Colorimetric approach to high-throughput mutation analysis

Nicole E. Benoit¹, David Goldenberg¹, Shirley X. Deng², Eli Rosenbaum¹, Yoram Cohen¹, Joseph A. Califano¹, William H. Shackelford², Xiao B. Wang², and David Sidransky¹

BioTechniques 38:635-639 (April 2005)

High-throughput genomic mutation screening for primary tumors has characteristically been expensive, labor-intensive, and inadequate to detect low levels of mutation in a background of wild-type signal. We present a new, combined PCR and colorimetric approach that is inexpensive, simple, and can detect the presence of 1% mutation in a background of wild-type. We compared manual dideoxy sequencing of p53 for eight lung cancer samples to a novel assay combining a primer extension step and an enzymatic colorimetric step in a 96-well plate with covalently attached oligonucleotide sequences. For every sample, we were able to detect the presence or absence of the specific mutation with a statistically significant difference between the sample optical density (OD) and the background OD, with a sensitivity and specificity of 100%. This assay is straightforward, accurate, inexpensive, and allows for rapid, high-throughput analysis of samples, making it ideal for genomic mutation or polymorphism screening studies in both clinical and research settings.

INTRODUCTION

In recent years, mutational analysis of primary tumors has yielded a host of genes that may play a role in disease development and carcinogenesis. Several genes have been linked to the development of solid tumors, notably, BRCA1 and BRCA2 (1,2), B-raf (3,4), k-ras (5,6), and p53 (7,8). Large screening studies to identify specific genetic alterations are difficult due to the typically high cost, low sensitivity, and laborious nature of the available assays (9,10). Newer techniques that address these issues are still only able detect a subset of the potential DNA mutations that may occur (11). We have designed an assay that combines PCR, a primer extension step, and a colorimetric detection method. This assay is simple, inexpensive, detects almost any variation of mutation including insertions, deletions, and point mutations, and can be completed in less than 1 day.

We analyzed eight different lung cancer samples, each with one of two common p53 mutations, by both dideoxy sequencing and a new approach combining a primer extension step and an enzymatic colorimetric step in a 96-well plate with covalently attached oligonucleotide sequences. The novelty of this assay lies in the primer extension step, which is modified by adding dNTPs with one dideoxy “terminator” base and a second base labeled with biotin. The dideoxy base assures the specific extension of only the mutated samples while terminating the extension of the wild-type samples. The biotin-labeled base enables the enzyme-linked colorimetric detection of the extended oligonucleotide. In each test, we were able to easily identify the mutated samples, even within the context of a wild-type background.

MATERIALS AND METHODS

All material used for this study was obtained with informed consent as per the institutional review board approved protocol at Johns Hopkins Medical Institutions (JHMI). Eight lung cancer samples were characterized for the presence of a p53 mutation. Primary lung tumor samples were microdissected to ensure at least 70% tumor content, and DNA was extracted as described elsewhere (12). The DNA was sequenced as previously described (13). Briefly, the 1.8-kb genomic product of p53 encompassing exons 5 through 9 was PCR-amplified, the PCR product was phenol:chloroform-extracted, and then sequenced by the standard ³²P end-labeled dideoxy NTP method. The sequenced products were run on a 6% polyacrylamide gel with 8 M urea, fixed, and exposed to film. Mutated samples were confirmed with a second PCR and sequencing reaction. PCR and sequencing primers are described elsewhere (14). Each of the mutated samples contained a missense mutation in the p53 gene. Four of the tumors had strong mutations (bands on sequencing gel) indicating a probable loss of one allele of p53, but the fifth sample had a weaker signal, indicating a greater percentage of wild-type DNA—either from more normal tissue in the microdissection or a heterozygous mutation.

