Mutation detection for the K-*ras* and P16 genes

Application

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Abstract

Mutations in the K-*ras* gene codon 12 region can lead to cancer, for example of the colon, pancreas, liver, spleen, stomach or lungs. The CDKN2A/P16 gene is a familial melanoma gene. Routine PCR and DNA sequencing methods can identify exactly which point mutation is present in patient tissue samples. Freshly frozen tumor sections direct from surgeries can be utilized, as well as archived paraffin-embedded specimens. Prior to DNA sequencing of K-*ras*, the nested PCR products are digested with a restriction enzyme and electrophoresed for quality and sizing purposes. A sample can be determined to be either wild-type or mutated simply by comparing the size of the PCR band to the size of the digested PCR band on a DNA chip. This analysis demonstrated the separation of PCR fragments from 135 bp to 106 bp. DNA sequencing is then utilized to verify the chip results. If a sample is shown to be mutated, sequencing can pinpoint the exact mutation. For P16 exon 3, the PCR's are electrophoresed on the Agilent 2100 bioanalyzer, purified, and sequenced. Heterozygous mutations can be resolved accurately within 10–15 % of base pair length using the Agilent 2100 bioanalyzer. The Lab-on-a-chip technology is a novel and important as well as rapid step in these diagnostic and quality control assays. In this Application Note we demonstrate how extra bands on Agilent's chip image correlate to mutated DNA sequences.

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Introduction

Technology that is currently available for SNP detection include the SpectruMedix platform, dHPLC, real-time PCR, DNA sequencing, the Nanogen cartridge platform, and the Affymetrix chip platform. We found that using the Agilent platform we could also detect single nucleotide polymorphisms, and verified this with sequencing analysis. The human K-*ras* gene is a member of the Ras family of GTPases[1]. Mutant, activated forms of Ras proteins, which are frequently observed in cancer, have an impaired GTPase activity rendering the protein resistant to inactivation by regulatory GAP proteins[2]. The ability to detect changes in the region of this gene which codes for activation is essential. For our purposes, this test was used to ultimately determine a cancer patient's eligibility into a clinical trial for a peptide vaccine. The normal form of codon 12 codes for glycine. Known mutations observed at codon 12 are: aspartic acid, valine, serine, cysteine, alanine, arginine, and asparagine. The PCR primers chosen amplified an initial product of 157 base pairs. The forward primer had a mismatch incorporated into it in order to create a BstNI site. This fragment was cut with BstNI, amplified (with an internal reverse primer), and cut again with BstNI[3]. The restriction enzyme's purpose was to trim away excess normal DNA sequences and enrich for any mutant sequences. In mutated samples, a BstNI site was not created, and therefore not recognized. Tumors will inherently contain normal tissue infiltrated throughout. Unless laser capture microdissection is incorporated, normal tissue cannot be removed by simple microtomy. Hence, we used BstNI to assist in normal DNA sequence removal. The second nested PCR amplification produced a 135-base pair band. If the sample was wild-type, BstNI recognized its site and trimmed the product to a 106 bp size. If the sample was mutated, the digested product remained at 135 bp.

For P16, a 198 bp fragment was generated. For normal samples, a single band appeared on the gel image. For mutated samples, a doublet band and sometimes a triplet band were observed. This corresponded 100 % with DNA sequence data. In instances of degraded or low amounts of initial DNA template, the quality of the PCR product was also viewed on the chip before wasting the reagents and time to sequence it. Often formalin-fixed tissues or lymph node metastases do not present good quality DNA template for PCR reactions. The chip images were a qualitative as well as a quantitative tool that indicated if a sample needed to be repeated to increase its yield. The resolution was also better on the Agilent 2100 bioanalyzer platform than on ethidium-stained agarose gels. Chips were safer to work with and preparation time was reduced dramatically.

Materials and methods

Tissues

Sample blocks were obtained through the National Cancer Institute's Naval Medical Oncology Branch. Ten 5-µm sections were deparaffinized and extracted using the Series III kit from Xtrana. For P16, DNA was received already extracted in barcoded 96-well plates. We quantitated the DNA via spectrophotometry and it was diluted to 10 ng for starting material.

