Digital PCR works by diluting a sample into many partitions and counting up the number of partitions in which a reaction occurs.

Table 1 | Commercial digital PCR offerings

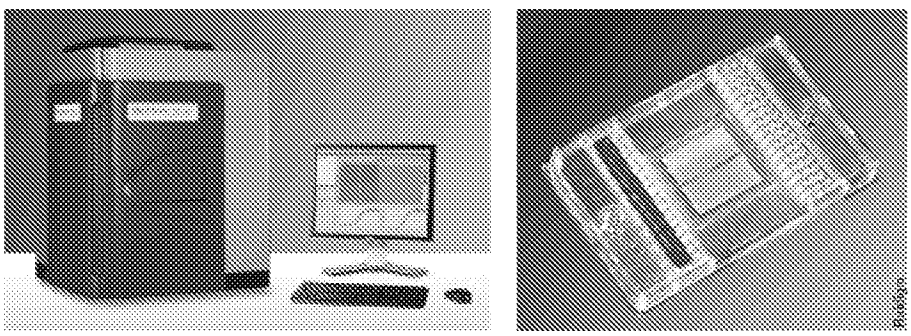
Vendor	Instruments and list price	Consumables and list price	Number and volume of partitions	Volumes required	qPCR capacity	Multiplexing
Fluidigm Corporation	BioMark HD: $200,000–$250,000	12 arrays per chip[a] (765 wells per array): $400 per chip (works in both EP1 and BioMark)	12-inlet chip: 9,180 partitions, 6 nl per partition	12-inlet chip: 8 µl of mix, ~4 µl of sample; 57% analyzed[b]	Yes	Can use up to 5 colors to detect 5 targets (assumes 5th color is ultraviolet)
	EP1: $100,000–$150,000	48 arrays per chip[a] (770 wells per array): $800 per chip (works in both EP1 and BioMark)	48-inlet chip: 36,960 partitions, 0.85 nl per partition	48-inlet chip: 4 µl of mix, ~2 µl of sample[b]	No	Can use up to 5 colors to detect 5 targets
Life Technologies	OpenArray RealTime PCR System and QuantStudio 12K Flex instrument: $140,000 and $90,000–$190,000, respectively	OpenArray plates[a] (64 holes per subarray): $150 per plate	Varies; 3,072 partitions per plate, 48 subarrays per plate, 33 nl per partition (machines run 3–4 plates at once)	100 µl of sample per plate (across 48 arrays)	Yes	Uses 2 colors of probes to detect 2 targets
Bio-Rad Laboratories	QX100 ddPCR System (machines to generate and read droplets): $89,000	8 samples per chip (14,000–16,000 droplets per sample): $3 per sample	Up to 96 samples per run (assumes manual pipetting into PCR plate); 1,344,000 partitions per run (assuming separate thermocycler runs 12 chips at once), 1 nl per partition	Up to 9 µl per sample (20,000 droplets made); an average of 70% read	No	Uses 2 colors to detect 2 targets
RainDance[c]	RainDrop Digital PCR (machines to generate, collect and read droplets): $100,000	8 samples per chip (up to 10,000,000 droplets per sample): $10–$30 per sample	8 samples per run; up to 80,000,000 partitions per run, 5 pl per partition	5–50 µl per sample	No	Uses 2 colors, but can use varying concentrations of probes to detect up to 10 targets

[a]Arrays can hold separate samples, or the same sample can be spread over multiple arrays. [b]For rare allele analysis, protocols are available to eliminate the dead volume. [c]Plans full commercial launch later this year.

dynamic range than digital PCR. For example, it can determine that transcripts of one gene are as much as a billion times more abundant than transcripts of another gene.

Also, qPCR experiments can routinely analyze hundreds of sequences per sample run. Eventually, Kubista believes that it will be possible to multiplex dPCR to examine perhaps as many as 100 reactions at once, but no one would consider measuring large numbers with digital PCR today, he says. And qPCR is already well-integrated into many researchers' labs. "We've been developing workflows for qPCR for 20 years." In contrast, the first full conference dedicated to applications of digital PCR is scheduled for October of this year (see http://www.healthtech.com/digital-pcr).

MicroRNA researcher Muneesh Tewari at the Fred Hutchinson Cancer Research Center uses digital PCR in situations where absolute quantification is important, such as when detecting low-abundance RNA. One advantage of digital PCR is that with more partitions, a greater volume of a dilute RNA sample can be analyzed, he says.



Fluidigm Corporation's BioMark HD System for digital PCR and qPCR.

Also, digital PCR does not require the calibration and internal controls necessary for qPCR. Instead, counts from replicate wells can simply be added together. "Thinking in terms of absolute copies is so intuitive," he says. Nonetheless, his lab currently performs more qPCR experiments than digital PCR experiments. qPCR has lower cost and higher throughput, says Tewari, and his staff is more familiar with it.

That's a typical situation, says Paul Pickering, head of the digital PCR business unit at Life Technologies. "Most customers are seeking to do RT-PCR and then, in the situations that they need it, they'll deploy digital PCR." In those cases, he says, "there are four attributes that customers value: sensitivity, specificity, precision of the answer and the fact that you can get an absolute count without needing to reference any other material."

Digital PCR on chips

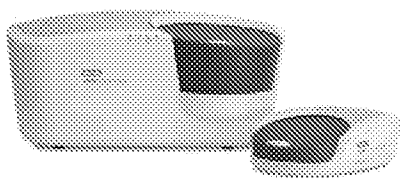
In 2006, Fluidigm became the first company to commercialize digital PCR. It offers two systems that mix samples with reagents, partition the reaction mixture, perform thermocycling and read

results within each partition. The simpler, cheaper EP1 machine detects only endpoints, that is, whether or not a reaction has occurred. The BioMark HD System, which also performs qPCR, can be set to monitor the course of a reaction and provide data that can eliminate false positives. Both systems use chips containing sophisticated microfluidics and tiny valves that partition samples into about 800 reactions, with either 12 or 48 samples per chip. If researchers want to run more reactions per sample, they can just double up arrays within chips or even double up on chips, says Ramakrishnan.

The company has developed another chip called the 200K with hundreds of thousands of partitions, and has licensed separate technology for chips with as many as a million partitions. However, plans to commercialize these technologies are on hold pending greater demand. "We can go up in terms of partitions, but we haven't found a huge pull from the market in going to that higher density," says Ramakrishnan.

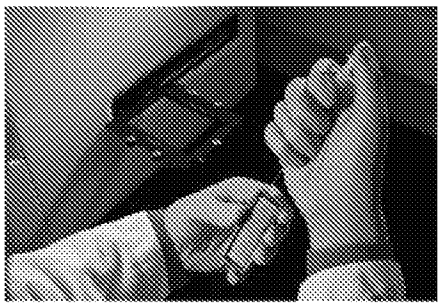
Life Technologies began offering digital PCR in 2009 after acquiring long-time collaborator BioTrove. It now sells two machines that can be used for both digital PCR and qPCR, the OpenArray and QuantStudio 12K Flex. These mix samples with reagents, load mixtures into reaction chambers, run amplification cycles and monitor reactions as they occur. The machines rely on plates that are roughly the size of a microscope slide and are essentially highly engineered peg boards with nano-sized holes; capillary forces and careful placement of hydrophilic and hydrophobic surfaces hold samples in place.

The OpenArray machine holds up to three plates, each containing 48 arrays with 64 partitions apiece. QuantStudio holds up to four plates and can also accept formats used in high-throughput qPCR experiments: TaqMan Array cards as well as 96- and 384-plate formats. "What we found is that a lot of our customers want the capability [for digital PCR] but aren't ready to jump in and say that that's the only thing that they have to do," says Pickering. Buying a machine that can do both, he says, is similar to the decision to purchase a hybrid gas and electric automobile rather than an electric-only vehicle.



Bio-Rad

Bio-Rad's QX100 droplet digital PCR System.



Life Technologies

Life Technologies' QuantStudio System for digital PCR and qPCR.

Digital PCR in droplets

Companies like Bio-Rad and RainDance sell machines that cannot perform qPCR but which offer many more partitions. In droplet digital PCR, reaction chambers are separated not by the walls of a well but by carefully titrated emulsions of oil, water and stabilizing chemicals. First, samples are put into a machine where they are mixed with all the necessary reagents and dispersed into tiny droplets. The droplets for each sample are transferred into tubes that can be placed in a thermocycler for PCR. Afterward, the tubes are transferred to a droplet reading machine, which functions like a flow cytometer to analyze each droplet for whether or not a reaction has occurred.

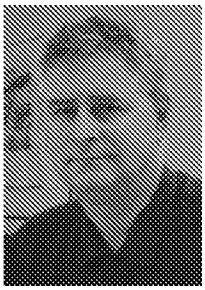
QuantaLife launched the first commercial digital droplet PCR system a year ago. In December 2011, the company was acquired by Bio-Rad Laboratories for $162 million, with promises for more cash if products hit certain milestones. All along, the goal was to develop an instrument that was both inexpensive and easy to use, says Bio-Rad marketing manager Mike Lucero, who was an early employee of QuantaLife. "We have two rules at the company: no chips and no lasers." He's betting that the low cost of consumables will set the company apart, he explains, holding up a clear, lightweight strip studded with sets of cups for holding collections of droplets, each narrower than a toothpaste cap. "This is less than $10," he says. "And it's because we took the time and effort to make it out of plastic."

Getting the chemistry for the droplets right was crucial, says Ben Hindson, one of QuantaLife's original employees and now a senior principal scientist at Bio-Rad. The droplets produced must remain a uniform size even if the temperature fluctuates slightly as they are generated. What's more, the droplets cannot burst or coalesce during handling, thermocycling and reading, and they also must maintain biocompatible conditions that support PCR. It takes 25 minutes to generate droplets for 96 samples, says Hindson, and one person running the system can analyze 3 sets of 96 samples a day. The technology has begun to appear in independent research; in a high-profile paper combining genomic, transcriptomic, proteomic and metabolomic data, digital droplet PCR developed by QuantaLife was used to detect differential expression of a variety of alleles[1].

Another digital PCR system has been developed by RainDance and is scheduled to launch later this year. The machines in

RainDance

RainDrop Source and RainDrop Sense machines for droplet digital PCR.



The most common applications of digital PCR at the TATAA Biocenter are standardizing qPCR assays, detecting copy number variations, detecting rare mutations and distinguishing differences between expression of nearly identical alleles, says Mikael Kubista.

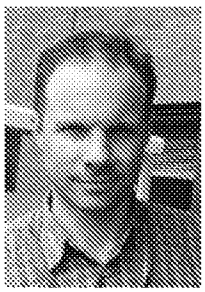
this system generate and read millions of picoliter-sized droplets, a feature that not only allows scientists to identify rarer alleles but also alleviates some of the need to dilute samples carefully. "With all those droplets, we can deal with a wide variety of different concentrations," explains company co-founder Darren Link. He dismisses competitors' claims that millions of partitions are more than most scientists will need. "Too many is never a problem, especially when you are talking about expression analysis," he says. "You don't want to run titrations to find the sweet spot of the dynamic range." Link also emphasizes that the system does not require any manual pipetting as droplets are moved between machines that make droplets, perform thermocycling and analyze droplets.

Researchers at RainDance and the University of Strasbourg and University Paris-Descartes reported that they could detect one mutant *KRAS* gene within 200,000 wild-type *KRAS* genes when the former was diluted into genomic DNA. The seven most common *KRAS* mutations were screened in two multiplex experiments: one examining the wild-type allele along with four mutations and one with the wild type alongside three mutations[8]. At AACR, RainDance presented results detecting cancer mutations in patient serum.

Thinking digitally

Digital PCR may not require the same kind of calibration and controls as qPCR, but there is still plenty of scope for artifacts, says Huggett. Working in tiny volumes and with single-molecule concentrations is a complicated engineering feat. "dPCR is at an early stage, so my advice would be to proceed with caution and be careful of sweeping statements," he says. For example, some researchers believe that digital PCR will be less susceptible than qPCR to enzyme-inhibiting substances that occur in some samples. For qPCR, the problem is that inhibitors increase the number of amplification cycles required to reach a given signal. But even though digital PCR does not count cycles, inhibitors could still be a problem if they cause false negatives by preventing reactions from occurring at all.

Some factors are particularly important to consider with digital PCR, says Kubista. "For example, it is really critical that the assay is well-performing, that you are confident that if there is a single target molecule [you] will see it. Not all assays are that good." In addition, researchers need to make sure that multiple sequences of interest do not appear on the same piece of DNA; otherwise, they cannot be separated into different partitions. (Also, if the positive partitions are clustered together rather than randomly dispersed, there is probably an issue with sample loading or analysis.)



"At the moment," says Jim Huggett, "digital PCR is a specialist approach that is much more costly than qPCR, and qPCR is suitable for the vast majority of applications."

Specificity is also an issue. Many assays will amplify products other than the sequence of interest, particularly if pseudogenes are present. Understanding rates of false positives is crucial when hunting for rare alleles. In these cases, most partitions will not contain the molecule of interest, and the number of false positives could dwarf the number of true positives. For these reasons, Kubista recommends a variety of control experiments. His center offers a kit called ValidPrime that amplifies just one copy of a gene per haploid genome and can be used to assess specificity.

Special consideration is warranted for the rarest alleles. If a sequence is only going to occur once in 50 microliters, says Pickering, it's essential to analyze more than 50 microliters of the sample. "No matter what the technique, if you haven't sampled enough volume to get what you're looking for, you're not going to detect it."

In applications for quantifying more abundant molecules, such as detecting copy number variants or measuring gene expression, researchers generally need to get a rough estimate of the concentration of their target of interest in order to make appropriate dilutions. Otherwise, too many partitions will contain multiple copies. (Statistics can compensate for this, but only to a limited extent.) If every partition shows a reaction, researchers cannot calculate the concentration of the original molecules, explains Kubista. "You get the best use of the chip by having 80% positive [partitions]. If the number rises above 90%, precision drops."

Monitoring the 'response curve', or how levels of DNA change over the course of amplification, can help eliminate false readings caused by nonspecific labeling of DNA sequences—a benefit that companies such as Fluidigm and Life Technologies, which provide such data, are keen to point out. Advocates of droplet digital PCR, however, believe that accurate measurements can be made with endpoint data alone, and cite the advantages of a greater number of partitions. "For allele-specific experiments, you may get false positives, but you can quantitate what that false positive rate is rather than infer from a curve," says Hindson.

Researchers should also consider all the steps that occur before digital PCR begins, says Kubista. As samples are processed, material is lost. Running controls in which a sequence is spiked in before processing can help determine how much sample is necessary, he says.

Although the experts urge care in designing digital PCR experiments, they are enthusiastic about its potential. As the technology matures and the costs come down, more researchers will learn to ask questions only digital PCR can answer, says Kubista. "There are a few applications today, and there will be more tomorrow."

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LightCycler® 1536 Application Note No. 2 ..... February 2012

Genotyping using digital PCR with the LightCycler® 1536 Real-Time PCR System to detect the *IDH1* mutation in plasma samples

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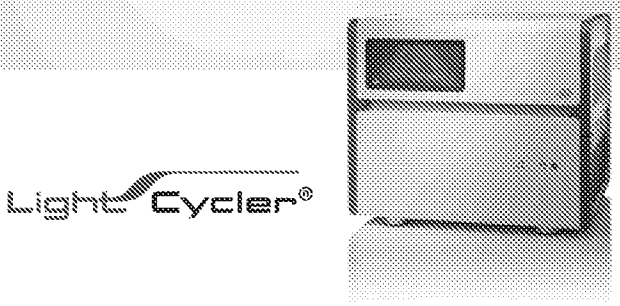

Introduction

Mutations in the gene encoding isocitrate dehydrogenase 1 (*IDH1*) have recently been identified in gliomas. *IDH1* mutations have rapidly been recognized as a powerful independent prognostic factor in subjects with glioma. Subjects with tumor cells with *IDH1* mutations were shown to have a longer life expectancy than subjects with a wild-type *IDH1* glioma (1-4). *IDH1* mutations are primarily found in gliomas, making them an excellent biomarker for this pathology (5-7).

Conventional PCR followed by Sanger sequencing is currently the gold standard in identifying *IDH1* mutations in tumor DNA. Typically, tumor samples are contaminated with normal tissue, diluting the total amount of genetic material present. Techniques are required to identify low level alterations in a background of wild-type DNA. This is particularly true for gliomas which are highly infiltrating tumors (8).

For this reason, unless the mutation exceeds a 20-25% abundance relative to wild-type alleles, conventional PCR followed by downstream methods, such as Sanger sequencing will not detect mutations in samples (9, 10). Enrichment methods are necessary to increase the mutant DNA to wild type DNA ratio. Tumor-cell enrichment methods, such as cell sorting or micro dissection, are however expensive and time-consuming options (10), making them impractical for routine use.

In this study we describe a novel genotyping assay to detect the *IDH1* mutation in free circulating tumor DNA. This *IDH1* genotyping assay uses digital PCR and the LightCycler® 1536 Real Time PCR System, after a first run of COLD-PCR (co-amplification at lower denaturation PCR).

1. Efficiency of COLD PCR

To determine if COLD-PCR can enhance the sensitivity of *IDH1* mutation detection, we compared the sensitivity of the two experimental protocols. Exon 4 of the *IDH1* gene was first amplified by conventional PCR. A second round of amplification was then performed either by PCR-HRM (high resolution melting), or by COLD-PCR HRM. We performed a serial dilution assay using a subjects DNA sample containing a R132H *IDH1* mutation at codon 132 as the source of the mutant allele. This mutation-containing gDNA sample was serially diluted into wild-type DNA to the following percentages: 25%, 10%, 8%, 5%, 4%, 2%, 1%, 0.5%, 0.25%, 0.1%, and 0.05%.

First round of amplification

PCR cycling conditions for the first round consisted of: +94°C for 5 min; 40 cycles of +94°C for 30s; +60°C for 1 min; and +72°C for 1.5 min; followed by final extension at +72°C for 7 min. Conventional PCR reactions contained: PCR Master Mix (Abgene), forward and reverse primers (Invitrogen), and gDNA template.

Second round of amplification

The second PCR amplification was performed using the LightCycler® 480 Real-Time PCR Instrument (Roche Diagnostics Corporation). Each reaction contained diluted PCR amplicons (1/1,000), primers and LightCycler® 480 HRM Master (Roche Applied Science). PCR HRM cycling conditions were as follows: +96°C, 10min; 40 cycles of +95°C, 30s; +60°C, 20s; +72°C, 20s. COLD-PCR HRM cycling conditions were as follows: +96°C , 10min; 20 cycles of +95°C, 15s; +60°C, 30s, then 30 cycles of +81°C, 15s; +60°C, 30s. After amplification, a melting curve program was performed by heating to +95°C for 1 min, cooling to +40°C for 1 min, and increasing the temperature to +95°C, while continuously measuring fluorescence using 25 acquisitions per 1°C.

2. Plasma DNA analysis

Step 1: DNA extraction

Blood samples were collected in EDTA-coated tubes and centrifuged immediately at 1,000g for 15 minutes at +4°C. Plasma was carefully collected and centrifuged again at 10,000g for 5 minutes at room temperature, and immediately aliquoted and stored at -80°C until use. DNA was extracted from of plasma using QIAamp® Circulating Nucleic Acid kit (Qiagen) and vacuum chamber (QIAvac24 Plus, Qiagen), according to the manufacturer's instructions. Once extracted, DNA samples were treated with RNase A (Invitrogen) to discard RNA carriers, and purified using NucleoSpin® Extract II (Macherey Nagel). DNA size and DNA concentration were analyzed using a 2100 Bioanalyzer and Agilent High Sensitivity DNA kit.

Step 2: Selective amplification of *IDH1* mutation by COLD PCR

Plasma DNA was amplified using a run of COLD PCR, as described above. PCR amplicons were then diluted by 1/1,000,000 with distilled water.

Step 3: Detection of *IDH1* mutation by digital PCR in plasma samples

Diluted COLD PCR products for each sample were assayed using a 1,536-well plate so that one plate corresponded to one individual subject. The following PCR master mix was used, containing RealTime Ready Probes Master 2X (Roche Applied Science), forward and reverse primers (Eurogentec), wild type and mutant LNA probes (Eurogentec), and diluted PCR amplicons. This mix was dispensed at 1µL/well across a LightCycler® 1536 Multiwell Plate using the Bravo Automated Liquid Handling Platform (Agilent Technologies). Following an initial denaturation step at +96°C during 1 min, qPCR cycling conditions on LightCycler® 1536 Instrument (Roche Applied Science) were carried out, as summarized in Figure 1.

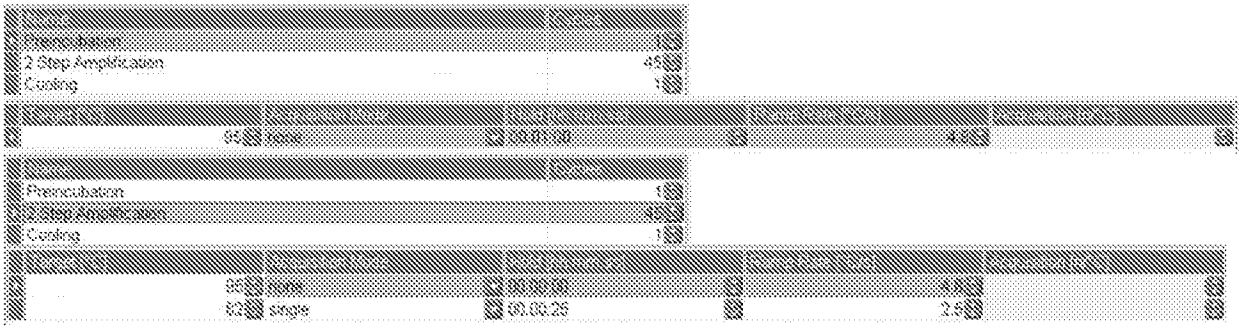

Figure 1: PCR program used for the detection of *IDH1* mutation in digital PCR conditions using the LightCycler® 1536 Real-Time PCR Instrument.

COLD PCR

High Resolution Melting (HRM) analysis

As a first step, we verified that the COLD PCR procedure effectively enriches mutant DNA. At the end of the second round of amplification, fluorescent melting curves were analyzed using LightCycler® 480 Gene Scanning Software V1.2.9 (Roche Applied Science). Amplification curves were analyzed following normalization, with respect to temperature shifting, automated grouping, and the inspection of difference plots.

The Grouping Software uses a curve shape-matching algorithm to identify wild type from mutant samples. The 0.2 value was chosen for grouping sensitivity in all experiments. Our findings show that the COLD-PCR HRM assay led to an approximately tenfold improvement in the *IDH1* mutation detection (see Figure 2B). In addition, compared to conventional PCR-HRM analysis, we found a detection threshold of 25% versus 2%, respectively (see Figure 2A).