¹The Johns Hopkins University School of Medicine, Baltimore and ²TrimGen Corporation, Sparks, MD, USA
Identical DNA samples were used for the colorimetric assay, performed using the Mutector™ Kit (www.trimgen.com; TrimGen, Sparks, MD, USA). A short, approximately 100-bp oligonucleotide, was PCR-amplified using specific PCR primers directed to the specific area of the known mutation. Five microliters of the PCR product (mixed with 45 µL buffer) were then added to a 96-well plate, which had been precoated with $10^{11}$ copies of covalently attached detection oligonucleotide specific to a known p53 mutation. The plate was heated to 95°C for 5 min, followed by at least 10 min at room temperature (20°–25°C). Following hybridization, the wells were washed twice with 250 µL wash buffer and blotted to remove excess liquid. Next, the Shifted Termination Assay (STA; International Patent pending) step was performed by adding the reaction mixture provided in the Mutector kit (including Taq DNA polymerase, buffer, and dNTPs with ddTTP), and biotin-labeled dCTP (1.6 µM final concentration; Invitrogen, Carlsbad, CA, USA). A total of 30 µL mixture was added to each well, and the plate was heated to 95°C for 2 min, 65°C for 45 min, and 95°C for 5 min, followed immediately with a cycling PCR consisting of 25 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and finished with 1 cycle of 95°C for 3 min. The plate was immediately aspirated and washed free of all reactants with 250 µL/well 0.1 M NaOH, followed by two additional washes using wash buffer. The streptavidin-horseradish peroxidase (HRP) complex was diluted with buffer, and 50 µL mixture were added to each well and hybridized for 20 min at room temperature. Each well was washed four times and again blotted to remove all liquid. Finally, 50 µL ABTS substrate [2,2′-azino-bis (3-ethylbenziazoline-6-sulfonic acid)] (blue-green color) were added to each well and incubated for 30 min at 25°C in the absence of light. Signal was measured with a 405-nm absorbance filter on a Sunset plate reader (Tecan, Maennedorf, Switzerland) and reported as an optical density (OD). A schematic drawing of the Mutector assay is shown in Figure 1, A–D. A color change indicating the presence of a mutation is often apparent after only a few minutes and can usually easily be seen with the naked eye (Figure 2). We completed each test in less than 7 h from initial PCR to final readout.

Serial dilution studies using p53 codon 158 mutated samples were performed to determine sensitivity thresholds for the assay. We diluted mutated DNA into wild-type DNA to 1%, 2%, 5%, 10%, 20%, and 50% mutated, as well as using 100% mutated alone and 100% wild-type alone (Figure 2).

![Figure 1. Representation of the Mutector assay.](image)

![Figure 2. Representational photograph of a completed assay.](image)
For each dilution, we used a Student’s t-test to determine the difference between the sample OD and wild-type. Significant differences were found (P < 0.05) between the mutated sample and wild-type sample, even at 1% dilution, with a mean OD for each mutated sample greater than 2-fold the OD of background wild-type.

The key to optimization of this assay lies in the selection of appropriate detection primer and modified bases for each mutation. The detection primer should be oriented either 5′ to 3′ or 3′ to 5′ to optimize the number of bases that can be incorporated into the mutant strand before sequence termination. It is designed to be about 30 bp long, with the free end of the primer the base just prior to the mutation (Figure 1B). The nucleotide complement to the wild-type base at the point of the mutation should always be used as the dideoxy “terminator” base. Using this base as the terminator ensures that the wild-type strands of hybridized PCR product will be minimally synthesized during the primer extension step, while still allowing the mutant DNA to extend (Figure 1C). The nucleotide chosen to be biotinylated should be the most heavily repeated base between the mutation and the next terminator base in the sequence. For example, in Figure 1, A–D, we show the extension of mutated sequence (C/A)TTCGTTGA, where the initial C is the mutated base, and A is the wild-type base. In this case, the chosen dideoxy base is T, as the complement to the wild-type A. This will effectively terminate the wild-type samples, while allowing the mutated samples to extend until the next A base, where the dideoxy T will terminate all samples. The next A in the sequence occurs eight bases after the mutation, and there are four T bases in that sequence. This makes the complementary A nucleotide the best candidate for biotinylation, since it occurs multiple times in the extended sequence and will ultimately provide the best color change.

