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$_4$SO$_4$, 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$_4$SO$_4$, 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,

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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′-CATTATTTTTATTATAAGGCCTGC-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
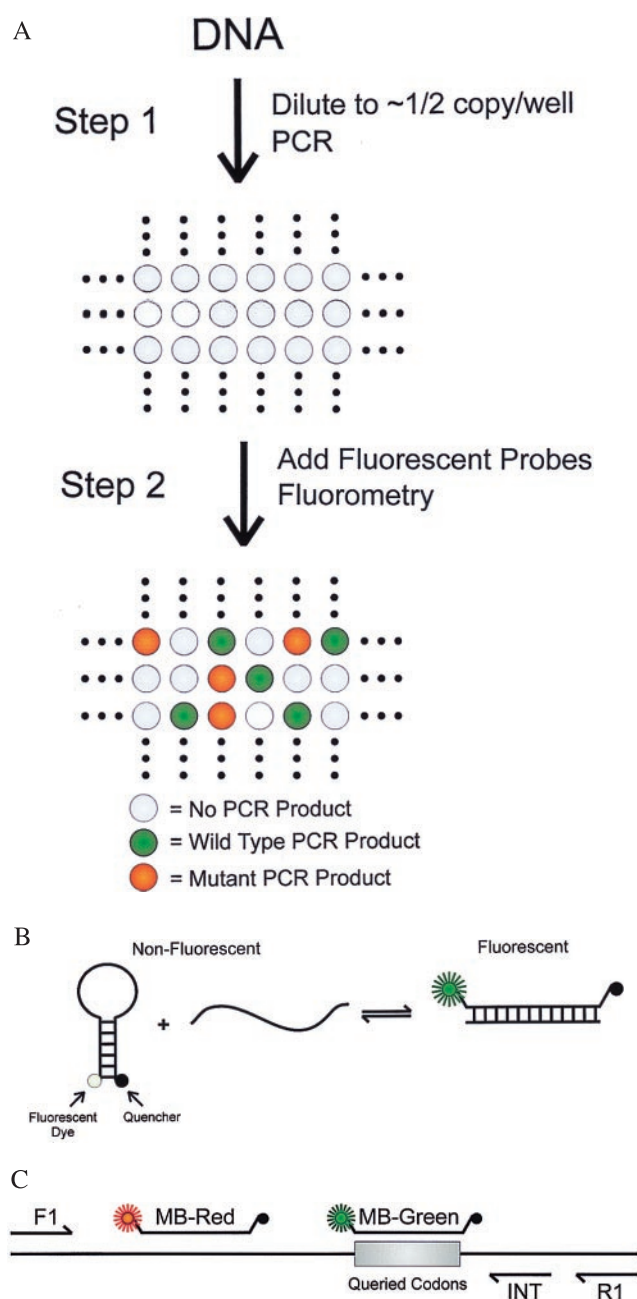

FIG. 1. Schematic of Dig-PCR. (*A*) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes that discriminate between WT and mutant alleles and subsequent fluorometry. (*B*) Principle of MB analysis. In the stem–loop configuration, fluorescence from a dye 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.

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 ($\approx$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
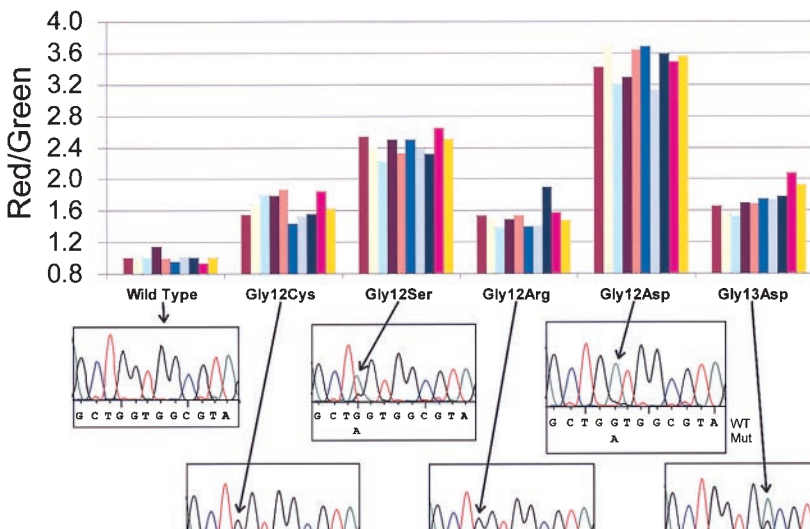

FIG. 2.   Discrimination between WT and mutant PCR products by MBs. Separate PCR products (*n* = 10), each generated from $\approx$50 genome equivalents of DNA of cells containing the indicated mutations of *c-Ki-Ras*, were analyzed with the MB probes described above. Representative examples of the PCR 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,

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an excess of a single internal primer (primer INT in Fig. 1*C*) 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).