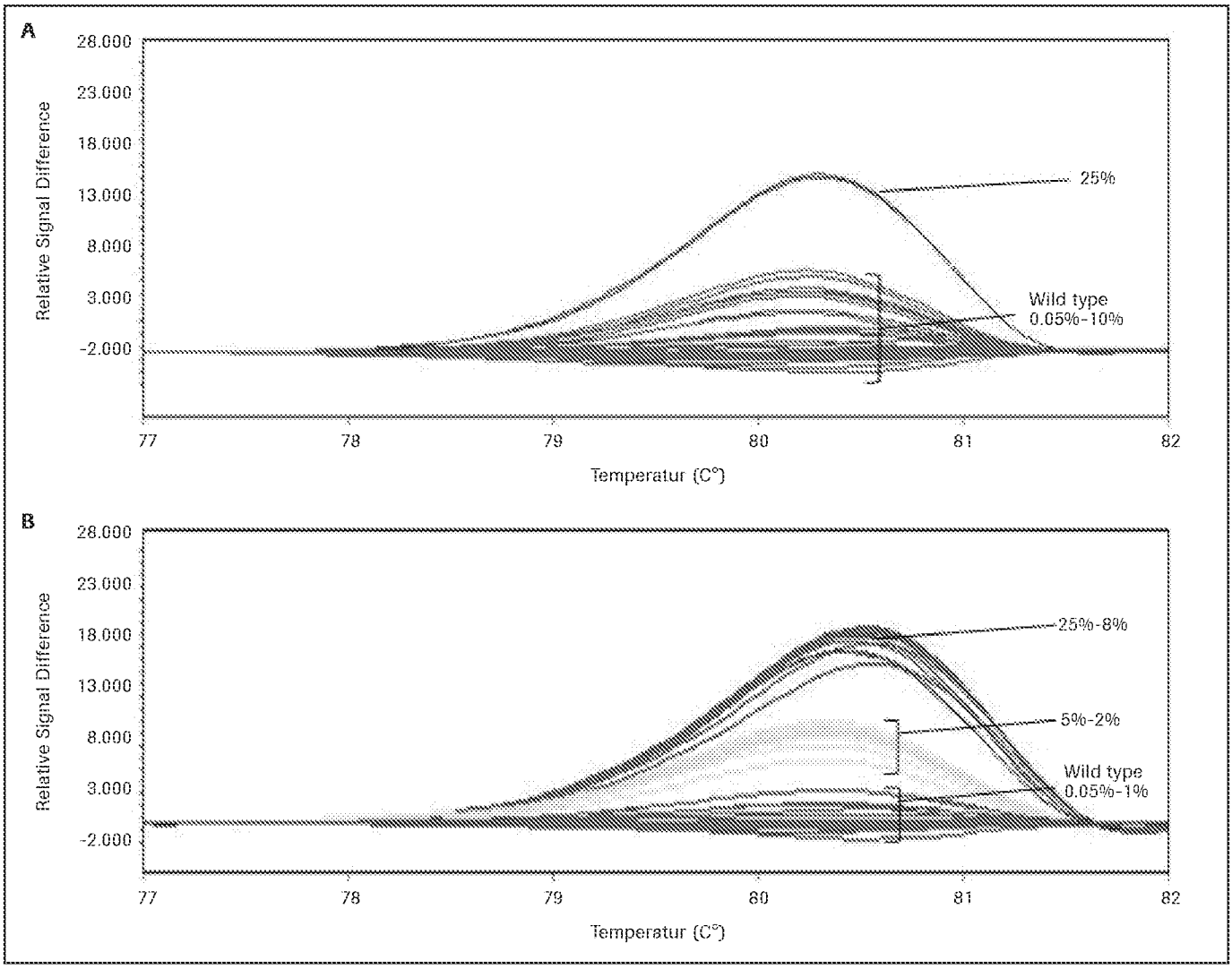

Figure 2: Comparison of conventional PCR HRM (A) and COLC PCR HRM (B) for the detection of *IDH1* mutation.

Detection of the IDH1 mutation in free circulating tumor DNA

We then used COLD PCR to detect the presence of the *IDH1* mutation in free circulating tumor DNA. After DNA extraction from blood, COLD PCR was followed by genotyping using digital PCR conditions with the LightCycler® 1536 Real Time PCR System (see Figure 3). The LightCycler® 1536 Instrument is most sensitive for the channel HEX, and thus used a HEX LNA probe for the mutant allele.

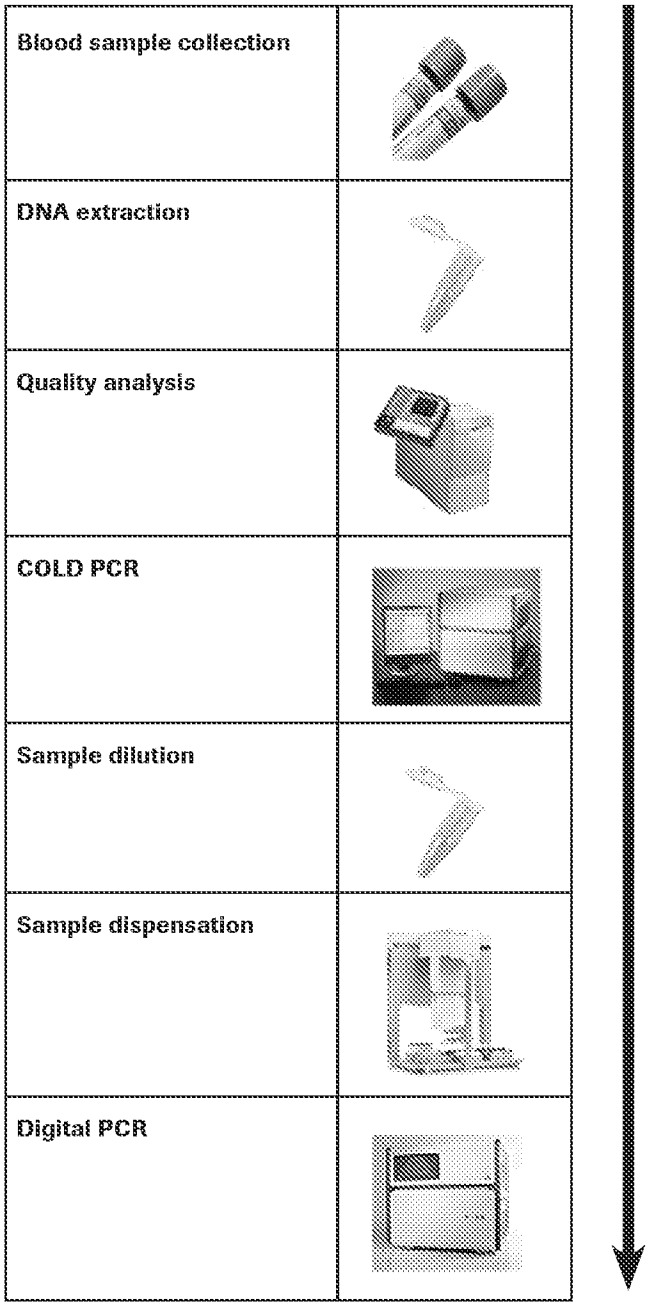

Figure 3: Workflow schematic for the detection of an *IDH1* mutation in the plasma of glioma subjects

Digital PCR for amplification of zero or one DNA copy in each well was performed when the total number of amplifications was between 100 and 1,200 out of the 1,536 wells of the *IDH1* wild type assay (see Figure 4).

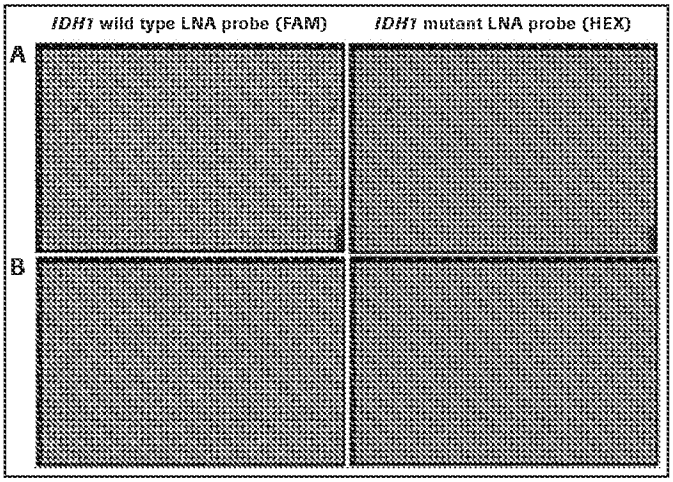

Figure 4: Representative heat maps obtained in an *IDH1* normal subject (A) and an *IDH1* mutant subject (B). Amplifications with positive and negative wells appear in green and in red, respectively.

We used the endpoint fluorescence (EPF) values generated by LightCycler® 1536 Software to analyze the obtained amplification data (see Figure 5). The threshold EPF of 0.1 was chosen for the mutant LNA probes (HEX), and an EPF threshold of 0.2 for wild type LNA probes (FAM). For an individual sample, the mutant amplification ratio was calculated to equal the number of informative wells with mutant probe divided by the number of informative wells with wild type probe.

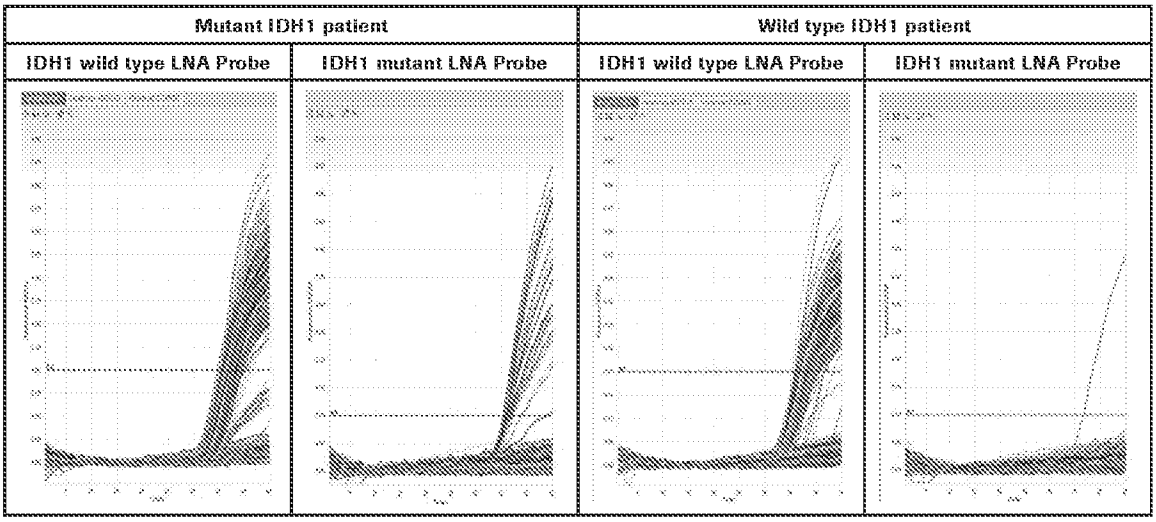

Figure 5: qPCR amplification curves obtained in *IDH1* normal subject (A) and an *IDH1* mutant subject (B). Amplifications curves using the wild type probe are shown in the left panel, and amplifications curves using the mutant probe are in the right panel.

Conclusions

Findings presented in this report show that combining two qPCR approaches can be useful for detecting mutant tumor DNA in plasma sample material. Performing COLD PCR as a first step enables selective amplification of the mutant allele in a background of wild type DNA. Here the use of the LightCycler® 480 Real Time PCR system is critical, because precise control of the denaturation temperature is ensured. The LightCycler® 1536 Real Time PCR System was then used for highly selective genotyping to detect the *IDH1* mutation using digital PCR conditions. This all-important second step also allows relative quantification of mutant tumor cell DNA in a blood sample. The method presented here can be successfully applied to other DNA biomarker research in the field of oncology.

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Product	Cat. No.	Pack Size
LightCycler® 480 Real-Time PCR Instrument, 96-well	05 015 278 001	1 instrument plus accessories
LightCycler® 1536 Real-Time PCR Instrument	05 334 276 001	1 instrument plus accessories
LightCycler® 1536 MultiWell Plate	05 358 639 001	10 x 10 plates
LightCycler® 480 MultiWell Plate	04 729 692 001	5 x 10 plates, with sealing foils
RealTime ready DNA Probes Master	05 502 381 001	5 x 1 mL
LightCycler® 480 HRM Master	04 707 494 001	5 x 1 mL, up to 500 reactions

Important note

Data included in this article are the sole responsibility of the authors that have published them, and these authors are responsible for following the respective local regulations for assay setup and validation.

Regarding the specific assay(s) described, Roche was neither involved in establishing the experimental conditions nor in defining the criteria for the performance of the specific assays. Roche therefore cannot take any responsibility for performance or interpretation of results obtained for the biological target parameter(s) described by the authors or other users using a similar experimental approach.

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The LightCycler® 1536 Real-Time PCR System are not intended for in vitro diagnostic use in the U.S.

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Review

EXPERT REVIEWS

The potential advantages of digital PCR for clinical virology diagnostics

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Digital PCR (dPCR), a new nucleic acid amplification technology, offers several potential advantages over real-time or quantitative PCR (qPCR), the current workhorse of clinical molecular virology diagnostics. Several studies have demonstrated dPCR assays for human cytomegalovirus or HIV, which give more precise and reproducible results than qPCR assays without sacrificing sensitivity. Here we review the literature comparing dPCR and qPCR performance in viral molecular diagnostic assays and offer perspective on the future of dPCR in clinical virology diagnostics.

KEYWORDS: accuracy • digital PCR • precision • sensitivity • viral quantitation

PCR technologies in viral diagnostics

Nucleic acid amplification techniques (NAATs) form the cornerstone of clinical viral diagnostics. PCR and quantitative or real-time PCR (qPCR) have been widely utilized to diagnose viral disease from plasma/serum, blood, cerebrospinal fluid and tissue samples. During qPCR, target template DNA is amplified with sequence-specific primers until it produces a signal that is supplied through a DNA intercalating dye or sequence-specific fluorescent probe. Higher levels of template DNA produce a fluorescent signal that is detectable after fewer cycles of PCR, and a standard curve is used to correlate the cycle giving a signal with a known quantity of DNA [1]. This technique has served the field well but has some drawbacks; namely, the necessity for a standard curve and the lack of universal standards of known quantity, both of which contribute to intra- and interlaboratory imprecision and lack of commutability.

Recently, a NAAT called digital PCR (dPCR) has become commercially available, and its utility in viral diagnostics has been explored by several clinical virology laboratories. dPCR uses the same primers and dyes/probes as qPCR, but partitions a single bulk reaction into thousands or millions of separate microliter to picoliter scale reactions [2,3]. Each of these reactions is cycled to the endpoint of the reaction, and each partition is read as positive or negative for target template DNA. Using Poisson statistics, the number of positive partitions is compared with the total number of partitions to give a direct readout or absolute quantitation of the number of target DNA template molecules in the reaction, abrogating the need for the standard curve traditionally used in qPCR [4,5].

Several commercial platforms with reasonable throughput are available for dPCR applications, making this a practical option for certain applications in clinical laboratories. Platforms from Fluidigm Corporation and Life Technologies utilize a plate format to partition reactions in separate wells, while platforms available from Bio-Rad Laboratories and RainDance use droplets formed by a water-in-oil emulsion to partition reactions (known as droplet dPCR, ddPCR). The per sample cost of Bio-Rad's ddPCR system is currently the lowest of all the systems at $3–5 per well. Details of these systems comparing number of partitions and estimated costs are reviewed elsewhere [6,7].

While qPCR is the current gold standard for molecular quantitation in viral diagnostics, dPCR offers several potential advantages over qPCR. Here we will review the literature addressing how dPCR could improve upon current viral diagnostic quantitation capabilities by increasing precision, reproducibility

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Table 1. Imprecision of ddPCR and quantitative PCR measured at several dilutions of cytomegalovirus-positive patient plasma sample.

Coefficient of variation (%)			
Copies/ml	ddPCR	qPCR	N
16,500	3.4	12.1	8
3830	8.0	17.8	8
1604	11.8	20.0	8
480	10.0	13.4	8

ddPCR: Droplet digital PCR; qPCR: Quantitative PCR.

and sensitivity, and by standardizing quantitation within the field. We will also discuss limitations of the current generation of dPCR platforms that may limit their widespread immediate adoption into the clinical laboratory.

dPCR exhibits precision superior to qPCR

The imprecision of typical clinical viral qPCR assays can be quite high, particularly at lower template copy numbers. Even within highly specialized laboratories, the coefficient of variation (CV, a measure of imprecision) can be 20–30% or higher [8–12]. The clinical impact of this variation in measurement is not fully known, but several studies on cytomegalovirus (CMV) and HIV have suggested that increases in viral load even at very low levels can be clinically significant [15–16]. For example, a study by Waggoner *et al.* comparing the performance of a commercially available CMV qPCR assay with the laboratory's reference protocol explored the clinical significance of low CMV viral loads (<200 copies/ml). In 22 episodes, viral load increased from <200 copies/ml to ≥200 copies/ml. All of these patients were eventually treated with antivirals, demonstrating that episodes of CMV viremia ultimately treated with antiviral therapy could be detected earlier using a more sensitive method [13]. Measurement precision at these levels could likewise be important in treatment decision making, to increase confidence that changes in day-to-day or week-to-week viral load measurements were biologically derived rather than assay derived. Given the inherent imprecision in qPCR assays, it is difficult to distinguish biologically relevant changes from measurement imprecision when viral load differences are less than one-half log.

One advantage of dPCR over qPCR in viral diagnostics is the potential for increased precision within- and between-runs. Several recent papers suggest that dPCR offers markedly higher precision than qPCR. In a study by Hindson and colleagues, ddPCR consistently displayed lower variation than qPCR for a set of synthetic miRNAs. Mean CVs were reduced 37–86% in ddPCR compared with qPCR for overall variation and reduced 48–72% for PCR-specific variation [17]. This study was performed on synthetic miRNAs, which while not directly relevant to viral diagnostics uses the same methods that would be relevant to implementation in a clinical virology laboratory. In the clinical realm, two additional studies, one on HIV and another on CMV, have demonstrated increased precision in ddPCR compared with qPCR. Both of these viruses are typically monitored longitudinally in infected patients, a situation where measurement variability between runs could mask clinically relevant changes in viral load. Strain and colleagues developed ddPCR assays targeting total HIV DNA (*pol* gene) and episomal 2-LTR circles in cells isolated from infected patients. After analysis of over 300 clinical samples, 150 of which were tested in triplicate with both ddPCR and qPCR, they observed a 4-fold decrease in variance for *pol* and a 20-fold decrease for 2-LTR using the ddPCR assays [18]. For CMV, a similar decrease in CV was observed at higher viral loads (≥4 $\log_{10}$) when comparing ddPCR and qPCR assays [19]. Data from our own laboratory's CMV ddPCR and qPCR assays agree with these observations. At 4 $\log_{10}$ copies/ml, CMV CVs decreased 4-fold for ddPCR compared with qPCR and at 3 $\log_{10}$ copies/ml decreased 1.5-fold (Table 1) [20]. While the analytical precision of ddPCR was greater than qPCR in this CMV study, no clinical advantage was found in a retrospective analysis of longitudinal samples from a cohort of 18 transplant patients [20]. However, the study size was small and the precision advantage of ddPCR might be more relevant in other clinical testing situations.

The increase in precision offered by ddPCR not only improves absolute measurement reproducibility as evidenced by HIV and CMV data, but also can be useful for ratiometric assays. One such ratiometric viral diagnostic application using this increased precision is a ddPCR assay to identify chromosomally integrated Human Herpesvirus 6 (ciHHV-6), which can be transmitted via the germ line and is present in about 1% of the population [21,22]. In ciHHV-6 individuals, HHV-6 is integrated in the telomeric region of chromosomes in every cell of the body [23]. This phenomenon complicates qPCR testing for active HHV-6 infection. In an individual with ciHHV-6, plasma samples (the typical specimen type used for viral load monitoring) are often positive for HHV-6 due to detection not of replicating virus but of genomic DNA from lysed cells, which carry a copy of HHV-6 [24]. Therefore, a ratiometric test for both HHV-6 and cellular DNA can be used to identify ciHHV-6, with an expected ratio of 1 HHV-6 genome per cell. Given the variation inherent in qPCR assays, this ratio cannot be determined with adequate precision to allow clinical diagnosis in ciHHV-6 individuals [25]. A ddPCR assay was recently developed that exhibits remarkable precision when assaying as few as 10^4 cells. Results for five independent runs using DNA from a human lymphoblastoid cell line containing 1 HHV-6 genome integrated on Chromosome 18 gave a mean and standard deviation of 0.96 ± 0.03 with a CV of 3%. Furthermore, the assay showed 100% sensitivity and specificity on cellular specimens from patients with ciHHV-6 as confirmed by fluorescence *in situ* hybridization [26].

In addition to increased precision or repeatability, some data suggest that ddPCR may also be more reproducible (less day-to-day variation). Comparison of ddPCR and qPCR quantitation of miRNAs determined that ddPCR improved day-to-day reproducibility by a factor of seven. Similarly, comparison of ddPCR and qPCR assays for CMV showed lower interassay

(between-run) CVs for ddPCR than for qPCR across multiple dilutions of a highly positive patient sample down to 150 copies/ml (Figure 1) [20].

dPCR & qPCR have comparable sensitivity

While recent work demonstrates a precision advantage of dPCR compared with qPCR, data on sensitivity comparisons are not as definitive. To date, no study has shown dPCR to be more or less sensitive than qPCR when equivalent amounts of DNA input are measured. A study comparing ddPCR and qPCR assays for CMV underscores the fact that sensitivity of any NAAT is determined primarily by the amount or concentration of nucleic acid input into reactions. One comparison of two laboratory-developed CMV assays suggested that qPCR showed 10-fold greater sensitivity than ddPCR. However, in this study, four times more volume of DNA was included in the qPCR (20 µl) assay than the ddPCR assay (5 µl), which accounts for much of the difference in limit of detection [19]. Our own laboratory's CMV ddPCR and qPCR comparison indicate that when equal amounts of DNA are included, sensitivity of the two PCR techniques is equivalent [20]. The miRNA study comparing ddPCR and qPCR likewise determined the two methods have comparable sensitivity [17]. Of note, while sensitivity of dPCR and qPCR can be equivalent, the dynamic linear range of dPCR is narrower than qPCR for most dPCR systems. This limitation derives from the number of partitions available in any particular system. For instance, Bio-Rad's ddPCR system has a linear range of 4–5 logs [4] compared with the 6 or 7 log range typically achievable in qPCR assays. However, a digital system with more partitions, such as RainDance's RainDrop, has a linear range up to 6 logs [27].

One approach to maximize dPCR sensitivity is that multiple wells or droplets can be 'merged' and analyzed as one metawell, to increase the amount of DNA that is interrogated for a certain template. This method can be utilized in dPCR but not qPCR because each reaction in dPCR is counted as positive or negative in a digital output, as opposed to the analog output of qPCR. Strain and colleagues demonstrated that this technique is feasible on HIV templates; they used 36 ddPCR wells (>500,000 droplets, each droplet representing ~1 nl reaction) to detect *pol* and 2-LTR amplicons down to 0.7 copies/10^6 cells [18].

dPCR may standardize quantitation

dPCR has great potential for increasing standardization within and across the multitude of clinical virology labs that utilize laboratory-developed assays. Interlaboratory commutability is a critical issue for providing meaningful viral load values across health care centers and within studies that set benchmark viral loads for treatment administration [28]. The WHO has created international standards and calibrants that can be used by labs across the world to set various laboratory-developed assays to the same value [29,30]. However, the drawback of these calibrants is that their values are set based on an average value obtained from multiple different laboratory assays. Recently, the National Institute for Standards and Technology created a standard material for CMV (SRM 2366) that is quantified by dPCR, to provide an absolute viral copy number, as opposed to an average based on many different separately calibrated qPCR assays. This DNA reference material can be used by different labs for calibration, method validation/verification and quality control or proficiency testing and is based on a precisely determined, known number of amplifiable DNA molecules [31].