RESULTS AND DISCUSSION

We evaluated eight lung cancer samples for the presence of a p53 mutation using two methods—standard dideoxy sequencing and a new, colorimetric assay using DNA. Our cohort of lung samples included three tumors with a codon 158 G/T mutation, two tumors with a codon 175 G/A mutation, and three wild-type tumors. Serial dilution studies done with p53 codon 158 mutations resulted in a mutation detection level of 1%; that is, one mutated cell could be reliably detected in a background of 100 wild-type cells (Figure 3). We established the assay threshold for detecting a mutation as a mean sample
OD of at least 2-fold over mean background OD (OD of known wild-type). We proceeded to perform the colorimetric assay on all of our eight samples at least three separate times. For four of our tumor samples, the mean sample OD was significantly greater than 2-fold increased over background (Figure 4). In the remaining case, there was a high percentage of wild-type DNA in the DNA sample, and the OD value was lower. However, since the fold increase of the sample OD was greater than 2-fold over wild-type, we were still able to detect the presence of the mutation using the established criteria.

Colorimetric studies have been used widely since the early 1960s for protein detection and chemical reactions (15), but it has only been in recent years that these assays have incorporated DNA analysis. Our assay is fast, relatively simple, and inexpensive, making it ideal for screening studies and large population characterizations. In our study, both the sensitivity and specificity of the colorimetric method was 100%. Also, we have reliably demonstrated that this assay is capable of detecting as low as 1% mutated DNA in a wild-type background. Our assay can also be used to detect the presence of a heterozygous mutation (data not shown), which can be clinically significant in some cases.

We believe that using an assay such as this will increase the efficacy of large screening studies where the mutation is known or is one of only a few possibilities. It is possible to individualize the reactions by changing the terminator base or by changing the detection oligonucleotide. The level of colorimetric detection can be further optimized by carefully choosing the dNTP base to be biotinylated, based on the sequence prediction of the target DNA. As we have shown, usually the detection of a specific mutation is extremely robust, allowing for a quick and convenient method of screening possibly large numbers of samples. By setting a cut-off threshold for mutations at an OD value greater than two times wild-type control OD, we have achieved 100% specificity and 100% sensitivity in our tests. Also, changing the biotinylated base, or increasing the number of primer extension PCR cycles, may further optimize samples with initially lower OD values.

The potential exists to use this assay for large studies to detect early markers of disease or to aid in diagnosis. Using known DNA alterations as risk indicators, this assay would be extremely useful for screening easily obtained samples from patients, such as sputum, urine, or blood. Another area of increasing interest for rapid screening techniques involves surgical specimens and also surgical margins (16). Characterizing samples for a particular mutation may also aid in treatment decisions. One recent study completed in our laboratory shows that this assay can be used to specifically detect B-raf mutations in fine-needle aspirates and tumor tissue from patients with papillary thyroid cancer, with a specificity approaching 100% (17). Our group has also used the Mutector assay to evaluate B-raf mutations in malignant melanoma (18). This assay is rapid, technically feasible for almost any level laboratory, and financially inviting. We were able to reproducibly complete the entire process in 1 day. The Mutector primer extension/colorimetric assay allows for easy detection of mutations from large numbers of samples, all within a reasonable time frame and budget.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Juna Lee for her assistance in the preparation of this manuscript. We are also grateful to James Wang for generously providing us with the assay kit and oligonucleotide-primed plates. This work was supported by Specialized Programs of Research Excellence grant no. P50 CA58184 from the National Cancer Institute for lung research and additional funding by Public Health Service grant no. 1 U01 CA84986 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, as part of The Early Detection Research Network.

COMPETING INTERESTS STATEMENT

X.B.W. is the President and W.H.S. is the Director of Business Development for TrimGen Corp., which manufactures the Mutector kits. All the other authors declare no competing interests.

REFERENCES


Address correspondence to David Sidransky, Johns Hopkins University School of Medicine, Division of Head and Neck Cancer Research, 818 Ross, 720 Rutland Avenue, Baltimore, MD 21205, USA. e-mail: dsidrans@jhmi.edu