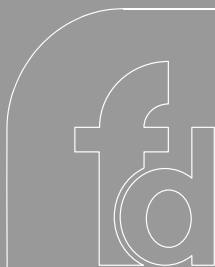


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Principle and applications of digital PCR

Gudrun Pohl and Ie-Ming Shih[†]

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 <i>KRAS</i> mutations in stool	<i>KRAS</i> mutations can be detected and quantified in stool DNA samples from colorectal cancer patients	[1]
Detection of <i>KRAS</i> mutational status	Low- and high-grade ovarian serous carcinoma develop through independent pathways	[18]
Analysis of <i>KRAS</i> and AI in APC genes	Mutations in <i>KRAS</i> and AI of APC occur in appendiceal adenomas	[12]
Detection of AI and <i>KRAS</i> mutations	High-grade ovarian serous carcinoma contain wild type <i>KRAS</i> 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 <i>BAT26</i> alterations in fecal DNA	Presence of <i>BAT26</i> 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 complementary 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 [1]. 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 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 [1].

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

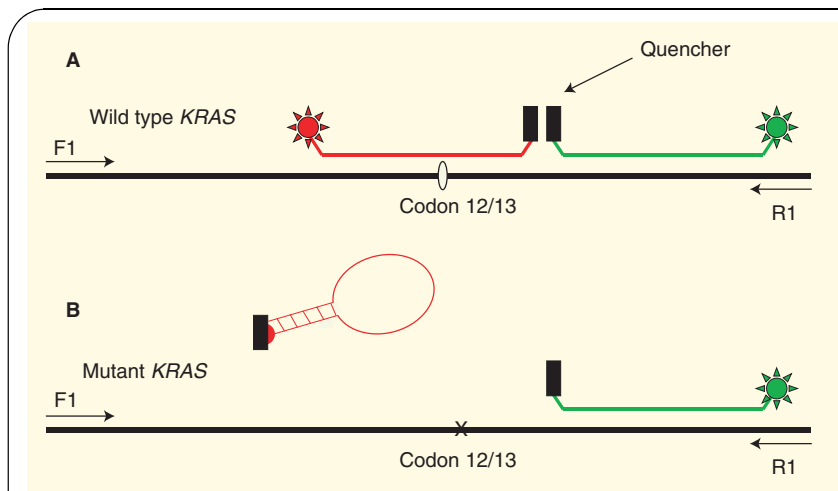


Figure 1. Design of molecular beacons and PCR primers for digital PCR analysis to detect *KRAS* mutations. Forward (F1) and reverse (R1) primers amplify exon 1 of the *KRAS* gene containing the relevant codons 12 and 13. Asymmetrical amplification generates single-stranded DNA complementary to the molecular 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].

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

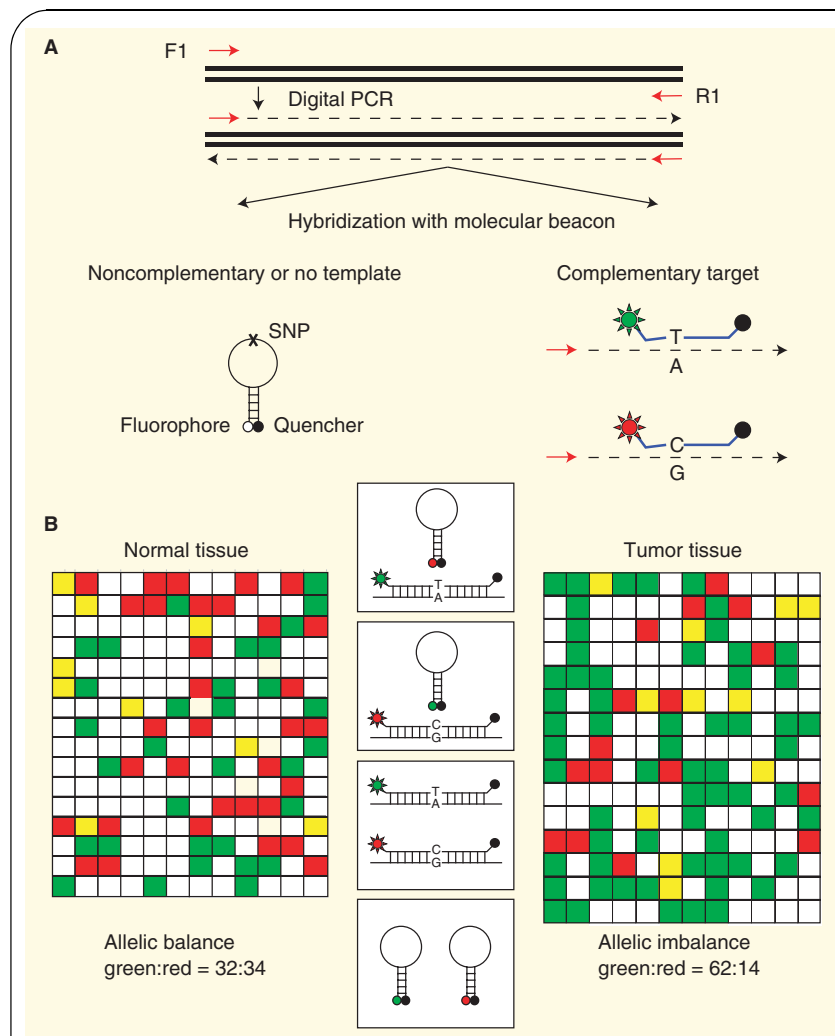


Figure 2. Digital PCR analysis to assess allelic imbalance. (A) Molecular beacon design. A pair of primers are designed to amplify approximately 100 bp of PCR product that contains a single nucleotide polymorphism (SNP) marker in the center region. The two beacons used for analysis of a specific SNP 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 complementary 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].

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

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