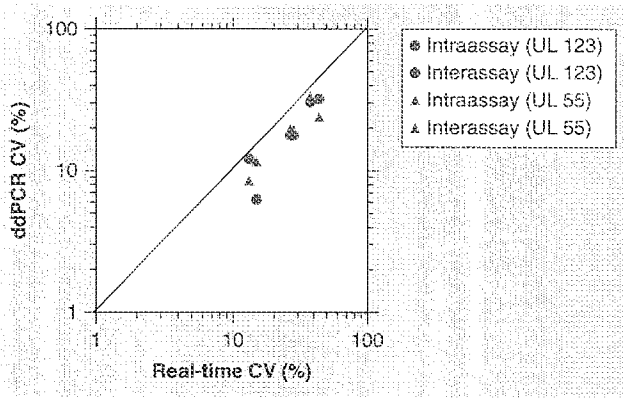

Figure 1. Comparison of within-run (intra-assay) and between-run (inter-assay) variability for ddPCR (UL55 and UL123 gene targets) and quantitative PCR utilizing three dilutions of a highly positive patient sample, each run in triplicate and repeated in four separate runs. Plot compares the CV obtained by ddPCR versus quantitative PCR for each sample and dilution.
ddPCR: Droplet digital PCR.
Data taken from [20].

dPCR is resistant to inhibition

dPCR also offers advantages such as increased tolerance to PCR inhibitory substances [32]compared with qPCR. In the clinical setting, Dingle *et al.* demonstrated that ddPCR was more refractory to inhibition than qPCR by testing substances such as heparin and SDS, common inhibitors in DNA extractions of clinical samples [33]. Greater than a half log increase in IC_{50} was observed for ddPCR compared with qPCR when heparin and SDS were spiked into CMV assays.

While the mechanism of increased tolerance to PCR inhibitors is not fully elucidated, one theory is that in ddPCR, the oil used to create the droplet emulsion may sequester some inhibitory substances away from the water phase PCR reaction. Also, as demonstrated by Dingle *et al.*, in a ddPCR reaction, delayed or reduced amplification efficiency per cycle due to partial inhibition can be visualized and does not affect the accurate quantitation of individual positive droplets [33]. In contrast, during qPCR, delayed or reduced amplification can result in significant underquantitation because the amplification reaction would differ in the inhibited sample reaction compared with the reactions for the standards. These data suggest that ddPCR may improve within- and between-lab reproducibility in inhibition-prone clinical specimens such as stool, sputum and tissues.

dPCR is less affected by target sequence variability

Another issue in viral clinical diagnostics that affects the commutability of quantitation between different assays is the sequence variability common across clinical strains of a single virus. Many different qPCR assays for the same virus may amplify different regions of the genome and each of these regions have their own level of sequence variability, which can lead to misquantitation if the sequence differences affect primer or probe binding [34]. Strain *et al.* showed that ddPCR may be less susceptible than qPCR to strain mismatches in primer and probe sequences [18]. They selected four patients with HIV *pol* sequences that diverged from the consensus primer/probe set and compared results from patient specific and consensus primers and probes using ddPCR or qPCR. Results from qPCR indicated that the consensus primer/probe set underestimated *pol* concentration by 10- to 100-fold in three of four patients and did not detect template at all in the fourth patient. In contrast, when the experiment was repeated by ddPCR, the underestimates were reduced in these patients (57% average underestimate reduction) [18], suggesting that ddPCR may help to overcome misquantitation resulting from viral sequence variability.

Quantitation by ddPCR may be less affected by sequence mismatches than qPCR for the same reason that partial inhibition is less problematic in ddPCR than in qPCR. When a mismatch occurs between the template DNA and the primer or probe set that does not completely abolish amplification, positive droplets are still detected, albeit at lower amplitudes (indicating less efficient reactions). These lower amplitude droplets are nevertheless counted as positive, resulting in accurate quantitation. However, in qPCR, less efficient amplification results in C_t values corresponding incorrectly to standard curve values based on highly efficient reactions, resulting in misquantitation.

Current drawbacks of dPCR over qPCR

dPCR is a promising newly commercialized NAAT with several potential advantages over qPCR for viral molecular diagnostics. However, dPCR is still an emerging technology that has only recently found application in the clinical virology laboratory. Therefore, several concerns with dPCR exist that remain to be overcome as clinical laboratories gain experience with this technique, and companies improve their systems with high-throughput molecular diagnostics in mind. While dPCR circumvents the need for a traditional standard curve, the lack of standard curve-based quantitation does not automatically guarantee better accuracy. Precisely and accurately defined calibrant materials or gold standards are still required to ensure commutability between molecular diagnostics laboratories. Different dPCR systems perform differently, with varied dynamic ranges and sensitivities defined by system-specific limitations on DNA input and microfluidics capabilities. In addition, dPCR assays rely upon the same amplification chemistry and primer/probe sets that are utilized in qPCR. While dPCR offers some advantages over qPCR in terms of increased tolerance to inhibitors and sequence mismatches, the accuracy of dPCR is still dependent upon the validity of the amplification assay utilized.

As mentioned above, some dPCR systems have a linear dynamic range logs lower than that of qPCR depending on the number of partitions utilized in each particular system. In addition, sample overload is a consideration, particularly in emulsion-based ddPCR. For example, up to 1 mg of cellular DNA may be added to a ddPCR reaction of the Bio-Rad system, but only after it has been digested (either mechanically or with a restriction enzyme). Without digestion, the viscosity of that amount of total DNA can interfere with droplet formation, resulting in inaccurate quantitation due to a dearth of appropriately sized droplets.

Furthermore, qPCR benefits from many years of implementation in molecular diagnostics laboratories. Companies have met the demand of laboratories to develop high-throughput, fast assay systems compatible with the quick turnaround desired for viral load monitoring (Figure 2). dPCR as it is implemented now is limited by the lower throughput and speed with which results can be generated. The ddPCR systems in particular require separate droplet generation, thermocycling and droplet reading steps. In our hands, a dPCR assay for a 96-well plate of CMV takes approximately five times longer from PCR reaction setup to result than our current clinical qPCR assay.

Many current dPCR systems are also 'open systems' with increased risk of contamination from run-to-run carryover or well-to-well transfer during a run. The Bio-Rad droplet system, for instance, requires mixing and pipetting of a maximum of eight reactions that are transferred to a droplet generation cartridge and are then transferred back to a 96-well plate before the reactions are sealed for thermocycling. With careful training and good technique, contamination can be avoided, but the system is not designed to ensure contamination-free operation by multiple clinical operators.

Finally, dPCR is currently more expensive than qPCR. Depending on the digital system implemented, the higher cost results from the throughput limitations inherent in many systems or from the microfluidics consumables required to partition reactions.

Expert commentary

Commercial dPCR systems have been available for only a few years, and in that period, multiple laboratories have demonstrated advantages of dPCR over traditional qPCR for viral molecular diagnostics. The precision of dPCR assays is markedly better than qPCR assays, with equivalent sensitivity, making dPCR a potentially attractive alternative diagnostic technique for monitoring viral load. Based on the superiority of dPCR for ratiometric assays, our laboratory now offers the first clinically validated viral ddPCR-based laboratory-developed test, which identifies chromosomally integrated HHV-6 [26]. Even without the throughput and speed of qPCR, dPCR surpasses qPCR as the diagnostic method of choice for certain assays requiring high precision. With the advent of more advanced automated systems, dPCR will become a more practical technique for routine viral clinical testing.

Moreover, the fundamental concept of dPCR, compartmentalization of a bulk reaction into many separate reactions, can

contribute to advancements in techniques outside of basic quantitation. Studies have already shown that compartmentalization can reduce the effects of inhibitors and even sequence dissimilarities. Compartmentalization also offers advantages for rare sequence detection, next-generation sequencing technologies and single cell analysis that will all become important in viral molecular diagnostics as applications advance.

Five-year view

dPCR is a powerful absolute quantitation method that has the potential to revolutionize how molecular virology diagnostics are performed. Although the throughput and cost of dPCR may not make it feasible or advantageous to replace qPCR with dPCR in molecular virology laboratories in the next 5 years, dPCR can be utilized to make great strides in standardizing calibrant materials within and between laboratories. CMV is the first virus to have a digitally quantitated standard material; more viruses will follow as the technology gains traction in the field. Even within a single laboratory, much time and expense are put toward maintaining standards. dPCR offers a reliable method of maintaining standard values over time, reducing problems associated with lot-to-lot variability and identifying potential quality control issues.

Within the next few years, dPCR systems will become more automated. Increasing automation will make them more attractive for use in routine diagnostic applications by increasing throughput and allowing 'closed systems' with decreased potentials for error or contamination from manual handling. As these systems advance, the sensitivity achieved with current dPCR assays may also increase. Sensitivity limitations derive mainly from the limited DNA input allowed by these systems, which can be improved by offering more concentrated reaction master mixes or increasing the number of partitions utilized.

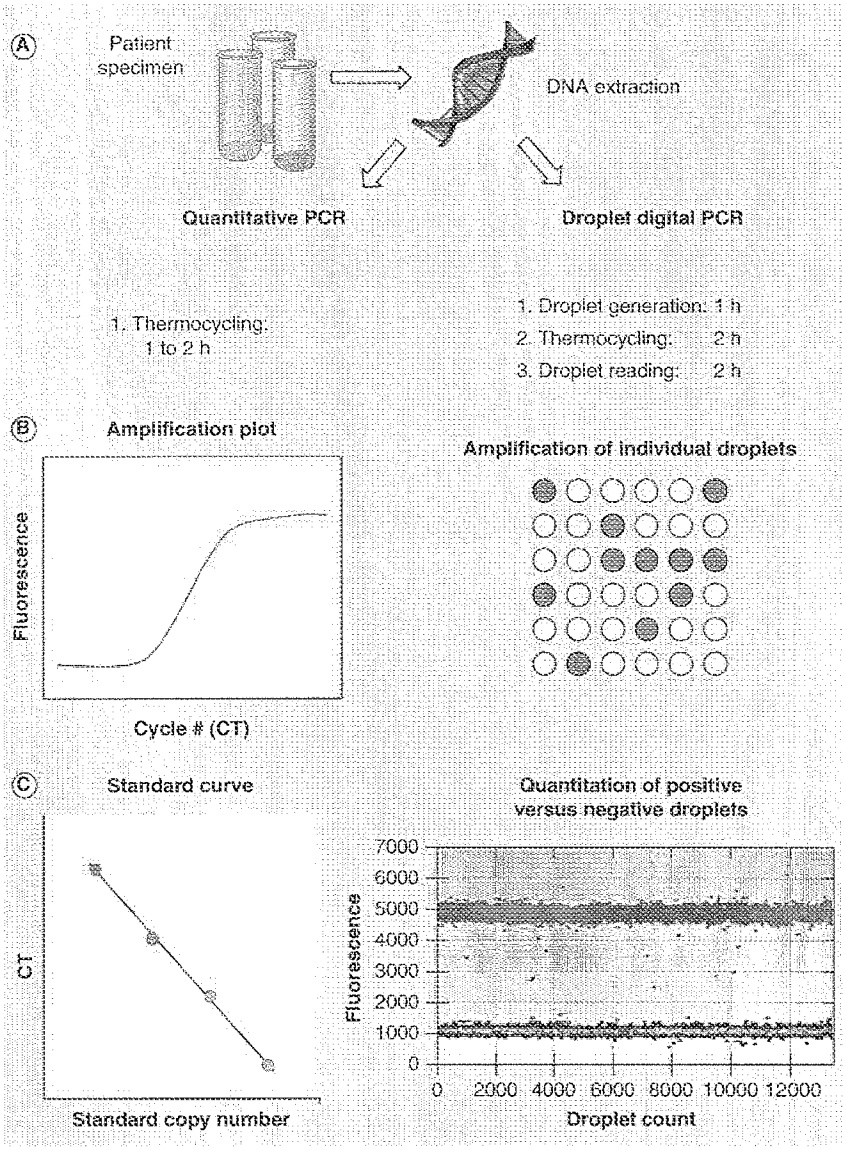

Figure 2. Workflow comparison for quantitative PCR versus droplet digital PCR. **(A)** Both methods begin with the same types of patient specimen and DNA extraction methods. **(B)** Reaction setup and cycling are significantly faster in quantitative PCR than current ddPCR systems. **(C)** Results for quantitative PCR are determined by comparing the C_T values from specimen amplification plots to a standard curve with known quantity standards set to copies per microliter of original specimen. Results for droplet digital PCR are in the form of a droplet plot that counts positive (green) and negative (gray) droplets to determine the absolute target quantity in copies per microliter of reaction. The plot shows individual droplet fluorescence for over 1300 individual droplets. The quantity of template in the original specimen can be calculated from the proportion of positive droplets.

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No writing assistance was utilized in the production of this manuscript.

Key issues

- Digital PCR(dPCR) is a newly commercialized nucleic acid amplification technology that will benefit viral molecular diagnostic testing and replace quantitive PCR (qPCR) testing in some situations.
- dPCR exhibits greater precision than qPCR with equivalent sensitivity.
- PCR inhibitors and sequence variability may be less of an issue in dPCR compared with qPCR.
- Accuracy and commutability may be improved with implementation of standards and calibrants quantified by dPCR.
- Throughput and cost will need to improve before dPCR competes with qPCR as the workhorse of clinical molecular virology labs.

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Review

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EXPERT REVIEWS

Noninvasive prenatal diagnosis of fetal aneuploidies and Mendelian disorders: new innovative strategies

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The application of recent technical developments, such as digital PCR or shot-gun sequencing, for the analysis of cell-free fetal DNA, have indicated that the long-sought goal of the noninvasive detection of Down syndrome may finally be attained. Although these methods are still cumbersome and not high throughput, they provide a paradigm shift in prenatal diagnosis, as they could effectively pronounce the end of invasive procedures, such as amniocentesis or chorionic villous sampling for the detection of such fetal anomalies. However, it remains to be determined how suitable these approaches are for the detection of more subtle fetal genetic alterations, such as those involved in hereditary Mendelian disorders (e.g., thalassemia and cystic fibrosis). New technical developments, such as microfluidics and reliable automated scanning microscopes, have indicated that it may be possible to efficiently retrieve and examine circulating fetal cells. As these contain the entire genomic complement of the fetus, future developments may include the noninvasive determination of the fetal karyotype.

KEYWORDS: cell-free fetal DNA/RNA • digital PCR • fetal cell • maternal blood • microfluidics • shot-gun sequencing

Although great strides have been made in the noninvasive determination of facile fetal genetic loci, such as Rhesus D in pregnancies at risk for hemolytic disease of the fetus and newborn, and fetal sex in pregnancies at risk for an X-linked disorder via the analysis of cell-free fetal DNA (cff-DNA) in maternal plasma/serum, the determination of more complex fetal genetic anomalies, such as those involved in Down syndrome, still rely on invasive procedures including amniocentesis of chorionic villous sampling [1,2]. Since these procedures pose a risk of fetal injury or loss, there is a need for safe efficacious alternatives.

A major driving force for the development of noninvasive prenatal diagnosis is the demographic change that has taken place in developed countries, whereby many pregnant women are over the age of 35 years, even if it was their first born. Since many couples elect to have only one child, they are naturally not keen to expose their long-desired baby to the risk of an invasive procedure.

A further complication is that despite incremental increases in the sensitivity and specificity of screening procedures for pregnancies bearing fetuses affected by chromosomal anomalies, such as Down syndrome, there has been very little net change in the number of live births of such affected babies in the past decade in certain countries, in contrast to others where the rate of such affected births has been halved [3,4]. Hence, a method needs to be developed that can alter this current state of affairs.

What is required?

Currently, a large proportion of pregnant women would automatically be judged to be at an increased risk of bearing a fetus with a chromosomal anomaly due to advanced maternal age; thus, the new system would need to be amenable to mass screening, akin to what is being undertaken using serum analytes in the first and second trimester of pregnancy. As such it should be:

- Simple and automatable
- Robust and high throughput
- Cost effective

- Permit the easy shipping and storage of samples, as it is likely to be off-site from where the sample is taken

In short, this is quite a challenge!

What options exist?

Historically, rare trafficking fetal cells in maternal blood presented the first target for the obtaining of fetal genetic material in a noninvasive manner [5]. This strategy was first attempted in 1979 by Bianchi and colleagues in the laboratory of Herzenberg, the developer of flow cytometry [6]. Following a few high-profile case reports in the mid- to late 1980s [7,8], which indicated that this system may permit the detection of fetal aneuploidies via the analysis of enriched fetal cells by FISH, the NIH funded a large-scale study to test the feasibility of this approach [9,10]. To date, this study, termed the National Institute of Health Fetal Cells Isolation Study (NIFTY), is still the largest multicenter analysis of its type. Unfortunately, the goal chosen by NIFTY was too lofty for the technology available at the time, and the degree of sensitivity and specificity attained was way below that required for clinical application (Bianchi *et al.* [2002]). Some questions also remain unanswered as to whether the fetal erythroblast is indeed the most suitable target cell, as they have dense compact nucleus with apoptotic character and may be impervious to FISH analysis [11,12].

Towards the end of the NIFTY trial, Dennis Lo and colleagues in Oxford, UK, made a startling discovery, by observing the presence of cff-DNA in the plasma and serum of pregnant women [13,14]. Not only was it easier to retrieve this material than to enrich for fetal cells, but it was much more abundant by a factor of 100- to 1000-fold [15]. As such, most attention in the past decade has been focused on the analysis of this new-found analyte, and the quest for fetal cells has faded somewhat into obscurity [1].

Is there resurgent interest in fetal cells?

In 2008, two independent publications suggested that all interest had not been lost in the enrichment and detection of fetal cells [16,17]. What is perhaps most surprising about these reports is that they were made by private companies and not by publicly funded research groups. This implies that, although many main stream research groups have largely given up hope on the use of fetal cells for noninvasive prenatal diagnosis, companies using private equity feel sufficiently motivated to follow this course as part of their business plan.

In the first of these reports, Seppo and colleagues at Ikonisys Inc., used an innovative automated scanning microscope system for the rapid and simple detection of putative target cells, identified by fluorescent staining for fetal or embryonic hemoglobin molecules and FISH for the X and Y chromosomes [16]. The Ikonisys system is different to other previous approaches, in that the microscope system is enclosed in an industrial box-like system, which includes a loader for 175 standard microscope slides. This has the advantage that the system can be placed anywhere in a standard diagnostic laboratory, without the need for dark-room facilities, normally required for FISH analysis. This system can be linked to a central computer network, thereby permitting off-site data analysis. This also permits several machines to be run in parallel. As the system features a simple user friendly interface and stand-alone 24/7 operation, it is clear that Ikonisys have factored the requirements of routine diagnostic laboratory use into their design.

In their first examination, they analyzed whole-blood samples for the presence of putative fetal erythroblast cells identified by XY-FISH, they determined that, on average, three XY-positive cells could be detected in samples taken in the first trimester of pregnancy, and approximately two such cells in second-trimester samples. On average, close to 4 million individual cells were scanned per case, indicating that 0.4–0.8 fetal cells were present per 1 million maternal cells. This is in good agreement with previous assessments.

If the samples were prepared by standard density-gradient centrifugation to remove the bulk of the erythrocytes, then the recovery of fetal cells was improved by between two- and three-fold – approximately two fetal cells per 1 million maternal cells. Unfortunately, a slight increase in the false-positive rate was also noted under such conditions.

It is of interest that the Ikonisys examination of more than 60 samples indicates that fetal cells could be reliably detected in 93% of cases, and that this was evident in both samples taken in the first and second trimesters of pregnancy. This pleasing result suggests that with a bit of optimization, this system may be suitable for use on all pregnancies. It also suggests that systems are now available that can reliably scan several million cells in a robust and automated manner for the presence of few or single-target cells – indeed a quantum leap forward in this technological arena – as such, systems were not available a few years ago.

What is not clear from this publication is the amount of time taken for the analysis of each case and how the issue for false-positives is to be addressed. What is clear, however, is that the system can be used for other diagnostic tasks, such as the detection of rare circulating tumor cells [18], thereby once again underscoring the similarity between these two fields.

In the second study, conducted by Huang and colleagues at Artemis Health Inc., use was made of a microfluidic filtration device to separate erythrocytes from other nucleated cells in maternal blood samples [17]. Similar systems have been examined previously; for instance, the Nanos system in Singapore, with the use of a dielectrophoresis (DEP) microfluidic device [19]. DEP is the movement of neutral particles induced by polarization in an asymmetric electric field. Manipulation can be performed on cells based on the differences in their dielectrophoretic properties.

The device developed by Artemis appears to be very effective in depleting erythrocytes from the maternal blood sample (>99.9% efficacy) [17]. What is also remarkable is that they claim to be able to retrieve a large number of putative target cells, namely erythroblasts, which, with an average recovery of 38 cells/ml maternal blood, is almost 20-times better than what could previously be achieved using the most highly optimized magnetic cell separation (MACS) approach [20]. It is also almost double the number we have previously been able to recover using a soy bean lectin system [21].

The high recovery may stem, in part, from the effective clearance of erythrocytes without the concomitant loss of target cells, as is the case when using other approaches, such a density-gradient centrifugation. Although there are still a number of questions that remain to be addressed, including whether the recovered cells are fetal or maternal, the time taken for sample processing and the number of samples that can be processed in parallel, this development is to be lauded and its progress closely monitored.

Although it is estimated that we may be able to recover only one or two fetal cells per million maternal cells, the accurate identification of the fetal origin of the cells using ε-globin would allow a pure population of fetal cells and, hence, pure fetal genomic DNA, to be recovered [22,23]. This could form the basis for automated scanning and recognition systems [24,25].

Laser microdissection and pressure catapulting (LMPC) is a rapidly emerging technology designed for the isolation of single cells for genomic analysis [26–28]. One such device is the Zeiss PALM MicroBeam® system, whereby integration of image-analysis platforms fully automates screening, identification and cell capture for downstream applications, such as whole-genome amplification, single-cell mRNA extraction, PCR-based technologies and microarray analyses [26]. This system has been used effectively for the isolation of single fetal erythroblasts for subsequent analysis by single-cell PCR [12]. It is not inconceivable that the very small numbers of fetal cells enriched from maternal blood may actually be sufficient for downstream analysis using the more modern single-cell analysis methods.

Consequently, these independent developments do strongly suggest that 'fetal cells are not dead yet' and that we are likely to see more commercially viable approaches appearing in the near future.

Problems besetting cell-free DNA

Even though cff-DNA is much more abundant than trafficking fetal cells, it only makes up a small fraction of the total (cf-DNA) in maternal plasma (3–10%) [15]. This fraction becomes even smaller in serum, as the amount of maternal cf-DNA increases by two- to three-fold due to dying cells releasing their nuclear DNA during the clotting procedure.

While the overt presence does not affect the analysis of facile fetal genetic loci completely absent form the maternal genome, such as the *RHD* gene in Rhesus d-negative pregnant women or the Y chromosome for fetal sex determination, it does become problematic when trying to discern fetal loci not so disparate from maternal ones [3,14]. The latter includes subtle alterations, such as point mutations, involved in Mendelian disorders, such as the hemoglobinopathies or cystic fibrosis, or alterations in gene dosage, such as the presence of an additional chromosome or part thereof in fetal aneuploidies, such as Down syndrome.

In these cases the large preponderance of maternal cf-DNA is problematic as, in essence, IT excludes the possibility of performing these analyses. In order to overcome this problem, several strategies have been explored.

Size fractionation

In our examination of whether any physical differences existed between maternal cf-DNA and cff-DNA fragments, we observed that fetal fragments were generally smaller in size than those of maternal origin (<300 vs >500 bp, respectively) [29]. This difference is probably attributable to differences in the apoptotic mechanisms involved in the release of cff-DNA by the syncytiotrophoblast, and that of maternal cf-DNA, which is largely of hemopoeitic origin, probably erythropoiesis. In this context, it is interesting to note that although the nuclear DNA in erythroblasts is cleaved prior to enucleation, this is not in the form of normal oligosomal apoptotic fragments but, rather, in large megabase fragments, which can only be resolved by pulse gel electrophoresis [30]. This feature was also observed in our analysis of total cf-DNA fragments [29].