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
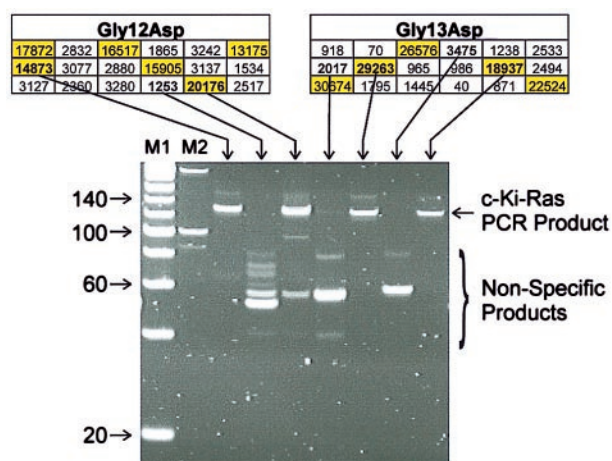

FIG. 3. Detecting Dig-PCR products with MB-RED. SFU of representative wells from an experiment employing colorectal cancer cells with Gly12Asp or Gly13Asp mutations of the *c-Ki-Ras* gene. Wells with values >10,000 SFU are shaded yellow. PAGE analyses of the PCR products from selected wells are shown. 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
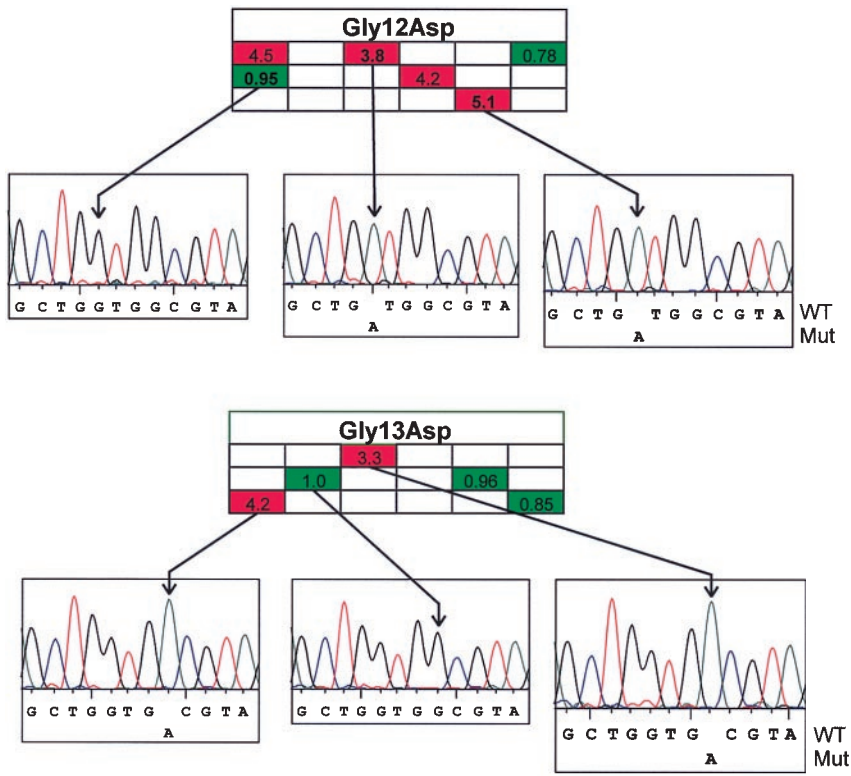
FIG. 4.   Discriminating WT from mutant PCR products obtained in Dig-PCR. RED/GREEN ratios were determined from the fluorescence of MB-RED and MB-GREEN as described in *Materials and Methods*. The wells shown are the same as those illustrated in Fig. 3. The sequences of PCR products from the indicated 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
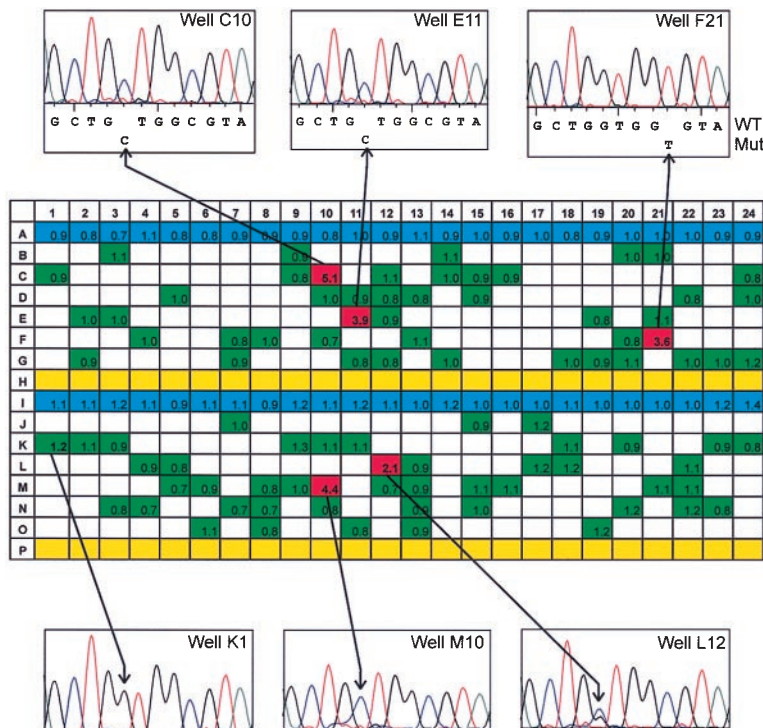

FIG. 5.   Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED, and the RED/GREEN ratios were 1.0 ± 0.1 (mean ±1 SD). The wells colored yellow 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

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