By exploiting this difference between maternal cf-DNA and cff-DNA fragments, we were able to selectively enrich for fetal fragments using conventional agarose gel electrophoresis [29]. These primary experiments showed that the cff-DNA fraction could be increased to over 30%, compared with less than 5% in the untreated sample. This increment permitted the detection of otherwise masked fetal loci, such as short-tandem repeats (STRs) or point mutations, such as those for achondroplasia or β-thalassemia [29,31,32]. Our analyses showed this feature held true for approaches using either real-time PCR or MALDI-TOF mass spectrometry [33,34].

The current problem with this approach is that no efficient method exists to perform the size fractionation [35]. Although useful for proof-of-principle experiments, the agarose gel electrophoresis approach requires large volumes of cf-DNA (extracted from 10–20 ml plasma) and is associated with a large degree of loss (~50%). As such, it will only become viable once more efficient methods for this process emerge, perhaps via the use of microfluidic devices.

Epigenetic differences

An alternative method to discriminate between fetal and maternal cf-DNA sequences is to use epigenetic differences between these two DNA species [36,37]. An example of a gene sequence that is hypomethylated in the placenta and hypermethylated in maternal blood cells is the *maspin* gene promoter [36]. By the use of bisulfite conversion, the unmethylated fetal cytosine nucleotide is converted to uralic (thymine), while the maternal methylated cytosine is left unchanged. The altered fetal allele can then be detected by mass spectrometry or real-time PCR. Since the *maspin* gene is located on chromosome 18, it was examined whether this approach could be used to detect fetal aneuploidies specific for this chromosome [38]. By targeting a SNP involving a methylated cytosine residue, it would be possible to use the epigenetic allele ratio to determine fetal ploidy. In their study, Tong and colleagues were, indeed, able to use this approach to discern trisomy 18 samples from euploid samples when using genomic placental DNA [38]. This distinction was, however, not absolute, and using the cut-off values proposed by the authors, three euploid samples would have been classified as being abnormal. Given this less than satisfactory state affairs when examining total genomic DNA, it is questionable how effective this approach would be for the analysis of cff-DNA in maternal plasma.

A major problem besetting the use of epigenetically modified DNA sequences is the use of a bisulfite conversion step, a procedure that is very aggressive and destroys a vast amount of template DNA; a major problem when dealing with limiting input DNA quantities. As such, alternative strategies need to be sought, perhaps such as the immunoprecipitation of methylated DNA sequences using antibodies specific for methylated cytosine residues [39].

Cell-free fetal mRNA

The discovery of cf-mRNA species of placental origin in maternal plasma opened up the way for an alternative strategy to the analysis of cff-DNA [40,41]. The major advantage of cff-RNA over cff-DNA is that it is possible to select for placenta-specific mRNA transcripts not expressed by any maternal tissues [42]. Hence, the analysis of cff-RNA is, in essence, very similar to the analysis of fetal genetic loci completely absent from the maternal genome (Rhesus D- or Y-chromosome-specific sequences) as it is not hindered by maternal background 'noise'.

In order to use such an approach for the determination of fetal ploidy, one has to select genes present on the chromosome of interest, for example, chromosome 21, and then ensure that the gene is only expressed in the placenta and not by maternal tissues [43]. An example of such a gene is *PLAC4*. For the determination of chromosomal ploidy, an approach similar to that used for *maspin* gene in trisomy 18 can be taken, namely the use of mass spectrometry to determine allelic ratios of a SNP locus in the *PLAC4* gene (Figure 1) [43].

Although the study conducted by Lo and colleagues was based on a small number of affected cases (n = 10), a remarkably clear difference between case and control samples (n = 56) was observed, yielding a sensitivity of 90% and specificity of 96%. The current drawback of this approach is that the fetus needs to be heterozygous for the SNP allele being interrogated. As such, almost 100 cases had to be excluded from the aforementioned investigation. A further problem is the labile nature of mRNA, which requires complex immediate post-phlebotomy handling and processing. Furthermore storage and shipping needs to be carried out at -70°C or on dry-ice, which is an additional burden. Regardless of these issues, this approach has been seized upon by Sequenom Inc., which is exploring the commercial viability.

Digital PCR & its affiliates

Digital PCR differs from other quantitative PCR approaches, such as real-time PCR, in that the PCR reaction is allowed to proceed to its plateau and a simple 'yes or no' answer is used to monitor the presence or absence of input template [44]. In order to use this system in a quantitative manner it is necessary to monitor each PCR reaction individually. Although initial exploration of such an approach were made in 1997 by Kalinina and colleagues [45] and later optimized by Vogelstein and Kinzler in 1999 [44], it was only with the introduction of microfluidic devices with several thousand individual reaction chambers, such as those developed by Fluidigm Inc., that 'digital PCR' came of age [46,47].

The use of this technology for the detection of fetal aneuploidies was published almost simultaneously by Fan and Quake [48] and the Hong Kong group of Dennis Lo [49]. In their examination, Fan and Quake used a similar PCR assay to the one we had previously established for the detection of trisomy 21 by Taqman real-time PCR on pure fetal genetic material obtained by invasive practises, in which we compared the dosage of the amyloid gene on chromosome 21 to that of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) on chromosome 12 [50]. In their analysis of pure fetal genetic material, they were similarly able to reliably discriminate between normal and aneuploid samples. In the Hong Kong, examination use was made both of a SNP locus and a gene dosage approach, obtaining results very analogous to those made by Fan and Quake (Figure 2).

What is of particular interest in these studies is that, they observed the ability to detect the presence of a fetal aneuploidy was still possible even if the fetal material only represented 10% of the total cf-DNA examined, provided that 4000 individual events were examined [48]. Hence, it may be possible to use this technology to detect fetal aneuploidies directly from cff-DNA in maternal plasma. Since subsequent studies using digital PCR have indicated that the amount of cff-DNA has been underestimated using real-time PCR, and may be as high as 10%, this facet could soon become a reality [51].

It is also likely that the digital PCR approach will be useful not only for the detection of fetal aneuploidies, but may also permit the noninvasive determination of single-gene disorders, such as the hemoglobinopathies [47,52]. Once again, it is evident that this technology may also be applicable to other clinical disciplines, such as oncology [53].

Although the current studies have focused on the use of microfluidic devices, such as those developed by Fluidigm Inc., it may be possible to use a somewhat 'cruder' approach termed 'beaming' for 'beads, emulsion, amplification and magnetics'. In this procedure developed by Diehl and colleagues, the input template DNA is first preamplified and then coupled to a streptavidin-coated bead via a primer containing a biotin tag [54]. The second round of PCR is then carried out in an emulsion phase, following which ration of the two DNA sequences of interest is monitored by flow cytometry using fluorescently labeled primers. By the use of such means, Diehl and colleagues have been able to monitor and quantify cancer-derived cf-DNA [55]. Although this method is technically more complex than straight-forward digital PCR, it does not require any expensive equipment or costly specialized microfluidic reaction chambers. It may, hence, emerge as an alternative for those with cash-strapped research budgets.

Shot-gun sequencing

Even though the digital PCR approach is quite promising, it is clear that the degree of accuracy required for the detection of a fetal aneuploidy is at the limit of current microfluidic devices, as they only have space for 12,000 individual reactions per chip [47]. For this reason an alternative was sought that offered a higher

degree of fidelity by examining more target molecules. Such a system was found in the Solexa/Illumina platform for shot-gun sequencing. In this system, very short tags from the entire genome are amplified and sequenced. In their pioneering study, Fan and colleagues obtained an average of 10 million 25-bp sequence tags per sample, which contained an average of 65,000 tags specific for chromosome 21 [56]. By using these sequence tags for digital PCR-like measure, they were able to discern nine cases of trisomy 21 from nine euploid cases with 100% accuracy, in an analysis of cff-DNA. They were furthermore able to detect two cases of trisomy 18 and one case of trisomy 13. In a parallel report, Chiu and colleagues were able to correctly discern 14 cases of trisomy 21 from 28 normal cases [57]. These two pivotal studies, therefore, strongly suggest that shot-gun sequencing may be the most suitable approach for the noninvasive detection of fetal aneuploidies.

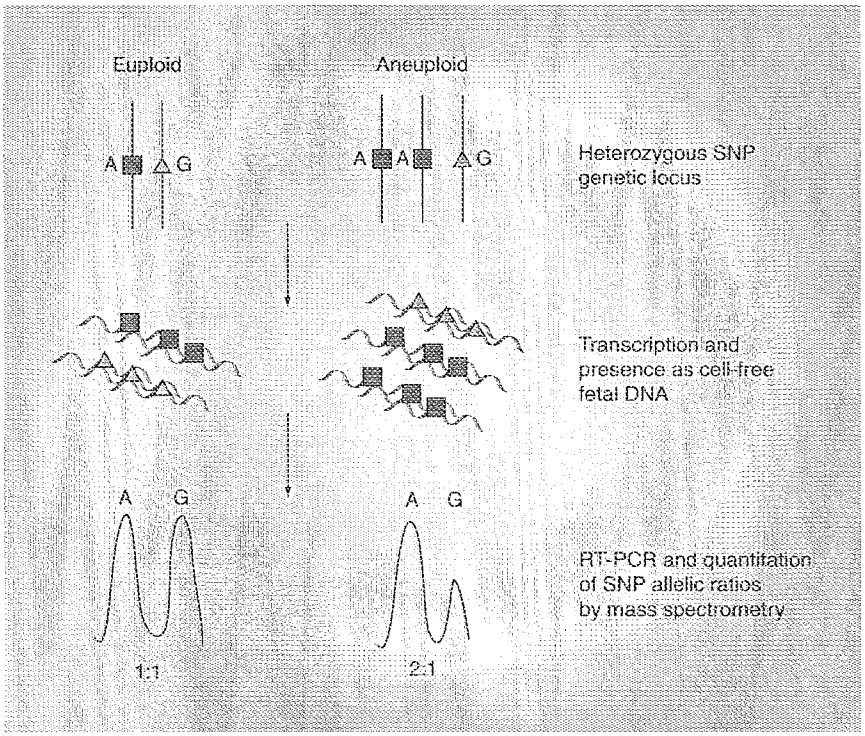

Figure 1. Mass spectrometry-based assay using placentally derived cell-free RNA for the determination of fetal aneuploidy.
RT: Real time; SNP: Single nucleotide polymorphism.

Other alternatives: urinary DNA?

Although the presence of cf-DNA in urine [58–60], other than that of kidney origin [61], has been a contentious issue, recent publication does suggest that 'transrenal DNA' may be another option for noninvasive prenatal diagnosis [62]. In this report by Shekhtman and colleagues, who were able to detect cff-DNA in maternal urine by using very short PCR amplicons (25–88 bp), observed that urinary cff-DNA fragments were very small, some specialized extraction and analytic procedures needed to be used in order to male cff-DNA in 78 out of detect them reliably [62]. In their report, they were able to detect 82 samples from women pregnant with a male fetus. On the other hand, Y-chromosome-specific signals were detected in 11 out of 91 samples where the pregnancy was with a female fetus. Hence, care needs to be taken to ensure that the maternal urine sample is not contaminated by male cells/DNA, probably as the result of intercourse.

In the analysis by the Hong Kong group of Dennis Lo, who pursued their investigations into cancer-derived cf-DNA in urine [63] by examining bone marrow-transplant recipients [64]. In this new study [65,66] they determined that both transplant-derived DNA and epithelial-like cells were present in recipient urine [64]. Furthermore, they concluded that the transplant-derived urinary DNA was derived from donor-derived stem cells, as these have settled in renal tubule niches.

As the persistence of transplacental trafficking of fetal cells with stem cell-like character has been reported on numerous occasions [67], and as these cell have been found to contribute to maternal tissues, especially in the capacity of tissue repair [68,69], it is unclear whether such cells could contribute to the phenomenon of transrenal cff-DNA.

Expert commentary & five-year view

Of all the systems tested to date, shot-gun sequencing used in a pseudodigital PCR mode appears to offer the most reliable detection of fetal aneuploidies, with truly amazing discrimination between affected cases and normal controls. The current problem with this approach is time and money, in that the processing and data analysis of each sample is a lengthy and costly affair (~US$700–1200 per case and only 16 samples/week/Illumina instrument). This revolutionary method, however, may offer the possibility of obtaining detailed karyotypic information by non-invasive means. It is, however, not clear if it will be useful for the analysis of Mendelian disorders, as the method relies on the analysis of a vast amount of genetic loci per chromosome targeted (in the order of 60,000 for chromosome 21), unlike the single/dual mutation involved in disorders, such as the hemoglobinopathies.

The optimization of digital PCR approaches by the employment of microfluidic devices that permit in excess of 30,000 single PCR reactions on a single chip may offer a cheaper alternative to the complex shot-gun sequencing approach. A drawback of these analyses is that the amount of cff-DNA needs to be estimated quite accurately in order to ensure that the template concentration is in the narrow range required for efficient digital PCR analysis (<1 copy/reaction/well).

Even though the fetal mRNA approach has been propagated to a large extent by Sequenom, it is not clear how suitable this system is to wide-scale application due to processing and shipping issues, a major concern when dealing with a labile analyte, such

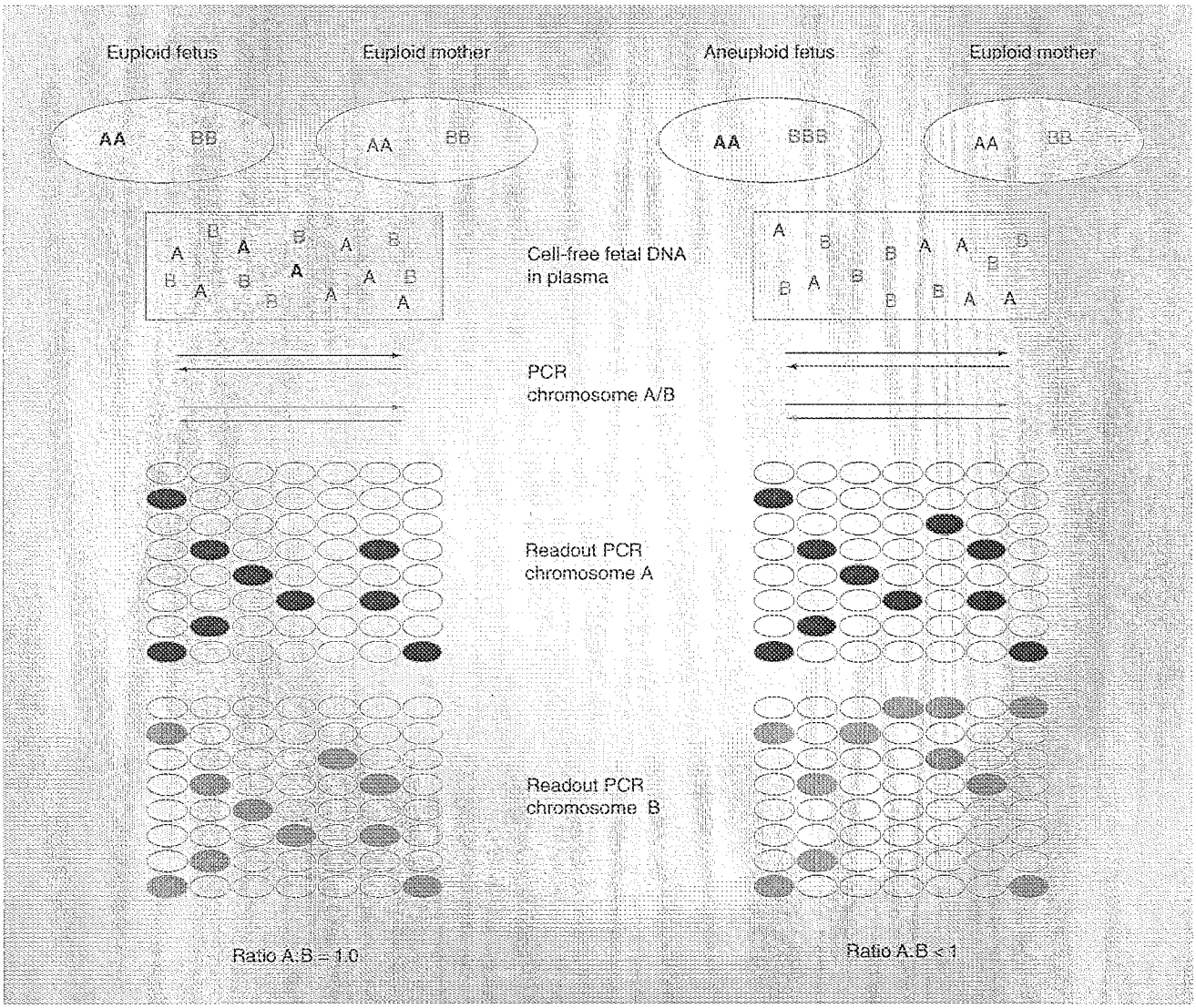

Figure 2. Digital PCR-based assay using cell-free fetal DNA for the determination of fetal aneuploidy.

as mRNA. In this context, it is worth noting that recent corporate statements from Sequenom indicate a possible move towards a DNA-based approach, perhaps involving epigenetic markers.

The resurgent interest in fetal cells suggests that these have not been buried, but are seriously being considered by commercial institutions. While it is unlikely that fetal cells will be offered for wide-spread screening, as could be done using cff-DNA/-RNA, the ability to examine the entire fetal genome offers up a realm of possibilities, which will not be possible via the cff-DNA route. As such, it is foreseeable that this route will be more restricted and costly.

As indicated earlier, it is likely that two strategies will emerge. In one instance, a quick and simple test will be offered that permits rapid screening for the most common fetal aneuploidies (e.g., chromosomes 13, 18 and 21). This is likely to occur using an approach developed by Sequenom that, by relying on its proprietary mass spectrometry technology, is geared for high-throughput analysis of several thousand samples per day. This could make the test cost effective, to the extent where it can compete with conventional screening approaches.

Even though digital PCR approaches are promising, and may be useful for the analysis of single-gene disorders, they currently require expensive equipment and analytic devices, with the microfluidic chambers used for each analysis still costing several hundred US dollars a piece. Furthermore, the cff-DNA sample needs to be diluted in such manner that approximately 0.6 copies are present per reaction well, a tedious and time-consuming exercise.

Shot-gun sequencing approaches are even more costly and time-consuming facets, which will restrict their use until a new generation of high-throughput devices becomes available. As discussed, the use of fetal cells is largely dependent on the speed of current progress in microfluidic enrichment tools, automated cell detection and retrieval, as well as analytic systems permitting whole-genome analysis from single or few cells.

As such, these latter options will be more costly and only offered to a select group, whilst the former employing high-throughput cff-DNA/DNA mass spectrometry-based strategies may end up replacing or complementing current screening procedures, such as ultrasound and concomitant serum analyte analysis.

As always, it pays to be aware of developments outside the immediate scope of this review, of which the most important is probably that of proteomics. Such approaches may lead to the development of a new generation of highly specific screening markers, which could be so effective that other approaches become commercially unviable. Consequently, researchers and clinicians active in prenatal diagnosis need to be prepared for rapid changes and developments in the next decade.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Remarkable developments have occurred in the past 2–3 years, largely due to the use of 'digital PCR' and 'shot-gun sequencing'.
- Technical developments now have to focus on economic, robust, high-throughput processes.
- 'Fetal cells are not dead yet', and may become a viable alternative via the use of microfluidics and automated scanning devices.
- Unlike cell-free DNA, fetal cells offer the possibility of examining the entire fetal genome.
- New screening markers developed by proteomics may challenge some of the 'quick and dirty' approaches.
- Detection of Mendelian disorders may remain complex, especially in cases where both parents share the same mutation.
- Urinary cell-free DNA may become a new tool of interest.
- The use of cell-free fetal DNA and fetal cells may also serve as potential markers for the prediction of preeclampsia or preterm labor.

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Recent advances in the analysis of fetal nucleic acids in maternal plasma

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Purpose of review

Noninvasive prenatal diagnosis can be achieved by analyzing cell-free fetal DNA in maternal plasma. The fact that circulating fetal DNA represents only a minor fraction of the DNA that is present in maternal plasma has presented analytical challenges for a number of applications. In this review, we discuss such challenges and how they have been resolved by recent developments in the field.

Recent findings

Digital molecular counting methods, such as digital PCR and massively parallel sequencing, have enabled high quantitative precision for maternal plasma DNA analysis. Noninvasive prenatal analysis of monogenic disease mutations has been achieved by identifying small quantitative differences between the mutant and wild-type alleles in maternal plasma. By measuring the small increment in the fractional concentrations of DNA derived from potentially aneuploid chromosomes in maternal plasma, fetal chromosomal aneuploidies have been detected with high diagnostic accuracies.

Summary

Recently, advances in molecular technologies have enhanced the diagnostic applications of maternal plasma DNA analysis for noninvasive prenatal diagnosis. We foresee that this technology could play an increasingly important role in prenatal investigations.

Keywords

circulating fetal DNA, digital PCR, massively parallel sequencing, noninvasive prenatal diagnosis

INTRODUCTION

Prenatal diagnosis has become an important part of obstetric care. Conventionally, prenatal genetic testing requires the collection of fetal genetic materials through invasive procedures such as chorionic villus sampling and amniocentesis, thus posing a risk of miscarriage [1]. Cell-free fetal DNA in maternal plasma has offered a noninvasive source of fetal genetic materials that can be conveniently collected by taking the mother's blood. The clinical applications of circulating fetal DNA have been continuously expanded since its discovery in 1997 [2]. Currently, in addition to paternally inherited sequences and mutations, circulating fetal DNA can also be used for detecting maternal alleles inherited by the fetuses, as well as fetal chromosomal aneuploidies [3,4].

DIRECT DETECTION OF PATERNAL SEQUENCES IN MATERNAL PLASMA

Maternal plasma contains both fetal and maternal DNA molecules. Initial studies of circulating fetal DNA have focused on paternally inherited fetal DNA sequences that are absent in the maternal genome [2]. One such application is for noninvasive fetal sex determination. The positive detection of chromosome Y DNA sequences in maternal plasma would indicate a male fetus. The fetal sex information is important for managing pregnancies at risk for X-linked diseases [5]. If a woman carries a female fetus, invasive diagnosis can be avoided because X-linked diseases rarely manifest in female individuals. Currently, noninvasive fetal sex determination using circulating fetal DNA has been implemented as routine service in a number of clinical centers [6,7]. According to a recent meta-analysis on the

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KEY POINTS

- The detection of maternally inherited fetal DNA in maternal plasma is technically challenging because of the interference from the maternal DNA background.
- Quantitative dosage analysis using molecular counting methods, such as digital PCR and massively parallel sequencing, has provided one approach for addressing this challenge.
- The noninvasive genotyping of fetal monogenic disease mutations using molecular counting methods, including autosomal and X-linked diseases, has been achieved.
- Large-scale studies showed that the noninvasive diagnosis of fetal aneuploidies is highly accurate by using massively parallel sequencing.
- Two proof-of-concept studies have demonstrated that fetal whole genome scanning is feasible.

published results between 1997 and 2011, the sensitivity and specificity for fetal sex detection were 95.4 and 98.6%, respectively [6]. Efforts to attempt to enhance the sensitivity of male fetal DNA detection in maternal plasma are still continuing [8] in order to allow the test to be applicable to very early pregnancies.

Fetal RhD blood group detection is another well developed example of a diagnostic application for circulating fetal DNA [9,10]. The presence of *RHD* gene sequences in the maternal plasma of a RhD-negative woman would indicate a RhD-positive fetus. Maternal administration of anti-RhD immunoglobulin would then be necessary in order to prevent sensitization. Clinical services for noninvasive fetal RhD determination using maternal plasma DNA have been available for over 10 years [11]. Recent reports indicated that the accuracy of the test was close to 100% [12,13].

QUANTITATIVE DOSAGE ANALYSIS OF MATERNAL PLASMA DNA

The major challenge of circulating fetal DNA analysis is caused by the large amount of background maternal DNA in maternal plasma. Fetal DNA constitutes approximately 10% of the total DNA in maternal plasma [14,15]. Hence, it is technically challenging to detect maternally inherited DNA sequences that are carried by the fetus.

Fetal monogenic diseases

To overcome this technical difficulty for fetal monogenic disease detection, an approach termed relative mutation dosage (RMD) was developed [16,17]. In the maternal plasma of a woman heterozygous for the disease-causing mutation, the contribution of fetal DNA to the maternal plasma DNA pool would lead to an increase in the total DNA concentration of the allele inherited by the fetus. Hence, the overrepresented allele observed in maternal plasma would be the allele that the fetus is inherited. However, the low fractional amount of fetal DNA in maternal plasma implies that the degree of overrepresentation is very small (Fig. 1) [18▪▪]. To obtain an analytical precision that would allow discrimination of the small concentration difference between the mutant and wild-type DNA, quantification based on molecular counting, such as digital PCR, has been employed. Digital PCR performed on a microfluidic platform involves the simultaneous analysis of thousands of PCRs, with each reaction containing either a single or no template DNA molecule [19]. The copy number of template DNA molecules in the sample is calculated by counting the number of positive reactions.

The RMD approach was first investigated in autosomal recessive diseases by using β-thalassemia and hemoglobin E disease as models [16]. For a pregnant woman heterozygous for a disease-causing mutation, if the fetus is homozygous for the mutant or the wild-type allele, an overrepresentation of the corresponding allele would be observed in maternal plasma. If the fetus is heterozygous for the mutation, there would be a dosage balance between the mutant and the wild-type allele (Fig. 1). Recently, the RMD approach has also been applied to determine the fetal inheritance of a sickle cell anemia mutation noninvasively using maternal plasma [20].

The application of RMD was further explored in X-linked recessive diseases [21]. As discussed above, the detection of chromosome Y DNA sequences in maternal plasma has provided a noninvasive means for fetal sex determination. Most of the female fetuses would either be unaffected or are asymptomatic carriers. However, the disease status of male fetuses would still be unknown if the genotype for the mutation cannot be confirmed. The RMD approach has, therefore, been used to determine whether the male fetus has inherited the X-linked mutation from the heterozygous mother. For X-linked diseases, a male fetus possesses only one chromosome X. Hence, there are only two observations in maternal plasma, that is, either the mutant or the wild-type allele is overrepresented, indicating an affected or a normal fetus, respectively (Fig. 1). This approach was successfully used to detect fetal mutation genotypes for hemophilia A and B noninvasively using maternal plasma [21].

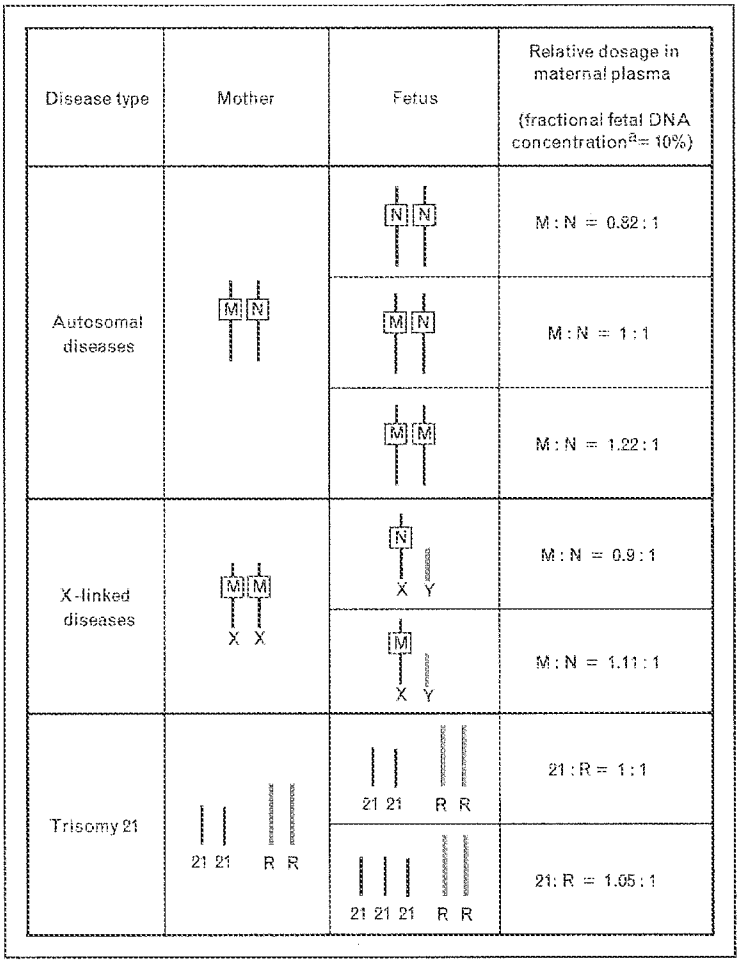

FIGURE 1. The relative dosage analysis in the scenarios of monogenic autosomal diseases, monogenic X-linked diseases, and trisomy 21. 21, chromosome 21; M, mutant allele; N, wild-type allele; R, reference chromosome, that is, the chromosome unaffected by aneuploidy; X, chromosome X; Y, chromosome Y. [a]A maternal plasma sample with fractional fetal DNA concentration of 10% was used for illustration. (Modified with permission from [18▪▪].

Fetal chromosomal abnormalities

In addition to monogenic diseases, the relative dosage analysis of maternal plasma DNA could theoretically be used for detecting the dosage of a potentially aneuploid chromosome possessed by the fetus. This strategy has been outlined for the noninvasive detection of fetal trisomy 21 [19]. One could compare the copy number of a locus on chromosome 21 with the copy number of another locus on an unaffected chromosome, that is, the reference chromosome, in maternal plasma. An overrepresentation of chromosome 21 DNA would indicate a trisomy 21 fetus, whereas a dosage balance would indicate a euploid fetus (Fig. 1). However, the deduction of fetal chromosome dosage is technically more difficult than fetal mutation analysis. As illustrated in Fig. 1, the degree of dosage imbalance in the scenario of trisomy 21 is smaller than that for the monogenic diseases. This suggests that more DNA molecules have to be analyzed in order to reach a precision that is able to discriminate such small dosage difference. To detect fetal trisomy 21 using a maternal plasma sample with a fractional fetal DNA concentration of 25%, it has been estimated that 7680 digital PCRs would have to be performed [19,22]. However, the amount of available template molecules is limiting due to the generally low concentration of cell-free DNA in maternal plasma. In the above example, in order to capture 7680 template molecules, approximately 15 ml of maternal blood would need to be drawn, which is at the limit of clinical acceptability. Hence, alternative methodology that could generate extra genetic information without additional input of DNA molecules would be desirable.

Random sequencing of maternal plasma DNA

Although plasma DNA molecules are partially degraded [23,24], they have been reliably analyzed

by massively parallel sequencing (MPS) [25,26]. MPS analyzes billions of DNA molecules originating from virtually the whole genome in a single run. By retrieving sequenced DNA molecules that are originated from the disease-relevant loci, noninvasive prenatal diagnosis could be performed.

Fetal trisomy 21 detection

MPS can be used to measure the proportional representation of a potentially aneuploid chromosome in maternal plasma and to determine whether the representation is abnormally high (e.g., in trisomy) or low (e.g., in monosomy). In proof-of-principle studies, MPS has been shown to be highly accurate for detecting the increased proportional representation of chromosome 21 DNA sequences in the plasma of pregnant women carrying trisomy 21 fetuses, when compared to those carrying euploid fetuses [25,26].

The accuracy of the MPS approach for noninvasive prenatal aneuploidy detection has been confirmed in many large-scale studies [27–32,33▪]. The reported detection sensitivities range from 98.6 to 100% and the specificities range from 97.9 to 100%. In order to increase the throughput of analysis for clinical implementation, DNA molecules from different plasma samples have been labeled with unique index sequences and analyzed by MPS in a multiplexed fashion. It has been shown that the accuracy of noninvasive fetal aneuploidy detection remained high even with such multiplexing [27,28].

Detection of fetal trisomies 18, 13, and other aneuploidies

In principle, fetal trisomies 18 and 13 could be detected noninvasively by the same approach using MPS. However, initial studies showed that the precision for measuring the proportional representation of chromosomes 13 and 18 in maternal plasma was suboptimal [25,26,34]. Such a large variance has been found to be related to the guanine and cytosine contents of chromosomes 18 and 13 [34,35], in which the number of sequenced reads obtained from the chromosomes is affected due to the possible intrinsic guanine and cytosine bias of library amplification before MPS [36]. Researchers therefore developed bioinformatic algorithms to correct for this guanine and cytosine bias. After the guanine and cytosine correction, the diagnostic sensitivities for noninvasive fetal trisomies 18 and 13 detection have dramatically improved [30,34,37]. Besides using a bioinformatic means, the amplification-associated guanine and cytosine bias could potentially be eliminated by a single molecule sequencing approach, in which DNA molecules are sequenced without prior library amplification [36]. We envision that with the advancement of new single molecule sequencing platforms, such as nanopore sequencing [38,39], the MPS of circulating nucleic acids would become simpler and more accurate.

In addition to aneuploidies involving an extra chromosome, the MPS approach has recently been demonstrated to be useful for the noninvasive prenatal diagnosis of monosomy X [32]. Besides the detection of supernumerary whole aneuploid chromsome, MPS has also been used to detect fetuses with trisomies 21 and 13 with robertsonian translocations [32,40], as well as a microdeletion on chromosome 12 [41]. Recently, the feasibility of the MPS-based noninvasive prenatal aneuploidy detection has also been tested in twin pregnancies [42]. Altogether, as most of these fetal aneuploidy situations were investigated only in a few individual cases (apart from trisomies 21, 13, and 18 mentioned above), their diagnostic accuracies would need to be confirmed in large-scale studies.

Fetal polymorphism and mutation detection

By analyzing maternal plasma DNA using MPS, researchers have been able to identify DNA sequences originated from the whole fetal genome [43,44]. Lo *et al.* [43] first demonstrated that with the use of the paternal genotype and maternal haplotype, a genome-wide genetic map of a fetus could be deduced from the maternal plasma DNA sequencing results. Recently, this approach has been confirmed by Kitzman *et al.* [44]. Through the use of parental genetic maps of increased resolution and deeper maternal plasma DNA sequencing, Kitzman *et al.* [44] have shown that the general approach described by Lo *et al.* [43] is scalable. Due to the increased resolution of the fetal genomic map that is deduced, Kitzman *et al.* [44] also attempted to demonstrate that the approach could be used, in principle, to detect fetal de-novo mutations with reasonable sensitivity but with a very low specificity. The most important message from the studies by Lo *et al.* [43] and Kitzman *et al.* [44] is that maternal plasma carries genetic information of the entire fetal genome, indicating that the noninvasive prenatal diagnosis of multiple genetic diseases in a single assay is theoretically possible. Indeed, Lo *et al.* [43] have demonstrated that the noninvasive prenatal diagnosis for β-thalassemia is possible using such an approach.

ENRICHING SELECTED REGIONS FOR TARGETED MASSIVELY PARALLEL SEQUENCING

The precision, and hence the accuracy, of measuring fetal chromosome and haplotype dosage depends on the number of DNA molecules analyzed by MPS.

The diagnostic accuracy could, therefore, be enhanced by sequencing more DNA molecules in maternal plasma. However, this approach is less suitable for clinical use because of the high sequencing cost and low throughput. In fact, in most of the current MPS studies on maternal plasma, the whole genome was randomly sampled. As the disease-related regions usually represent only a proportion of the genome, one could make the argument that it might perhaps be more cost-effective to selectively enrich DNA molecules originating from these target regions before MPS.

Liao *et al.* [45] demonstrated the use of targeted MPS for analyzing maternal plasma DNA, using 3 Mb of exonic regions on chromosome X as a model system. Sequence-specific nucleic acid probes were used to capture and enrich DNA molecules of the targeted regions. The authors showed that with 12 million DNA reads sequenced, the reads covering the targeted regions were enriched by 213-fold. More importantly, the proportional amount of maternal and fetal DNA did not alter after enrichment, suggesting that the quantitative dosage information was not significantly distorted by the enrichment process.

Recently, the feasibility of targeted MPS for noninvasive prenatal aneuploidy detection has been investigated [46,47]. To detect fetal trisomies 13 and 21 with maternal plasma, Stumm *et al.* [46] utilized a commercially available target-capturing probe set to capture DNA sequences from the exonic regions along the whole genome. However, the enrichment efficiency of using this probe set would depend on the relative length of the targeted regions that are located in the relevant chromosomes. The authors have not reported whether the sequenced reads covering the aneuploid chromosomes were enriched by this method [46]. As an alternative approach, Liao *et al.* [47] designed a probe set to enrich chromosome 7, 13, 18, and 21 DNA sequences in maternal plasma. They have, thus, increased the read coverage of the targeted chromosomes by 225-fold [47]. The authors then explored an allelic ratio strategy for noninvasive fetal trisomy 21 detection. They analyzed informative single nucleotide poymorphisms (SNPs) in which the mother was homozygous and the fetus was heterozygous for the SNP genotypes. Theoretically, in the maternal plasma of a woman carrying a trisomic fetus, the relative amount of the fetal allele to the allele shared by the mother and fetus would deviate from that of a euploid pregnancy. The accuracy of this method depends on the availability of sufficient allelic read counts in the plasma samples [47].

In yet another targeted MPS strategy, Sparks *et al.* [48] used a target amplification method to selectively amplify hundreds of loci on chromosomes 18 and 21 prior to MPS. The authors have applied this method to calculate the risk for trisomies 18 and 21 noninvasively using maternal plasma. They developed a risk-scoring algorithm that adjusted the MPS result with fractional fetal DNA concentration of the plasma sample, and integrated the risk factors of maternal age and gestational age [48,49]. The relative contributions of the DNA sequencing results, the fractional fetal DNA concentrations, and integrated risk factors to the performance of the final test result are not entirely clear [48]. It would be interesting to compare the relative robustness of the method described by Sparks *et al.* [48] with methods based on random sequencing [27,29], as the latter methods do not appear to require the incorporation of the fractional fetal DNA concentration and integrated risk factors to arrive at a robust prediction of aneuploidy risk.

CLINICAL IMPLEMENTATION OF NONINVASIVE FETAL ANEUPLOIDY SCREENING

Owing to the promising diagnostic performance, the MPS-based noninvasive fetal aneuploidy detection has been rapidly introduced as a clinical service in the United States, Mainland China, and Hong Kong. Currently, despite the continual cost reduction, MPS is still a relatively expensive technology. Hence, a balance between the running cost and the rate of aneuploidy cases being identified has to be considered for clinical implementation. In practice, one implementation option is to provide the MPS-based noninvasive prenatal diagnosis to high-risk women as identified by biochemical and ultrasound screening. Women who are subsequently positive for the noninvasive diagnosis are indicated for invasive confirmatory tests. This new screening pathway could significantly reduce the number of invasive procedures and the associated miscarriages [33▪,50].

CONCLUSION

In summary, with the development of very precise quantitative dosage analysis by molecular counting technologies, the noninvasive prenatal diagnosis of monogenic diseases and fetal aneuploidies has progressed rapidly in recent years. Such progress has allowed a genome-wide fetal genetic map and indeed the entire fetal genome to be assembled noninvasively. We foresee that noninvasive prenatal diagnosis would play an increasingly important role in future prenatal testing. In this regard, ethical and social issues concerning the clinical practice of

noninvasive prenatal diagnosis should be actively discussed [51,52].

Acknowledgements

None.

Conflicts of interest

The authors have filed patent applications and hold patents on the analysis of fetal nucleic acids in maternal plasma. Part of this patent portfolio has been licensed to Sequenom. Y.M.D. Lo is a consultant to and holds equities in, Sequenom.

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- ▪ of special interest
- ▪▪ of outstanding interest

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DIGITAL AMPLIFICATION

The U.S. government retains certain rights in this invention by virtue of its support of the underlying research, supported by grants CA 43460, CA 57345, and CA 62924 from the National Institutes of Health.

The disclosure of all claimed priority applications is expressly incorporated herein.

TECHNICAL FIELD OF THE INVENTION

This invention is related to diagnostic genetic analyses. In particular it relates to detection of genetic changes and gene expression.

BACKGROUND OF THE INVENTION

In classical genetics, only mutations of the germ-line were considered important for understanding disease. With the realization that somatic mutations are the primary cause of cancer, and may also play a role in aging, new genetic principles have arisen. These discoveries have provided a wealth of new opportunities for patient management as well as for basic research into the pathogenesis of neoplasia. However, many of these opportunities hinge upon detection of a small number of mutant-containing cells among a large excess of normal cells. Examples include the detection of neoplastic cells in urine, stool, and sputum of patients with cancers of the bladder, colorectum, and lung, respectively. Such detection has been shown in some cases to be possible at a stage when the primary tumors are still curable and the patients asymptomatic. Mutant sequences from the DNA of neoplastic cells have also been found in the blood of cancer patients. The detection of residual disease in lymph nodes or surgical margins may be useful in predicting which patients

might benefit most from further therapy. From a basic research standpoint, analysis of the early effects of carcinogens is often dependent on the ability to detect small populations of mutant cells.

Because of the importance of this issue in so many settings, many useful techniques have been developed for the detection of mutations. DNA sequencing is the gold standard for the detection of germ line mutations, but is useful only when the fraction of mutated alleles is greater than ~20%. Mutant-specific oligonucleotides can sometimes be used to detect mutations present in a minor proportion of the cells analyzed, but the signal to noise ratio distinguishing mutant and wild-type (WT) templates is variable. The use of mutant-specific primers or the digestion of polymerase chain reaction (PCR) products with specific restriction endonucleases are extremely sensitive methods for detecting such mutations, but it is difficult to quantitate the fraction of mutant molecules in the starting population with these techniques. Other innovative approaches for the detection of somatic mutations have been reviewed. A general problem with these methods is that it is difficult or impossible to independently confirm the existence of any mutations that are identified.

Thus there is a need in the art for methods for accurately and quantitatively detecting genetic sequences in mixed populations of sequences.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for determining the presence of a selected genetic sequence in a population of genetic sequences.

It is another object of the present invention to provide molecular beacon probes useful in the method of the invention.

These and other objects of the invention are achieved by providing a method for determining the presence of a selected genetic sequence in a

population of genetic sequences. A biological sample comprising nucleic acid template molecules is diluted to form a set of assay samples. The template molecules within the assay samples are amplified to form a population of amplified molecules in the assay samples of the set. The amplified molecules in the assay samples of the set are then analyzed to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence. The first number is then compared to the second number to ascertain a ratio which reflects the composition of the biological sample.

Another embodiment of the invention is a method for determining the ratio of a selected genetic sequence in a population of genetic sequences. Template molecules within a set comprising a plurality of assay samples are amplified to form a population of amplified molecules in each of the assay samples of the set. The amplified molecules in the assay samples of the set are analyzed to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence. At least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence. The first number is compared to the second number to ascertain a ratio which reflects the composition of the biological sample.

According to another embodiment of the invention, a molecular beacon probe is provided. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 16 base pairs which has a T_m of 50-51 C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

A second type of molecular beacon probe is provided in another embodiment. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 19-20 base pairs and has a T_m of 54-56 C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

Another embodiment provides the two types of molecular beacon probes, either mixed together or provided in a divided container as a kit.

The invention thus provides the art with the means to obtain quantitative assessments of particular DNA or RNA sequences in mixed populations of sequences using digital (binary) signals.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A, 1B, 1C. Schematic of experimental design. (Fig. 1A) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes which discriminate between WT and mutant alleles and subsequent fluorometry. (Fig. 1B) Principle of molecular beacon analysis. In the stem-loop configuration, fluorescence from a dye at the 5' end of the oligonucleotide probe is quenched by a Dabcyl group at the 3' end. Upon hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence. Modified from Marras *et al.* (Fig. 1C) Oligonucleotide design. Primers F1 and R1 are used to amplify the genomic region of interest. Primer INT is used to produce single stranded DNA from the original PCR products during a subsequent asymmetric PCR step (see Materials and Methods). MB-RED is a Molecular Beacon which detects any appropriate PCR product, whether it is WT or mutant at the queried codons. MB-GREEN is a Molecular Beacon which preferentially detects the WT PCR product.

FIG. 2. Discrimination between WT and mutant PCR products by Molecular Beacons. Ten separate PCR products, each generated from ~25 genome equivalents of genomic DNA of cells containing the indicated mutations of *c-Ki-Ras*, were analyzed with the Molecular Beacon probes described in the text. Representative examples of the PCR products used for Molecular Beacon analysis were purified and directly sequenced. In the cases with Gly12Cys (SEQ ID NO: 11) and Gly12Arg (SEQ ID NO: 10) mutations, contaminating non-neoplastic cells within the tumor presumably accounted for the relatively low ratios. In the cases with Gly12Ser (SEQ ID NO: 8) and Gly12Asp (SEQ ID NO: 12), there were apparently two or more alleles of mutant *c-Ki-Ras* for every WT allele (SEQ ID NO: 7); both these tumors were aneuploid. Analysis of the Gly13Asp mutation is also shown (SEQ ID NO: 9).

FIG. 3. Detecting Dig-PCR products with MB-RED. Specific Fluorescence Units of representative wells from an experiment employing colorectal cancer cells with Gly12Asp or Gly13Asp mutations of the *c-Ki-Ras* gene. Wells with values >10,000 are shaded yellow. Polyacrylamide gel electrophoretic analyses of the PCR products from selected wells are shown. Wells with fluorescence values <3500 had no PCR product of the correct size while wells with fluorescence values >10,000 SFU always contained PCR products of 129 bp. Non-specific products generated during the large number of cycles required for Dig-PCR did not affect the fluorescence analysis. M1 and M2 are molecular weight markers used to determine the size of fragments indicated on the left (in base pairs).

FIG. 4. Discriminating WT from mutant PCR products obtained in Dig-PCR. RED/GREEN ratios were determined from the fluorescence of MB-RED and MB-GREEN as described in Materials and Methods. The wells shown are the same as those illustrated in Fig. 3. The sequences of PCR products from the

indicated wells were determined as described in Materials and Methods. The wells with RED/GREEN ratios >3.0 each contained mutant sequences while those with RED/GREEN ratios of ~1.0 contained WT sequences. WT c-Ki-Ras (SEQ ID NO: 7), Gly12Asp (SEQ ID NO: 13), and Gly13Asp (SEQ ID NO: 9) were analyzed.

FIG. 5. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED and the RED/GREEN ratios were 1.0 +/- 0.1 (mean +/- 1 standard deviation). The wells colored yellow contained no template DNA and each was negative with MB-RED (i.e., fluorescence <3500 fluorescence units.). The other wells contained diluted DNA from the stool sample. Those registering as positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis. The sequence of WT *c-Ki-Ras* in well (SEQ ID NO: 7), and mutant *c-Ki-Ras* in wells C10, E11, M10, and L12 (SEQ ID NO: 14), and well F21 (SEQ ID NO: 15) were analyzed.

DETAILED DESCRIPTION OF THE INVENTION

The method devised by the present inventors involves separately amplifying small numbers of template molecules so that the resultant products have a proportion of the analyte sequence which is detectable by the detection means chosen. At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type (WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques.

The method requires analyzing a large number of amplified products simply and reliably. Techniques for such assessments were developed, with the output providing a digital readout of the fraction of mutant alleles in the analyzed population.

The biological sample is diluted to a point at which a practically usable number of the diluted samples contain a proportion of the selected genetic sequence (analyte) relative to total template molecules such that the analyzing technique being used can detect the analyte. A practically usable number of diluted samples will depend on cost of the analysis method. Typically it would be desirable that at least 1/50 of the diluted samples have a detectable proportion of analyte. At least 1/10, 1/5, 3/10, 2/5, 1/2, 3/5, 7/10, 4/5, or 9/10 of the diluted samples may have a detectable proportion of analyte. The higher the fraction of samples which will provide useful information, the more economical will be the overall assay. Over-dilution will also lead to a loss of economy, as many samples will be analyzed and provide no signal. A particularly preferred degree of dilution is to a point where each of the assay samples has on average one-half of a template. The dilution can be performed from more concentrated samples. Alternatively, dilute sources of template nucleic acids can be used. All of the samples may contain amplifiable template molecules. Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules.

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ~0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This

sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a G to T transversion at the second position of codon 12 of *c-Ki-Ras*), are expected to occur in < 1 in 50 of these polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred *in vivo*. Significance can be established through rigorous statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Digital Amplification is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene can be easily determined using photoluminescent probes specific for each of the PCR products generated. Similarly, Digital Amplification can be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers which are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One photoluminescent probe would then be used to detect PCR products from the reference transcript and a second photoluminescent probe used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which

the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic status when two mutations are observed upon sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (*vs.* both occurring in one allele), cloning of PCR products is generally performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Digital Amplification are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR provide an excellent alternative to use of molecular beacons. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Digital Amplification may prove useful for these applications when the expected differences are small, (*e.g.*, only ~2-fold, such as occurs with allelic imbalances.)

Application	Example	Probe 1 Detects:	Probe 2 Detects:
Base substitution mutations	Cancer gene mutations in stool, blood, lymph nodes	mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	normal or translocated alleles	translocated allele
Gene amplifications	Determine presence or extent of amplification	sequence within amplicon	sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	minor exons	common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	first transcript	reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

The ultimate utility of Digital Amplification lies in its ability to convert the intrinsically exponential nature of PCR to a linear one. It should thereby prove useful for experiments requiring the investigation of individual alleles, rare variants/mutations, or quantitative analysis of PCR products.

In one preferred embodiment each diluted sample has on average one half a template molecule. This is the same as one half of the diluted samples having one template molecule. This can be empirically determined by amplification. Either the analyte (selected genetic sequence) or the reference genetic sequence can be used for this determination. If the analysis method being used can detect analyte when present at a level of 20%, then one must dilute such that a significant number of diluted assay samples contain more than 20% of analyte. If the analysis method being used requires 100% analyte to detect, then dilution down to the single template molecule level will be required.

To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. More preferably the dilution will be to between 0.1 and 0.6, more preferably to between 0.3 and 0.5 of the assay samples yielding an amplification product.

The digital amplification method requires analysis of a large number of samples to get meaningful results. Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. As in any method, the accuracy of the determination will improve as the number of samples increases, up to a point. Because a large number of samples must be analyzed, it is desirable to reduce the manipulative steps, especially sample transfer steps. Thus it is preferred that the steps of amplifying and analyzing are performed in the same receptacle. This makes the method an *in situ*, or "one-pot" method.

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As shown in the examples, the method can be used to find a tumor mutation in a population of cells which is not purely tumor cells. As described in the

examples, a probe for a particular mutation need not be used, but diminution in binding to a wild-type probe can be used as an indicator of the presence of one or more mutations. Chromosomal translocations which are characteristic of leukemias or lymphomas can be detected as a measure of the efficacy of therapy. Gene amplifications are characteristic of certain disease states. These can be measured using digital amplification. Alternatively spliced forms of a transcript can be detected and quantitated relative to other forms of the transcript using digital amplification on cDNA made from mRNA. Similarly, using cDNA made from mRNA one can determine relative levels of transcription of two different genes. One can use digital amplification to distinguish between a situation where one allele carries two mutations and one mutation is carried on each of two alleles in an individual. Allelic imbalances often result from a disease state. These can be detected using digital amplification.

Biological samples which can be used as the starting material for the analyses may be from any tissue or body sample from which DNA or mRNA can be isolated. Preferred sources include stool, blood, and lymph nodes. Preferably the biological sample is a cell-free lysate.

Molecular beacon probes according to the present invention can utilize any photoluminescent moiety as a detectable moiety. Typically these are dyes. Often these are fluorescent dyes. Photoluminescence is any process in which a material is excited by radiation such as light, is raised to an excited electronic or vibronic state, and subsequently re-emits that excitation energy as a photon of light. Such processes include fluorescence, which denotes emission accompanying descent from an excited state with paired electrons (a "singlet" state) or unpaired electrons (a "triplet" state) to a lower state with the same multiplicity, *i.e.*, a quantum-mechanically "allowed" transition. Photoluminescence also includes phosphorescence which denotes emission accompanying descent from an excited triplet or singlet state to a lower state of

different multiplicity, *i.e.*, a quantum mechanically "forbidden" transition. Compared to "allowed" transitions, "forbidden" transitions are associated with relatively longer excited state lifetimes.

The quenching of photoluminescence may be analyzed by a variety of methods which vary primarily in terms of signal transduction. Quenching may be transduced as changes in the intensity of photoluminescence or as changes in the ratio of photoluminescence intensities at two different wavelengths, or as changes in photoluminescence lifetimes, or even as changes in the polarization (anisotropy) of photoluminescence. Skilled practitioners will recognize that instrumentation for the measurement of these varied photoluminescent responses are known. The particular ratiometric methods for the analysis of quenching in the instant examples should not be construed as limiting the invention to any particular form of signal transduction. Ratiometric measurements of photoluminescence intensity can include the measurement of changes in intensity, photoluminescence lifetimes, or even polarization (anisotropy).

Although the working examples demonstrate the use of molecular beacon probes as the means of analysis of the amplified dilution samples, other techniques can be used as well. These include sequencing, gel electrophoresis, hybridization with other types of probes, including TaqMan™ (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif), pyrene-labeled probes, and other biochemical assays.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Step 1: PCR amplifications. The optimal conditions for PCR described in this section were determined by varying the parameters described in the Results. PCR was performed in 7 ul volumes in 96 well polypropylene PCR plates (RPI). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 1 uM primer F1, 1 uM primer R1, 0.05 units/ul Platinum Taq polymerase (Life Technologies, Inc.), and "one-half genome equivalent" of DNA. To determine the amount of DNA corresponding to one-half genome equivalent, DNA samples were serially diluted and tested via PCR. The amount that yielded amplification products in half the wells, usually ~1 pg of total DNA, was defined as "one-half genome equivalent" and used in each well of subsequent Digital Amplification experiments. Fifty ul light mineral oil (Sigma M-3516) was added to each well and reactions performed in a HybAid Thermal cycler at the following temperatures: denaturation at 94° for one min; 60 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 70° for five minutes. Reactions were read immediately or stored at room temperature for up to 36 hours before fluorescence analysis.

EXAMPLE 2

Step 2: Fluorescence analysis. 3.5 ul of a solution with the following composition was added to each well: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. This fluorescence in wells without template was typically 10,000 to

20,000 fluorescence "units", with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for one minute; 10 - 15 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 60° for five minutes. The plates were then incubated at room temperature for at least 20 minutes and fluorescence measured as described above. The fluorescence readings obtained were stable for several hours. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio exhibited by the positive controls (25 genome equivalents of DNA from normal cells, as defined in Materials and Methods). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could not be reliably determined from experiments in which they were tested by hybridization to relatively short complementary single stranded oligonucleotides, and that actual PCR products had to be used for validation.

EXAMPLE 3

Oligonucleotides and DNA sequencing. Primer F1: 5'-CATGTTCTAATATAGTCACATTTTCA-3' (SEQ ID NO: 1); Primer R1: 5'-TCTGAATTAGCTGTATCGTCAAGG-3' (SEQ ID NO: 2); Primer INT: 5'-TAGCTGTATCGTCAAGGCAC-3' (SEQ ID NO: 3); MB-RED: 5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3' (SEQ ID NO: 4); MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'. (SEQ ID NO: 5).

Molecular Beacons were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link. All were dissolved at 50 uM

in TE (10 mM Tris, pH 8.0/ 1 mM EDTA) and kept frozen and in the dark until use. PCR products were purified using QIAquick PCR purification kits (Qiagen). In the relevant experiments described in the text, 20% of the product from single wells was used for gel electrophoresis and 40% was used for each sequencing reaction. The primer used for sequencing was 5'-CATTATTTTTATTATAAGGCCTGC-3' (SEQ ID NO: 6). Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

EXAMPLE 4

Principles underlying experiment. The experiment is outlined in Fig. 1A. First, the DNA is diluted into multiwell plates so that there is, on average, one template molecule per two wells, and PCR is performed. Second, the individual wells are analyzed for the presence of PCR products of mutant and WT sequence using fluorescent probes.

As the PCR products resulting from the amplification of single template molecules should be homogeneous in sequence, a variety of standard techniques could be used to assess their presence. Fluorescent probe-based technologies, which can be performed on the PCR products "*in situ*" (i.e., in the same wells) are particularly well-suited for this application. We chose to explore the utility of one such technology, involving Molecular Beacons (MB), for this purpose. MB probes are oligonucleotides with stem-loop structures that contain a fluorescent dye at the 5' end and a quenching agent (Dabcyl) at the 3' end (Fig. 1B). The degree of quenching via fluorescence energy resonance transfer is inversely proportional to the 6^{th} power of the distance between the Dabcyl group and the fluorescent dye. After heating and cooling, MB probes reform a stem-loop structure which quenches the fluorescent signal from the dye. If a PCR product whose sequence is complementary to the loop sequence is present during the heating/cooling cycle, hybridization of the MB

to one strand of the PCR product will increase the distance between the Dabcyl and the dye, resulting in increased fluorescence.

A schematic of the oligonucleotides used for Digital Amplifications shown in Fig. 1C. Two unmodified oligonucleotides are used as primers for the PCR reaction. Two MB probes, each labeled with a different fluorophore, are used to detect the PCR products. MB-GREEN has a loop region that is complementary to the portion of the WT PCR product that is queried for mutations. Mutations within the corresponding sequence of the PCR product should significantly impede the hybridization of it to the MB probe. MB-RED has a loop region that is complementary to a different portion of the PCR product, one not expected to be mutant. It thus should produce a signal whenever a well contains a PCR product, whether that product is WT or mutant in the region queried by MB-GREEN. Both MB probes are used together to simultaneously detect the presence of a PCR product and its mutational status.

Practical Considerations.

Numerous conditions were optimized to define conditions that could be reproducibly and generally applied. As outlined in Fig. 1A, the first step involves amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a product resulting from one set of primers is used as template in a second reaction employing internal primers. As many applications of digital amplification are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating and optimized concentrations of dNTP's, primers, buffer components,

and temperature. The conditions specified in Examples 1-3 were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (~2.5 hr), the number of cycles used was high and excessive compared to the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in Fig 1A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same queried sequence. For example, in the *c-Ki-Ras* gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the

queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16 base pair loop, of melting temperature (Tm) 50-51°, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56°, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of *c-Ki-Ras* are shown in Fig. 2. In this experiment, fifty copies of genomic DNA equivalents were diluted into each well prior to amplification. Each of six tested mutants yielded ratios of RED/GREEN fluorescence that were significantly in excess of the ratio obtained with DNA from normal cells (1.5 to 3.4 in the mutants compared to 1.0 in normal DNA; $p < 0.0001$ in each case, Student's t-Test). The reproducibility of the ratios can be observed in this figure. Direct DNA sequencing of the PCR products used for fluorescence analysis showed that the RED/GREEN ratios were dependent on the relative fraction of mutant genes within the template population (Fig. 2). Thus, the DNA from cells containing one mutant *C-Ki-Ras* allele per every two WT *c-Ki-Ras* allele yielded a RED/GREEN ratio of 1.5 (Gly12Arg mutation) while the cells containing three mutant *c-Ki-Ras* alleles per WT allele exhibited a ratio of 3.4 (Gly12Asp). These data suggested that wells containing only mutant alleles

(no WT) would yield ratios in excess of 3.0, with the exact value dependent on the specific mutation.

Though this mode is the most convenient for many applications, we found it useful to add the MB probes after the PCR-amplification was complete (Fig. 1). This allowed us to use a standard multiwell plate fluorometer to sequentially analyze a large number of multiwell plates containing pre-formed PCR products and bypassed the requirement for multiple real time PCR instruments. Additionally, we found that the fluorescent signals obtained could be considerably enhanced if several cycles of asymmetric, linear amplification were performed in the presence of the MB probes. Asymmetric amplification was achieved by including an excess of a single internal primer (primer INT in Fig. 1C) at the time of addition of the MB probes.

EXAMPLE 5

Analysis of DNA from tumor cells. The principles and practical considerations described above was demonstrated with DNA from two colorectal cancer cell lines, one with a mutation in *c-Ki-Ras* codon 12 and the other in codon 13. Representative examples of the MB-RED fluorescence values obtained are shown in Fig. 3. There was a clear biphasic distribution, with "positive" wells yielding values in excess of 10,000 specific fluorescence units (SFU, as defined in Materials and Methods) and "negative" wells yielding values less than 3500 SFU. Gel electrophoreses of 127 such wells demonstrated that all positive wells, but no negative wells, contained PCR products of the expected size (Fig. 3). The RED/GREEN fluorescence ratios of the positive wells are shown in Fig. 4. Again, a biphasic distribution was observed. In the experiment with the tumor containing a Gly12Asp mutation, 64% of the positive wells exhibited RED/GREEN ratios in excess of 3.0 while the other 36% of the positive wells exhibited ratios ranging from 0.8 to 1.1. In the case of the tumor with the Gly13Asp mutation, 54% of the positive wells exhibited RED/GREEN ratios >3.0 while the other positive wells yielded ratios ranging from 0.9 to 1.1. The PCR products from 16 positive wells were used as sequencing templates (Fig. 4). All the wells yielding a ratio in excess of 3.0 were found to contain mutant c-Ki-Ras fragments of the expected sequence, while WT sequence was found in the other PCR products. The presence of homogeneous WT or mutant sequence confirmed that the amplification products were usually derived from single template molecules. The ratios of WT to mutant PCR products determined from the Digital Amplification assay was also consistent with the fraction of mutant alleles inferred from direct sequence analysis of genomic DNA from the two tumor lines (Fig. 2).

Digital Analysis of DNA from stool. As a more practical example, we analyzed the DNA from stool specimens from colorectal cancer patients. A representative result of such an experiment is illustrated in Fig. 5. From previous analyses of stool specimens from patients whose tumors contained *c-Ki-Ras* gene mutations, we expected that 1% to 10% of the *c-Ki-Ras* genes purified from stool would be mutant. We therefore set up a 384 well Digital Amplification experiment. As positive controls, 48 of the wells contained 25 genome equivalents of DNA (defined in Materials and Methods) from normal cells. Another 48 wells served as negative controls (no DNA template added). The other 288 wells contained an appropriate dilution of stool DNA. MB-RED fluorescence indicated that 102 of these 288 experimental wells contained PCR products (mean +/- s.d. of 47,000 +/- 18,000 SFU) while the other 186 wells did not (2600 +/- 1500 SFU). The RED/GREEN ratios of the 102 positive wells suggested that five contained mutant c-Ki-Ras genes, with ratios ranging from 2.1 to 5.1. The other 97 wells exhibited ratios ranging from 0.7 to 1.2, identical to those observed in the positive control wells. To determine the nature of the mutant *c-Ki-Ras* genes in the five positive wells from stool, the PCR products were directly sequenced. The four wells exhibiting RED/GREEN ratios in excess of 3.0 were completely composed of mutant c-Ki-Ras sequence (Fig. 5B). The sequence of three of these PCR products revealed Gly12Ala mutations (GGT to GCT at codon 12), while the sequence of the fourth indicated a silent C to T transition at the third position of codon 13. This transition presumably resulted from a PCR error during the first productive cycle of amplification from a WT template. The well with a ratio of 2.1 contained a ~1:1 mix of WT and Gly12Ala mutant sequences. Thus 3.9% (4/102) of the *c-Ki-Ras* alleles present in this stool sample contained a Gly12Ala mutation. The mutant alleles in the stool presumably arose from the colorectal cancer of the patient, as direct sequencing of PCR products

generated from DNA of the cancer revealed the identical Gly12Ala mutation (not shown).

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Genetics

Digital PCR

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ABSTRACT **The identification of predefined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. Here, we describe an approach for transforming the exponential, analog nature of the PCR into a linear, digital signal suitable for this purpose. Single molecules are isolated by dilution and individually amplified by PCR; each product is then analyzed separately for the presence of mutations by using fluorescent probes. The feasibility of the approach is demonstrated through the detection of a mutant *ras* oncogene in the stool of patients with colorectal cancer. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.**

In classical genetics, only mutations of the germ line were considered important for understanding disease. With the realization that somatic mutations are the primary cause of cancer (1) and may also play a role in aging (2, 3), new genetic principles have arisen. These discoveries have provided a wealth of opportunities for patient management as well as for basic research into the pathogenesis of neoplasia. However, many of these opportunities hinge on detection of a small number of mutant-containing cells among a large excess of normal cells. Examples include the detection of neoplastic cells in urine (4), stool (5, 6), and sputum (7, 8) of patients with cancers of the bladder, colorectum, and lung, respectively. Such detection has been shown in some cases to be possible at a stage when the primary tumors are still curable and the patients asymptomatic. Mutant sequences from the DNA of neoplastic cells have also been found in the blood of patients with cancer (9–11). The detection of residual disease in lymph nodes or surgical margins may be useful in predicting which patients might benefit most from further therapy (12–14). From a basic research standpoint, analysis of the early effects of carcinogenesis often depends on the ability to detect small populations of mutant cells (15–17).

Because of the importance of this issue in so many settings, many useful techniques have been developed for the detection of mutations. DNA sequencing is the gold standard for the detection of germ-line mutations but is useful only when the fraction of mutated alleles is greater than ≈20% (18, 19). Mutant-specific oligonucleotides sometimes can be used to detect mutations present in a minor proportion of the cells analyzed, but the signal-to-noise ratio distinguishing mutant and wild-type (WT) templates is variable (20–22). The use of mutant-specific primers and the digestion of PCR products with specific restriction endonucleases are extremely sensitive methods for detecting such mutations, but it is difficult to quantitate the fraction of mutant molecules in the starting population with these techniques (23–28). Other innovative approaches for the detection of somatic mutations have been reviewed (29–32). A general problem with these methods is that it is difficult or impossible to confirm independently the existence of any mutations that are identified.

We therefore sought to develop an approach to the problem that would overcome some of the aforementioned difficulties. The strategy described in this paper involves separately amplifying individual template molecules so that the resultant PCR products are completely mutant or completely WT. The homogeneity of these PCR products makes them easy to distinguish with existing techniques. Such separate amplifications are only useful in a practical sense, however, if a large number of them can be assessed simply and reliably. Techniques for such assessments were developed, with the output providing a digital readout of the fraction of mutant alleles in the analyzed population. A variety of applications for this technology are foreseeable.

MATERIALS AND METHODS

Step 1: PCR Amplifications. The optimal conditions for PCR described in this section were determined by varying the parameters described in *Results*. PCR was performed in 7-μl volumes in 96-well polypropylene PCR plates (Marsh Biomedical Products, Rochester, NY). The composition of the reactions was 67 mM Tris (pH 8.8), 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 6% (vol/vol) DMSO, 1 μM primer F1, 1 μM primer R1, 0.05 units/μl Platinum *Taq* polymerase (Life Technologies, Grand Island, NY), and one-half genome equivalent of DNA (see below for description of primers). To determine the amount of DNA corresponding to one-half genome equivalent, DNA samples were serially diluted and tested via PCR. The amount that yielded amplification products in half of the wells, usually ≈1.5 pg of total DNA, was defined as one-half genome equivalent and used in each well of subsequent digital PCR (Dig-PCR) experiments. Light mineral oil (50 μl; Sigma M-3516) was added to each well, and reactions were performed in a HybAid Thermal cycler (Middlesex, U.K.) at the following temperatures: denaturation at 94° for 1 min; 60 cycles of 94° for 15 s, 55° for 15 s, 70° for 15 s; and 70° for 5 minutes. Reactions were analyzed immediately or stored at room temperature for up to 36 h before fluorescence analysis.

Step 2: Fluorescence Analysis. The following solution (3.5 μl) was added to each well: 67 mM Tris (pH 8.8), 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 6% (vol/vol) DMSO, 5 μM primer INT, 1 μM molecular beacon (MB)-GREEN, 1 μM MB-RED, and 0.1 units/μl Platinum *Taq* polymerase. The plates were centrifuged for 20 s at 6,000 × *g*, and fluorescence was read at excitation/emission wavelengths of 485/530 nm for MB-GREEN and 530/590 nm for MB-RED. The fluorescence in wells without template was typically 10,000 to 20,000 specific fluorescence units (SFU), with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for 1 min; 10–15 cycles of 94° for 15 s, 55° for 15 s, 70° for 15 s; 94° for 1 min; and 60° for 5 min. The plates were then incubated at room temperature for 10–60 min, and fluorescence was measured as described

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Abbreviations: Dig-PCR, digital PCR; MB, molecular beacon; SFU, specific fluorescence unit; WT, wild-type.

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above. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio of the positive controls (25 genome equivalents of DNA from normal cells, as defined above). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could not be determined reliably from experiments in which they were tested by hybridization to relatively short complementary single-stranded oligonucleotides and that actual PCR products had to be used for validation.

Oligonucleotides and DNA Sequencing. Primer F1 was 5′-CATGTTCTAATATAGTCACATTTTCA-3′; primer R1 was 5′-TCTGAATTAGCTGTATCGTCAAGG-3′; primer INT was 5′-TAGCTGTATCGTCAAGGCAC-3′; MB-RED was 5′-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3′; MB-GREEN was 5′-fluorescein-CACGGGAGCTGGTGGCG-TAGCGTG-Dabcyl-3′. MBs (33, 34) were synthesized by Midland Scientific, and other oligonucleotides were synthesized by Gene Link (Thornwood, NY). All were dissolved at 50 μM in TE buffer (10 mM Tris, pH 8.0/1 mM EDTA) and kept frozen and in the dark until use. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, CA). In the relevant experiments described in the text, 20% of the product from single wells was used for gel electrophoresis, and 40% was used for each sequencing reaction. The primer used for sequencing was 5′-CATTATTTTATTATAAGGCCTGC-3′. Sequencing was performed by using fluorescently labeled Applied Biosystems Big Dye terminators and an Applied Biosystems 377 automated sequencer.

RESULTS

Principles Underlying Dig-PCR. The two steps comprising Dig-PCR are outlined in Fig. 1*A*. First, the DNA is diluted into multiwell plates so that there is, on average, one template molecule per two wells, and PCR is performed. Second, the individual wells are analyzed for the presence of PCR products of mutant and WT sequence by using fluorescent probes.

As the PCR products resulting from the amplification of single template molecules should be homogeneous in sequence, a variety of standard techniques could be used to assess their presence (see Introduction). Fluorescent probe-based technologies, which can be performed on the PCR products "*in situ*" (i.e., in the same wells), are particularly well suited for this application (31, 33–40). We chose to explore the utility of one such technology, involving MBs, for this purpose (33, 34). MB probes are oligonucleotides with stem–loop structures that contain a fluorescent dye at the 5′ end and a quenching agent (Dabcyl) at the 3′ end (Fig. 1*B*). The degree of quenching via fluorescence energy resonance transfer is inversely proportional to the sixth power of the distance between the Dabcyl group and the fluorescent dye (41). After heating and cooling, MB probes reform a stem–loop structure that quenches the fluorescent signal from the dye. If a PCR product whose sequence is complementary to the loop sequence is present during the heating/cooling cycle, hybridization of the MB to one strand of the PCR product will increase the distance between the Dabcyl and the dye, resulting in increased fluorescence.

A schematic of the oligonucleotides used for Dig-PCR is shown in Fig. 1*C*. Two unmodified oligonucleotides are used as primers for the PCR reaction. Two MB probes, each labeled with a different fluorophore, are used to detect the PCR products. MB-GREEN has a loop region that is complementary to the portion of the WT PCR product that is tested for mutations. Mutations within the corresponding sequence of the PCR product should impede its hybridization to the MB probe significantly (33, 34). MB-RED has a loop region that is complementary to a different portion of the PCR product, one not expected to be mutant. It thus should produce a signal whenever a well contains

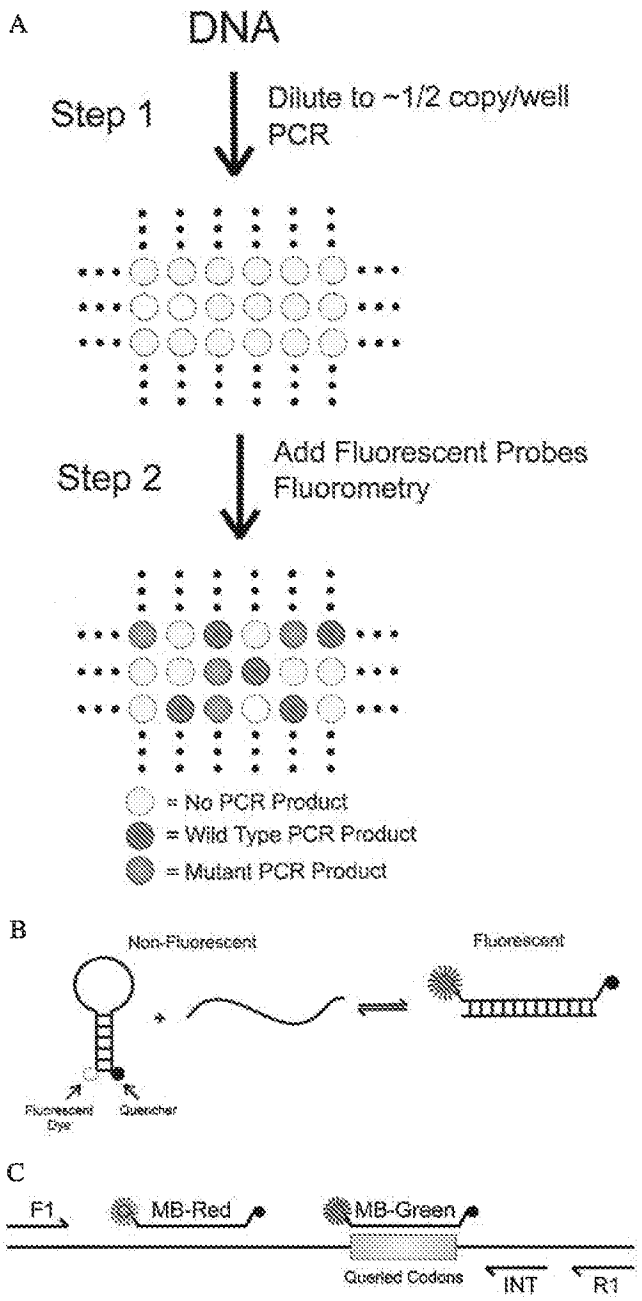

FIG. 1. Schematic of Dig-PCR. (*A*) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes that discriminate between WT and mutant alleles and subsequent fluorometry. (*B*) Principle of MB analysis. In the stem–loop configuration, fluorescence from a dye at the 5′ end of the oligonucleotide probe is quenched by a Dabcyl group at the 3′ end. On hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence (modified from Marras *et al.*; ref. 56). (*C*) Oligonucleotide design. Primers F1 and R1 are used to amplify the genomic region of interest. Primer INT is used to produce single-stranded DNA from the original PCR products during a subsequent asymmetric PCR step (see *Materials and Methods*). MB-RED is an MB that detects any appropriate PCR product, whether it is WT or mutant at the queried codons. MB-GREEN is an MB that preferentially detects the WT PCR product.

a PCR product, whether that product is WT or mutant in the region tested by MB-GREEN. Both MB probes are used together to detect the presence of a PCR product and its mutational status simultaneously.

Practical Considerations. Numerous conditions were optimized to define conditions that could be reproducibly and

generally applied to Dig-PCR-based projects. As outlined in Fig. 1*A*, the first step of Dig-PCR involves PCR-amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a PCR product resulting from one set of primers is used as template in a second PCR employing internal primers (42, 43). Because many applications of Dig-PCR are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating (44, 45) and of optimized concentrations of dNTPs, primers, buffer components, and temperature. The conditions specified in *Materials and Methods* were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (≈2.5 h), the number of cycles used was high and excessive compared with the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in Dig-PCR involves the detection of these PCR products. It was necessary to modify the standard MB probe approach in order for it to function efficiently in Dig-PCR applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the tested sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be identified. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same tested sequence. For example, in the *c-Ki-Ras* gene example explored here, 12 different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least 7 of these are observed in naturally occurring human cancers. To detect all 12 mutations as well as the WT sequence with individual MBs would require 13 different probes. Inclusion of such a large number of MB probes would raise the background fluorescence and the cost of the assay. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the tested sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16-bp loop with a melting temperature of 50–51° and a 4-bp stem of sequence 5′-CACG-3′ were optimal. For MB-RED probes, the same stem with a 19- to 20-bp loop with a melting temperature of 54–56° proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of *c-Ki-Ras* are shown in Fig. 2. In this experiment, 50 genome equivalents of DNA were added into each well before amplification. Each of six tested mutants yielded ratios of RED/GREEN fluorescence that were significantly in excess of the ratio obtained with DNA from normal cells (1.5 to 3.4 in the mutants, compared with 1.0 in normal DNA; $P < 0.0001$ in each case, Student's t test). The reproducibility of the ratios can be observed in Fig. 2. Direct DNA sequencing of the PCR products used for fluorescence analysis showed that the RED/GREEN ratios depended on the relative fraction of mutant genes within the template population (Fig. 2). Thus, the DNA from cells containing one mutant *c-Ki-Ras* allele per every two WT *c-Ki-Ras* alleles yielded a RED/GREEN ratio of 1.5 (Gly12Arg mutation), whereas the cells containing three mutant *c-Ki-Ras* alleles per WT allele had a ratio of 3.4 (Gly12Asp). These data suggested that wells containing only mutant alleles (no WT) would yield ratios in excess of 3.0, with the exact value dependent on the specific mutation.

Fluorescent probes such as those of the MB type are generally included in the PCR mix and followed in real time. Though this mode is the most convenient for many applications, we found it useful to add the MB probes after the PCR amplification was complete (Fig. 1). This procedure allowed us to use a standard multiwell plate fluorometer to analyze sequentially a large number of multiwell plates containing preformed PCR products and bypassed the requirement for multiple real-time PCR instruments. Additionally, we found that the fluorescent signals obtained could be considerably enhanced if several cycles of asymmetric, linear amplification were performed in the presence of the MB probes. Asymmetric amplification was achieved by including

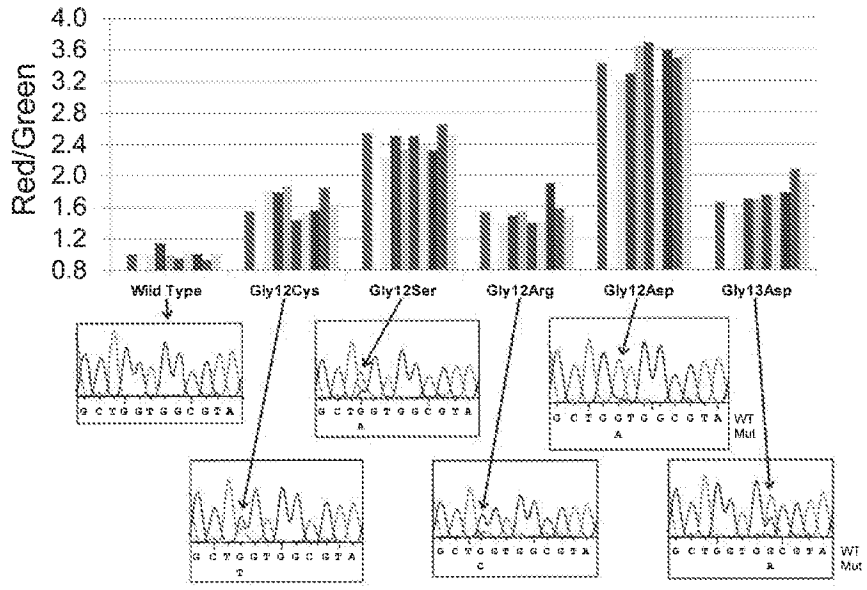

FIG. 2. Discrimination between WT and mutant PCR products by MBs. Separate PCR products (n = 10), each generated from ≈50 genome equivalents of DNA of cells containing the indicated mutations of *c-Ki-Ras*, were analyzed with the MB probes described above. Representative examples of the PCR products used for MB analysis were purified and sequenced directly. In the cases with Gly12Cys and Gly12Arg mutations, contaminating nonneoplastic cells within the tumor presumably accounted for the relatively low ratios. In the cases with Gly12Ser and Gly12Asp, there were apparently two or more alleles of mutant *c-Ki-Ras* for every WT allele; both these tumors were aneuploid.

an excess of a single internal primer (primer INT in Fig. 1C) at the time of addition of the MB probes.

Dig-PCR on DNA from Tumor Cells. The principles and practical considerations described above were illustrated with DNA from two colorectal cancer cell lines, one with a mutation in *c-Ki-Ras* codon 12 and the other in codon 13. Representative examples of the MB-RED fluorescence values obtained are shown in Fig. 3. There was a clear biphasic distribution, with "positive" wells yielding values in excess of 10,000 SFU and "negative" wells yielding values less than 3,500 SFU. Gel electrophoreses of 127 such wells indicated that all positive wells but no negative wells contained PCR products of the expected size (Fig. 3). The RED/GREEN fluorescence ratios of the positive wells are shown in Fig. 4. Again, a biphasic distribution was observed. In the experiment with the tumor containing a Gly12Asp mutation, 64% of the positive wells had RED/GREEN ratios in excess of 3.0, whereas the other 36% of the positive wells had ratios ranging from 0.8 to 1.1. In the case of the tumor with the Gly13Asp mutation, 54% of the positive wells had RED/GREEN ratios >3.0, whereas the other positive wells yielded ratios ranging from 0.9 to 1.1. The PCR products from 16 positive wells were used as sequencing templates (Fig. 4). All the wells yielding a ratio in excess of 3.0 were found to contain mutant *c-Ki-Ras* fragments of the expected sequence, whereas WT sequence was found in the other PCR products. The presence of homogeneous WT or mutant sequence confirmed that the amplification products usually were derived from single template molecules. The ratios of WT to mutant PCR products determined from the Dig-PCR assay were also consistent with the fraction of mutant alleles inferred from direct sequence analysis of genomic DNA from the two tumor lines (Fig. 2).

Dig-PCR on DNA from Stool. As a more practical example of the intended use of Dig-PCR, we analyzed the DNA from stool specimens of patients with colorectal cancer. A representative result of such an experiment is illustrated in Fig. 5. From previous analyses of stool specimens from patients whose tumors contained *c-Ki-Ras* gene mutations, we expected that 1–10% of the *c-Ki-Ras* genes purified from stool would be mutant. We therefore set up a 384-well Dig-PCR experiment. As positive controls, 48 of the wells contained 25 genome equivalents of DNA (defined in *Materials and Methods*) from normal cells. Another 48 wells served as negative controls (no DNA template added). The other 288 wells contained an appropriate dilution of stool DNA. MB-RED fluorescence indicated that 102 of these 288 experimental wells contained PCR products (mean ± SD of 47,000 ± 18,000 SFU), whereas the other 186 wells did not (2,600 ± 1,500 SFU). The RED/GREEN ratios of the 102 positive wells suggested that five contained mutant *c-Ki-Ras* genes, with ratios ranging from 2.1 to 5.1. The other 97 wells had ratios ranging from 0.7 to 1.2, identical to those observed in the positive-control wells. To determine the nature of the mutant *c-Ki-Ras* genes from stool in the five positive wells, the PCR products were sequenced directly. The four wells with RED/GREEN ratios in excess of 3.0 were completely composed of mutant ras sequence (Fig. 5). The sequence of three of these PCR products indicated Gly12Ala mutations (GGT to GCT at codon 12), whereas the sequence of the fourth indicated a silent C-to-T transition at the third position of codon 13. This transition presumably resulted from a PCR error during the first productive cycle of amplification from a WT template. The well with a ratio of 2.1 contained an ≈1:1 mix of WT and Gly12Ala mutant sequences. Thus 3.9% (4 of 102) of the *c-Ki-Ras* alleles present in this stool sample contained a Gly12Ala mutation. The mutant alleles in the stool presumably arose from the colorectal cancer of the patient, as direct sequencing of PCR products generated from DNA of the cancer identified the identical Gly12Ala mutation (not shown).

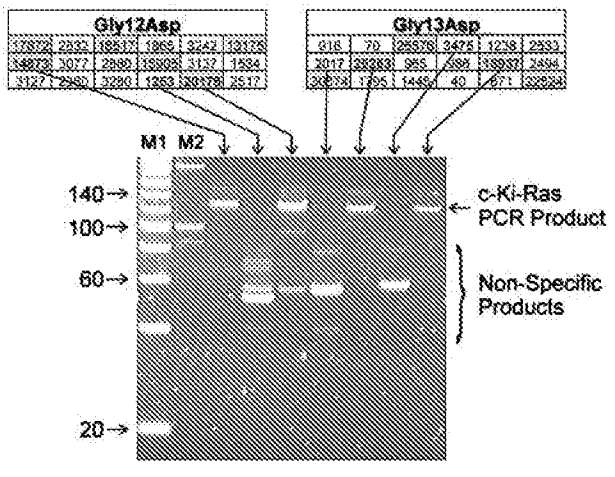

FIG. 3. Detecting Dig-PCR products with MB-RED. SFU of representative wells from an experiment employing colorectal cancer cells with Gly12Asp or Gly13Asp mutations of the *c-Ki-Ras* gene. Wells with values >10,000 SFU are shaded yellow. PAGE analyses of the PCR products from selected wells are shown. Wells with fluorescence values <3,500 SFU had no PCR product of the correct size, whereas wells with fluorescence values >10,000 SFU always contained PCR products of 129 bp. Nonspecific products generated during the large number of cycles required for Dig-PCR did not affect the fluorescence analysis. M1 and M2 are molecular length markers used to determine the size of fragments (indicated on the left in base pairs).

DISCUSSION

Dig-PCR represents another example of the power of PCR, in combination with more recently developed detection technologies, to provide opportunities for genetic analysis. There are several precedents for the approach described here. For example, PCR-amplification from single cells isolated by physical separation or dilution has been used to address a variety of interesting biologic questions (46–49). Gel electrophoretic and sequence analysis of single alleles, produced by amplification of diluted DNA or from cloning of PCR products, has also proven useful in several areas of investigation (43, 48, 50–53). *In situ* amplification of single alleles by using rolling-circle amplification represents another exciting strategy for extracting genetic data that would be impossible to obtain from more standard analyses of bulk DNA populations (54).

Dig-PCR can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. The 384-well PCR plates are commercially available, and 1,536-well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ≈0.1% level. It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was <0.3%, i.e., in control experiments with DNA from normal cells, none of 340 wells containing PCR products had RED/GREEN ratios >3.0. Any individual mutation (such as a G-to-C transversion at the second position of codon 12 of *c-Ki-Ras*) is expected to occur in <1 in 50 polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques described in the Introduction, provides unequivocal validation of a prospective mutation; a significant fraction of the mutations found in individual wells should be identical if the mutation occurred *in vivo*. Significance can be established through rigorous statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Dig-PCR

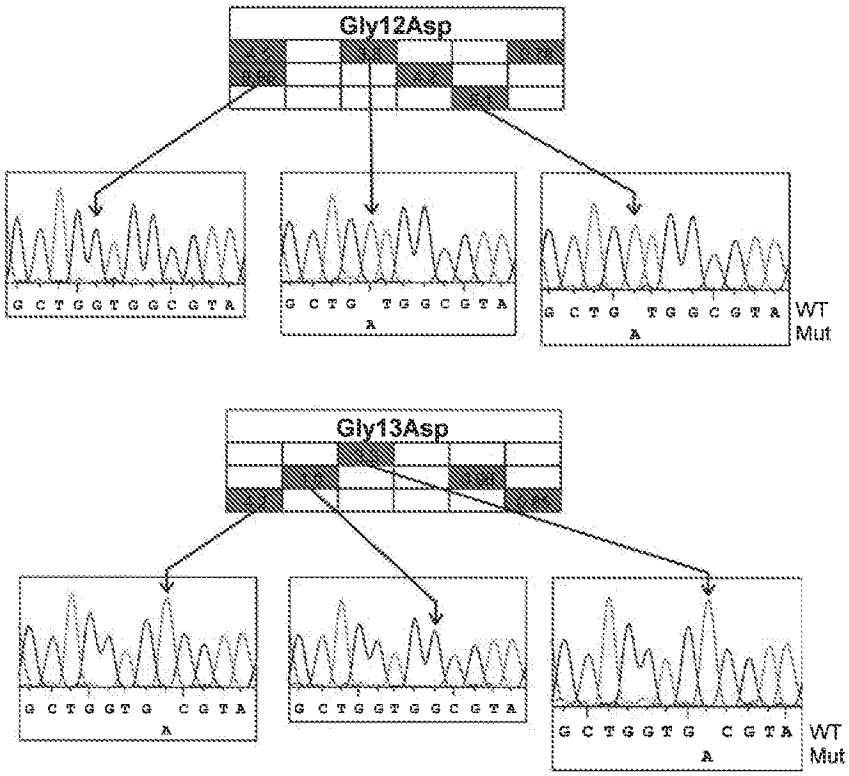

FIG. 4. Discriminating WT from mutant PCR products obtained in Dig-PCR. RED/GREEN ratios were determined from the fluorescence of MB-RED and MB-GREEN as described in *Materials and Methods*. The wells shown are the same as those illustrated in Fig. 3. The sequences of PCR products from the indicated wells were determined as described in *Materials and Methods*. The wells with RED/GREEN ratios >3.0 each contained mutant sequences, whereas those with RED/GREEN ratios of ≈1.0 contained WT sequences.

experiments can be determined precisely through performance of Dig-PCR on DNA templates from normal cells.

Dig-PCR is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene could be determined easily by using fluorescent probes specific for each of the PCR products generated. Similarly, Dig-PCR could be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers that are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One fluorescent probe would then be used to detect PCR products from the reference transcript, and a second fluorescent probe would be used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic

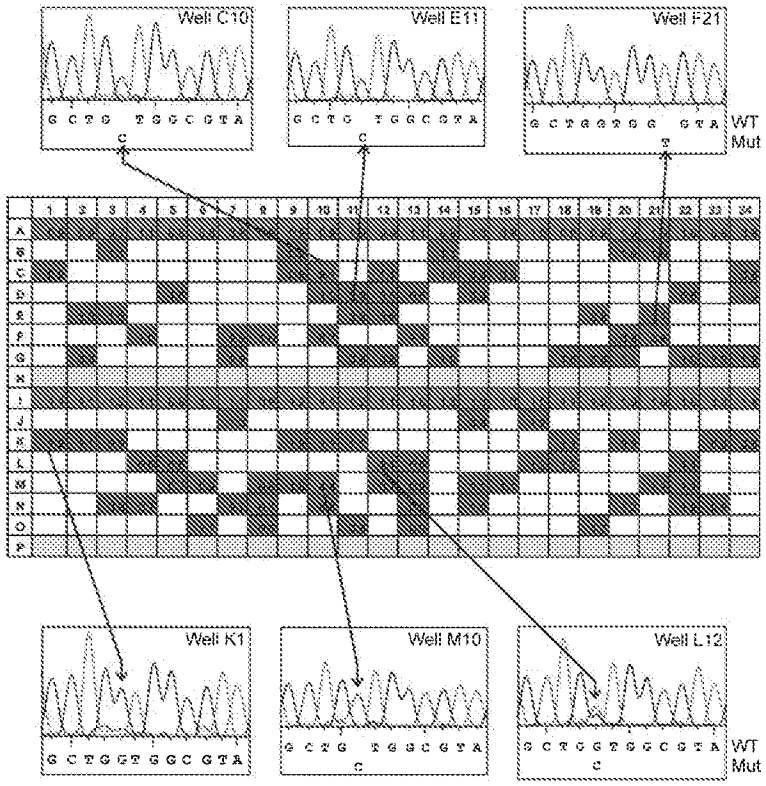

FIG. 5. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED, and the RED/GREEN ratios were 1.0 ± 0.1 (mean ±1 SD). The wells colored yellow contained no template DNA, and each was negative with MB-RED (i.e., fluorescence <3,500 SFU.). The other 288 wells contained diluted DNA from the stool sample, prepared by alkaline extraction (57). Those registering positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis.

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

status when two mutations are observed in the sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (vs. both occurring in one allele), cloning of PCR products generally is performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Dig-PCR are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR (31, 33–38, 40) provide an excellent alternative to Dig-PCR. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Dig-PCR may prove useful for these applications when the expected differences are small (e.g., only ≈2-fold, as with allelic imbalances; ref. 55).

The ultimate utility of Dig-PCR lies in its ability to convert the intrinsically exponential nature of PCR to a linear one. It should thereby prove useful for experiments requiring the investigation of individual alleles, rare variants/mutations, or quantitative analysis of PCR products.

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PTO/SB/31 (02-14)
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NOTICE OF APPEAL FROM THE EXAMINER TO THE PATENT TRIAL AND APPEAL BOARD

Docket Number (Optional): 001107.00989

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In re Application of: USP 6,440,706

Application Number	Filed
90/012,894	June 17, 2013

For: Digital Amplification

Art Unit	Examiner
3991	Bruce R. Campell

Applicant hereby **appeals** to the Patent Trial and Appeal Board from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 800

☐ Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ _____

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/Sarah A. Kagan/
Signature

Sarah A. Kagan
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202.824.3000
Telephone number

September 9, 2014
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 41.31. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11, 1.14 and 41.6. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	90012894
Filing Date:	17-Jun-2013
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	6,440,706 B1
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Attorney Docket Number:	001107.00989

Filed as Large Entity

ex parte reexam Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Notice of Appeal	1401	1	800	800
Post-Allowance-and-Post-Issuance:				
Extension of Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**800**

Electronic Acknowledgement Receipt

EFS ID:	20082289
Application Number:	90012894
International Application Number:	
Confirmation Number:	8442
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	6,440,706 B1
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00989
Receipt Date:	09-SEP-2014
Filing Date:	17-JUN-2013
Time Stamp:	10:34:25
Application Type:	Reexam (Patent Owner)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$800
RAM confirmation Number	9396
Deposit Account	190733
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Amendment-to-FOA.pdf	177198 53a6c0a38eeb8958da1466bd942ee29dbb133a57	yes	19

Multipart Description/PDF files in .zip description

Document Description	Start	End
Response After Final Action	1	1
Claims	2	8
Applicant Arguments/Remarks Made in an Amendment	9	19

Warnings:

Information:

2	Oath or Declaration filed	Executed-Declaration-Lapidus.pdf	120902 a61e684545ca43c0d0cc611af12b4add082217c3	no	3

Warnings:

Information:

3	Miscellaneous Incoming Letter	Lapidus-Exhibit.pdf	350594 40d058be7d2d074bcc97f2fd882b8c10da4a0a8d	no	6

Warnings:

Information:

4	Oath or Declaration filed	Executed-Declaration-Shih.pdf	330706 27d6e5ca9fda29fe99f246556caf763f306472ec	no	9

Warnings:

Information:

5	Miscellaneous Incoming Letter	Shih-Exhibits2.pdf	21468818 c667b723893e6bdddc02d0a7f6808c2af0915461	no	190

Warnings:

Information:

6	Notice of Appeal Filed	Notice-of-Appeal.pdf	211208 efb6bc6a59ca402a54efc5092e710dc98f33f199	no	2

Warnings:

Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	29589 59ed63b0a580ca585223a6a60236c72744a49c1d	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	22689015	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re *Ex Parte* Reexamination:	)	Group Art Unit: 3991
	)	
U.S. Patent No. 6,440,706	)	Docket No. 001107.00989
	)	
Control No. 90/012,894	)	Confirmation No: 8442
	)	
Reexam Filing Date: June 17, 2013	)	Examiner: Bruce R. Campell

For: DIGITAL AMPLIFICATION

CERTIFICATE OF SERVICE

The undersigned certifies that a complete copy of the Responsive Amendment to Final Office Action filed in the U.S. Patent and Trademark Office on September 9, 2014, has been mailed via first class mail to the third party requester this day at the following address:

Life Technologies Corporation
Attn: IP Department
5791 Van Allen Way
Carlsbad, CA 92008

/Sarah A. Kagan/
Sarah A. Kagan
Registration No. 32,141

Dated: September 9, 2014

Banner & Witcoff, Ltd.
Customer No. 11332

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re *Ex Parte* Reexamination:	)	Group Art Unit: 3991
U.S. Patent No. 6,440,706	)	Docket No. 001107.00989
Control No. 90/012,894	)	Confirmation No: 8442
Reexam Filing Date: June 17, 2013	)	Examiner: Bruce R. Campell
For: DIGITAL AMPLIFICATION	)	

DECLARATION OF STANLEY N. LAPIDUS

I, Stanley N. Lapidus, declare:

1. I am the President, CEO, and Founder of SynapDx located at Four Hartwell Place, Lexington, MA 02421.
2. I have a Bachelor of Science degree in Electrical Engineering from The Cooper Union for the Advancement of Science and Art.
3. A true and correct copy of my curriculum vitae is attached to this Declaration as Exhibit 1.
4. I have been retained as an expert consultant by Esoterix Genetic Laboratories in connection with the reexamination of U.S. Patent No. 6,440,706 (the '706 patent).
5. I am inventor on certain patents at issue in a related litigation matter in the United States District Court for the Middle District of North Carolina, Greensboro Division (*Esoterix Genetics Laboratories v. Life Technologies Corporation*, Case No. 12-CV-411).
6. Laboratory Corporation of America is a minority investor in SynapDx.
7. I was a Founder and former President and CEO of Exact Sciences Corporation, and I currently own a small number of shares in Exact, which I purchased on the open market.
8. I have reviewed the '706 patent, including the claims, which I understand is related to determining the ratio of a selected genetic sequence in a population of genetic sequences using a method generally referred to as digital polymerase chain reaction (PCR), a term coined by Dr. Bert Vogelstein and Dr. Kenneth Kinzler and adopted by the industry.

9. I have been actively engaged in the field of biotechnology research and development for over 27 years. I was recently elected to the College of Fellows of the American Institute of Medical and Biological Engineering, an organization whose Fellows are said to represent the top 2% of the medical and biological engineering community.
10. I was conducting research in this area at the time that Drs. Vogelstein and Kinzler invented and first presented their research on digital PCR.
11. Digital PCR was a brilliant innovation that made a tremendous impact on the field, particularly for generating quantitative data concerning rare genetic sequences. When Drs. Vogelstein and Kinzler first described digital PCR, I, and others skilled in the art, were genuinely surprised by the success of the method and even considered it to be an audacious method to try. Digital PCR was not obvious at the time of its invention to those of us skilled in the art. Researchers in this area immediately appreciated the significance of this invention and its capabilities.
12. Digital PCR met a previously unmet need in the art. Many publications were directed to how to determine mutant to wild-type genetic ratios and the like, but none suggested digital PCR, which allowed for the quantification of rare sequences, including rare mutations or alleles, in a population of sequences, through the use of digital enumeration by spatial separation.
13. Digital PCR was a substantial improvement over other methods used at the time to determine the ratio of mutant or rare sequences to wild type sequences in a sample. Methods in use at the time included cytometry, fluorescence in situ hybridization (FISH), counting, amplification-refractory mutation system (ARMS), and gel-based methods. These methods are distinctly different than digital PCR, which worked better than the methods in use at the time Drs. Vogelstein and Kinzler invented digital PCR.
14. Digital PCR is still in use today. A number of companies have marketed or are currently marketing products for use in digital PCR methods, including, for example, Life Technologies.
15. All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true; and further that these statements were made

with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the patent.

Full Name of Declarant: STANLEY N. LAPIDUS

Declarant's Signature: 

Date: August 25, 2014

Electronic Acknowledgement Receipt

EFS ID:	20083966
Application Number:	90012894
International Application Number:	
Confirmation Number:	8442
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	6,440,706 B1
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00989
Receipt Date:	09-SEP-2014
Filing Date:	17-JUN-2013
Time Stamp:	12:13:14
Application Type:	Reexam (Patent Owner)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Reexam Certificate of Service	Certificate-of-Service.PDF	78450 c657e1594eb83b73d4f6e5426a0fa743d322a32f	no	1

Warnings:	
Information:	

2	Oath or Declaration filed	Executed-Declaration-Lapidus.PDF	115924 27ba2090372d1d1538691595bafc2f0fdfdbac34	no	3

Warnings:	
Information:	
Total Files Size (in bytes):	194374

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
90/012,894	06/17/2013	6,440,706 B1	001107.00989	8442

11332 7590 10/03/2014

Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

EXAMINER	
CAMPELL, BRUCE R	
ART UNIT	PAPER NUMBER
3991	

MAIL DATE	DELIVERY MODE
10/03/2014	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
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Alexandria, VA 22313-1450
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LIFE TECHNOLOGIES CORPORATION

ATTN: IP DEPARTMENT

5791 VAN ALLEN WAY

CARLSBAD, CA 92008

EX PARTE REEXAMINATION COMMUNICATION TRANSMITTAL FORM

REEXAMINATION CONTROL NO. *90/012,894*.

PATENT NO. *6,440,706 B1 E*.

ART UNIT *3991*.

Enclosed is a copy of the latest communication from the United States Patent and Trademark Office in the above identified *ex parte* reexamination proceeding (37 CFR 1.550(f)).

Where this copy is supplied after the reply by requester, 37 CFR 1.535, or the time for filing a reply has passed, no submission on behalf of the *ex parte* reexamination requester will be acknowledged or considered (37 CFR 1.550(g)).

Notice of Intent to Issue Ex Parte Reexamination Certificate	Control No.	Patent Under Reexamination	
	90/012,894	6,440,706 B1 E	
	Examiner	Art Unit	AIA (First Inventor to File) Status
	BRUCE CAMPELL	3991	No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☒ Prosecution on the merits is (or remains) closed in this *ex parte* reexamination proceeding. This proceeding is subject to reopening at the initiative of the Office or upon petition. *Cf.* 37 CFR 1.313(a). A Certificate will be issued in view of
   (a) ☒ Patent owner's communication(s) filed: *9/9/2014*.
   (b) ☐ Patent owner's failure to file an appropriate timely response to the Office action mailed: _____.
   (c) ☐ Patent owner's failure to timely file an Appeal Brief (37 CFR 41.31).
   (d) ☐ The decision on appeal by the ☐ Board of Patent Appeals and Interferences ☐ Court dated _____
   (e) ☐ Other: _____.
2. The Reexamination Certificate will indicate the following:
   (a) Change in the Specification: ☐ Yes ☒ No
   (b) Change in the Drawing(s): ☐ Yes ☒ No
   (c) Status of the Claim(s):
      (1) Patent claim(s) confirmed: _____.
      (2) Patent claim(s) amended (including dependent on amended claim(s)): *See Continuation Sheet*
      (3) Patent claim(s) canceled: _____.
      (4) Newly presented claim(s) patentable: _____.
      (5) Newly presented canceled claims: _____.
      (6) Patent claim(s) ☐ previously ☐ currently disclaimed: _____
      (7) Patent claim(s) not subject to reexamination: 13, 17, 18, 33-37, 45, 49, 50.
3. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
4. ☒ Note the attached statement of reasons for patentability and/or confirmation. Any comments considered necessary by patent owner regarding reasons for patentability and/or confirmation must be submitted promptly to avoid processing delays. Such submission(s) should be labeled: "Comments On Statement of Reasons for Patentability and/or Confirmation."
5. ☐ Note attached NOTICE OF REFERENCES CITED (PTO-892).
6. ☐ Note attached LIST OF REFERENCES CITED (PTO/SB/08 or PTO/SB/08 substitute).
7. ☐ The drawing correction request filed on _____ is: ☐ approved ☐ disapproved.
8. ☐ Acknowledgment is made of the priority claim under 35 U.S.C. § 119(a)-(d) or (f).
   a)☐ All b)☐ Some* c)☐ None of the certified copies have
   ☐ been received.
   ☐ not been received.
   ☐ been filed in Application No. _____.
   ☐ been filed in reexamination Control No. _____.
   ☐ been received by the International Bureau in PCT Application No. _____.
   * Certified copies not received: _____.
9. ☐ Note attached Examiner's Amendment.
10. ☐ Note attached Interview Summary (PTO-474).
11. ☐ Other: _____.

All correspondence relating to this reexamination proceeding should be directed to the **Central Reexamination Unit** at the mail, FAX, or hand-carry addresses given at the end of this Office action.

Bruce Campell
Primary Examiner
Art Unit: 3991

cc: Requester (if third party requester)

Continuation of (2) Patent claim(s) amended: 1-12,14-16,19-32,38-44, 46-48, 51-64.

STATEMENT OF REASONS FOR PATENTABILITY AND/OR CONFIRMATION

The following is an examiner's statement of reasons for patentability and/or confirmation of the claims found patentable in this reexamination proceeding:

Neither Li nor Zhang anticipates the claims because neither reference discloses analysis of nucleic acids by diluting isolated or cell free nucleic acids to produce a set of assay samples. Neither reference in combination with Jeffreys renders the claims obvious. The experiment depicted in Li Fig.1 is a method for determining the relative abundance of normal and sickle cell anemia cells in a mixed population of cells, which is the same as, or analogous to, "determining the ratio of a selected genetic sequence in a population of genetic sequences," but Li does not suggest any method for making this determination using “bulk” DNA isolated from a population of cells. Li was interested in gene mapping, not determining the ratio of a selected genetic sequence in a population of genetic sequences. Jeffreys diluted bulk DNA into samples containing the equivalent of a single genome and showed that it is possible to amplify a single copy of a DNA sequence. However Jeffreys’ main concern appears to have been testing the limits of PCR sensitivity. Jeffreys did not appreciate that a set of assay samples could be used as a proxy for a set of individual cells, with the proportion of assay samples in which a genetic sequence is amplified being roughly equivalent to the proportion of cells in a population containing that sequence (i.e., it “reflects the composition of the biological sample”). In order to rely on equivalence as a rationale supporting an obviousness rejection, the equivalency must be recognized in the prior art (MPEP 2144.06(II)). While the method described in the ‘706 patent allowed the inventors to determine that about 4% of c-Ki-Ras alleles in a stool sample were mutated (see Example 5), Jeffreys does not suggest any way to analyze the data he produced so as to determine the frequency of an allele in a population of nucleic acid sequences. Jeffreys was concerned with DNA fingerprinting, i.e. comparing two DNA samples to determine whether they are identical, which is not analogous to determining the ratio of a selected genetic sequence in a population of genetic sequences. The studies reported in Zhang were intended to

determine whether genomic analysis could be performed using DNA from a single cell; Zhang does not envision determining the ratio of a selected genetic sequence in a population of genetic sequences.

The remaining references applied under 35 U.S.C. 103 do not suggest “digital" amplification of nucleic acid sequences. Kalinina, Burg and Chou are directed to technical details of the PCR amplification and detection of PCR products, as recited in the dependent claims. Trümper shows that PCR can be performed on cDNA produced from mRNA isolated from single cells. Trümper, Pontén, Kanzler, Gravel and Schwab disclose certain genetic abnormalities (as recited in the dependent claims) whose frequency can be determined by digital PCR, but do not suggest the concept of digital PCR itself.

Further evidence of non-obviousness (i.e. “secondary considerations”) has been presented in the declarations under 37 C.F.R. 1.132 filed with the response of September 9, 2014.

Stanley Lapidus declares that digital PCR was a “brilliant innovation” which met an unmet need in the art, and whose success surprised those skilled in the art (Lapidus declaration ¶¶ 11-12). Mr. Lapidus further declares that digital PCR is an improvement over other methods in use at the time of the invention and that companies have marketed products for use in performing digital PCR (¶¶ 13-14).

Ie-Ming Shih declares that digital PCR, as the term is used in the ‘706 patent, has been used clinically to detect and quantify mutations, to detect allelic imbalances and loss of heterozygosity and to quantitatively detect gene expression in tumor tissues, blood and stool samples (Shih declaration, ¶¶ 11-13 and exhibit 3). The *PNAS* publication upon which the ‘706 patent is based is frequently cited by others and scientific conferences devoted to digital PCR methods have been held (¶¶ 14-17 and exhibits 4-8). Digital PCR has enabled the detection of fetal genetic abnormalities by measuring allelic imbalances in fetal DNA circulating in maternal plasma (¶¶ 18-20 and exhibits 9-11, 16, 17). A number of researchers report that digital PCR is more sensitive and more precise than other PCR methods (¶¶ 21-24 and exhibits 12-15).

Several manufacturers have developed and marketed instruments designed to implement digital PCR (¶¶ 25, 27, 28 and exhibits 12-14).

Any comments considered necessary by PATENT OWNER regarding the above statement must be submitted promptly to avoid processing delays. Such submission by the patent owner should be labeled: "Comments on Statement of Reasons for Patentability and/or Confirmation" and will be placed in the reexamination file.

/Bruce Campell/
Patent Reexamination Specialist
Central Reexamination Unit 3991

/Padmashri Ponnaluri/
Patent Reexamination Specialist
Central Reexamination Unit 3991

/Deborah D Jones/
Supervisory Patent Examiner, Art Unit 3991

IN THE CLAIMS

Please amend the following claims as indicated by the status identifier. Patent claims under reexamination but not amended are indicated as "original." Patent claims not subject to reexamination are not shown.

1. (Amended) A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

diluting isolated nucleic acid template molecules [in] isolated from a biological sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.

2. (Original) The method of claim 1 wherein the step of diluting is performed until at least one-tenth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required for the step of analyzing to determine the presence of the selected genetic sequence.

3. (Amended) The method of claim 1 wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product of at least one of the selected and reference genetic sequences when subjected to a polymerase chain reaction.

4. (Original) The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 10 nucleic acid template molecules containing the reference genetic sequence.

5. (Original) The method of claim 1 wherein the step of diluting is performed until all of the

assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 100 nucleic acid template molecules containing the reference genetic sequence.

6. (Original) The method of claim 1 wherein the biological sample is cell-free.

7. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 10.

8. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 50.

9. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 100.

10. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 500.

11. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 1000.

12. (Original) The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

13. (Not subject to reexamination)

14. (Original) The method of claim 1 wherein the step of analyzing employs gel electrophoresis.

15. (Original) The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

16. (Amended) The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid [probe] probes.

17-18. (Not subject to reexamination)

19. (Original) The method of claim 1 wherein the step of amplifying employs a single pair of primers.

20. (Original) The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.

21. (Original) The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

22. (Original) The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

23. (Original) The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

24. (Original) The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.

25. (Original) The method of claim 1 wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.

26. (Original) The method of claim 1 wherein the selected genetic sequence is a translocated allele.

27. (Original) The method of claim 1 wherein the selected genetic sequence is a wild-type allele.

28. (Original) The method of claim 1 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

29. (Original) The method of claim 1 wherein the selected genetic sequence is a rare exon sequence.

30. (Original) The method of claim 1 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

31. (Original) The method of claim 1 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

32. (Original) The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

33-37. (Not subject to reexamination)

38. (Twice amended) A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

distributing cell-free nucleic acid template molecules from a biological sample to form a set comprising a plurality of assay samples;

amplifying the nucleic acid template molecules [within a set comprising a plurality of assay samples] to form a population of amplified molecules in [each of the] individual assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the

composition of the biological sample.

39. (Original) The method of claim 38 wherein the number of assay samples within the set is greater than 10.

40. (Original) The method of claim 38 wherein the number of assay samples within the set is greater than 50.

41. (Original) The method of claim 38 wherein the number of assay samples within the set is greater than 100.

42. (Original) The method of claim 38 wherein the number of assay samples within the set is greater than 500.

43. (Original) The method of claim 38 wherein the number of assay samples within the set is greater than 1000.

44. (Original) The method of claim 38 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

45. (Not subject to reexamination)

46. (Original) The method of claim 38 wherein the step of analyzing employs gel electrophoresis.

47. (Original) The method of claim 38 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

48. (Amended) The method of claim 38 wherein the step of analyzing employs hybridization to at least two nucleic acid [probe] <u>probes</u>.

49-50. (Not subject to reexamination)

51. (Original) The method of claim 38 wherein the step of amplifying employs a single pair of primers.

52. (Original) The method of claim 38 wherein the step of amplifying employs a polymerase which is activated only after heating.

53. (Original) The method of claim 38 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

54. (Original) The method of claim 38 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

55. (Original) The method of claim 38 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

56. (Original) The method of claim 38 wherein the template molecules are obtained from a body sample selected from the group consisting of stool, blood, and lymph nodes.

57. (Original) The method of claim 38 wherein the template molecules are obtained from a body sample of a leukemia or lymphoma patient who has received anti-cancer therapy, said body sample being selected from the group consisting of blood and bone marrow.

58. (Original) The method of claim 38 wherein the selected genetic sequence is a translocated allele.

59. (Original) The method of claim 38 wherein the selected genetic sequence is a wild-type allele.

60. (Original) The method of claim 38 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

61. (Original) The method of claim 38 wherein the selected genetic sequence is a rare exon sequence.

62. (Original) The method of claim 38 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

63. (Original) The method of claim 38 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

64. (Original) The method of claim 38 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	90012894	6,440,706 B1 ET AL.
	Examiner	**Art Unit**
	BRUCE CAMPELL	3991

CPC		
Symbol	**Type**	**Version**

CPC Combination Sets				
Symbol	**Type**	**Set**	**Ranking**	**Version**

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	53	
/BRUCE CAMPELL/ Primary Examiner.Art Unit 3991	9/12/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	90012894	6,440,706 B1 ET AL.
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	BRUCE CAMPELL	3991

US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION														
CLASS	SUBCLASS	CLAIMED									NON-CLAIMED					
435	91.2	C	1	2	N	15 / 09 (2006.01.01)										
CROSS REFERENCE(S)		C	1	2	Q	1 / 68 (2006.01.01)										
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)															
435	7.1	91.1	6.12													
536	22.1	23.1	24.3	24.31	24.32											
536	24.33															

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	53	
/BRUCE CAMPELL/ Primary Examiner.Art Unit 3991	9/12/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	90012894	6,440,706 B1 ET AL.
	Examiner	**Art Unit**
	BRUCE CAMPELL	3991

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	53	
/BRUCE CAMPELL/ Primary Examiner.Art Unit 3991	9/12/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

Reexamination	Application/Control No.	Applicant(s)/Patent Under Reexamination
	90012894	6,440,706 B1 ET AL.
	Certificate Date	Certificate Number
		C1

Requester Correspondence Address:	☐ Patent Owner	☒ Third Party

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LITIGATION REVIEW ☒	/BC/ (examiner initials)	09/15/2014 (date)
Case Name		Director Initials
Esoterix Genetic Laboratories, Llc et al v. Life Technologies		
U.S. District - North Carolina Middle 1:12cv1173		

COPENDING OFFICE PROCEEDINGS	
TYPE OF PROCEEDING	NUMBER
1. none	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	90012894	6,440,706 B1 ET AL.
	Examiner	Art Unit
	BRUCE CAMPELL	3991

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
reviewed prosecution history of 09/613,826	7/12/13	/BC/
google: gene amplification in cancer review	7/24/13	/BC/

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

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CONFIRMATION NO. 8442

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
90/012,894	06/17/2013 RULE	435	3991	001107.00989

APPLICANTS

INVENTORS

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LIFE TECHNOLOGIES CORPORATION, CARLSBAD, CA

**** CONTINUING DATA ***************************

This application is a REX of 09/613,826 07/11/2000 PAT 6440706
which claims benefit of 60/146,792 08/02/1999

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /BRUCE R CAMPELL/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
				64	5

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TITLE

Digital Amplification

FILING FEE RECEIVED		
12000	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

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(12) EX PARTE REEXAMINATION CERTIFICATE (10360th)

United States Patent
Vogelstein et al.

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(45) Certificate Issued: Oct. 24, 2014

(54) **DIGITAL AMPLIFICATION**

(75) Inventors: **Bert Vogelstein**, Baltimore, MD (US); **Kenneth W. Kinzler**, BelAir, MD (US)

(73) Assignee: **The Johns Hopkins University**, Baltimore, MD (US)

Reexamination Request:
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Issued:	**Aug. 27, 2002**
Appl. No.:	**09/613,826**
Filed:	**Jul. 11, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/146,792, filed on Aug. 2, 1999.

(51) **Int. Cl.**
C12N 15/09 (2006.01)
C12Q 1/68 (2006.01)

(52) **U.S. Cl.**
USPC **435/91.2**; 435/7.1; 435/91.1; 435/6.12; 536/22.1; 536/23.1; 536/24.3; 536/24.31; 536/24.32; 536/24.33

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

To view the complete listing of prior art documents cited during the proceeding for Reexamination Control Number 90/012,894, please refer to the USPTO's public Patent Application Information Retrieval (PAIR) system under the Display References tab.

Primary Examiner — Bruce Campell

(57) **ABSTRACT**

The identification of pre-defined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. The exponential, analog nature of the polymerase chain reaction is transformed into a linear, digital signal suitable for this purpose. Single molecules can be isolated by dilution and individually amplified; each product is then separately analyzed for the presence of mutations. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

Claims **1**, **3**, **16**, **38** and **48** are determined to be patentable as amended.

Claims **2**, **4-12**, **14-15**, **19-32**, **39-44**, **46**, **47** and **51-64**, dependent on an amended claim, are determined to be patentable.

Claims **13**, **17**, **18**, **33-37**, **45**, **49** and **50** were not reexamined.

1. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

diluting *isolated* nucleic acid template molecules **[**in**]** *isolated from* a biological sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.

3. The method of claim **1** wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product *of at least one of the selected and reference genetic sequences* when subjected to a polymerase chain reaction.

16. The method of claim **1** wherein the step of analyzing employs hybridization to at least two nucleic acid **[**probe**]** *probes*.

38. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

distributing cell-free nucleic acid template molecules from a biological sample to form a set comprising a plurality of assay samples;

amplifying *the nucleic acid* template molecules **[**within a set comprising a plurality of assay samples**]** to form a population of amplified molecules in **[**each of the**]** *individual* assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.

48. The method of claim **38** wherein the step of analyzing employs hybridization to at least two nucleic acid **[**probe**]** *probes*.

* * * * *