PCR

Nested PCR was performed for K-*ras* with final reaction concentrations of 0.25 µM each primer[3] (Invitrogen), 0.2 mM dNTPs (Roche), 1X PCR buffer (Roche), 1.25 U Taq polymerase (Roche), HPLC-grade water (Fisher), and Amp Enhancer (Xtrana). 50 µl were added to the bound DNA on the Xtrana tubes. For samples not extracted using the Xtrana kit, the Amp Enhance solution was eliminated and solubilized template was incorporated. This reaction was cycled 18 times using ABI's 9700 thermal cycler under the following conditions: 94 ºC for 45 seconds, 55 ºC for 1 minute, and 72 ºC for 90 seconds. This was purified using the Wizard kit (Promega). The eluant was used as template in a BstNI reaction with BSA (New England BioLabs). This reaction was purified using the MinElute kit (Qiagen). Then an aliquot was used in the second round of PCR; the same conditions as above, but for 40 cycles. Again, the amplicon was purified and subjected to the same restriction enzyme digest/purification. At this point the samples were run on the Agilent 2100 bioanalyzer and submitted for DNA sequencing. For the P16 gene, the final reaction concentrations were 0.1 µM of each primer[4] (Invitrogen), with 0.2 mM dNTPs (Invitrogen), 1 unit of Platinum Taq High Fidelity, 1X PCR buffer (Invitrogen), 3 mM magnesium sulfate, 3 % DMSO (Sigma), 10 ng of DNA template, and HPLC-grade water (Fisher) in a total volume of 50 µl. An initial 3-minute denaturation at 95 ºC was followed by 40 cycles at the following conditions, again on ABI's 9700 thermal cycler: 95 ºC for one minute, 58 ºC for 1 minute, and 72 ºC for 1 minute. A ten minute final extension at 72 ºC was also incorporated. Amplicons were freed of excess primers and nucleotides using ExoSap (USB), analyzed on the Agilent 2100 bioanalyzer, and submitted for DNA sequencing. Sequencing reactions were purified/de-salted using Sephadex G50 (Sigma).

Analysis of PCR-products

Amplified products were electrophoresed on Agilent's 2100 bioanalyzer. The DNA 1000 LabChip® kit was used in accordance with manufacturer's instructions. Briefly, 9 µl of the gel dye mixture was added to the chip well labeled "G." This was pressurized for one minute throughout the chip with the syringe attachment provided. Then 9 µl of the gel dye mixture was added to the other two chip wells labeled with "G." 1 µl of ladder was added to the ladder well, followed by 5 µl of the gel dye mixture. This was pipetted up and down several times to mix. 5 µl of the markers were added to each of the twelve sample wells. 1 µl of each sample was added to their corresponding wells on the chip. The chip was vortexed for one minute on the IKA vortex adapter provided at the recommended setting. The chip was placed in the Agilent 2100 bioanalyzer and the double-stranded DNA 1000 assay software was run. Twelve samples could be run in a 30-40 minute time frame. Data was saved to our server and analyzed using the Agilent analysis software package provided.

Results and discussion

Our established PCR assays enable the analysis of two gene regions relevant to cancer. The significance of these diagnostics relies on accurate interpretation of visual data. First, we compared mutant versus wild type samples for K-*ras* codon 12. The sizing differences, as well as the intensity of heterozygous bands would be difficult to discern on a standard agarose gel, not to mention time-consuming. Applying the microfluidic technology of Agilent's equipment, we were able to easily confirm our data with that of DNA sequencing. The sizing and quantitation offered by the software were invaluable to eliminating any guesswork previously involved in this assay. Figure 1 displays wild-type (lanes 10 and 11) versus mutated samples (lanes 4-7), as well as a heterozygous mutation (lanes 2 and 3). PCR products are in even-numbered lanes, and PCR products subjected to BstNI digests are in odd-numbered lanes. The ladder is in lane 1 and water negative controls are in lanes 8 and 9.

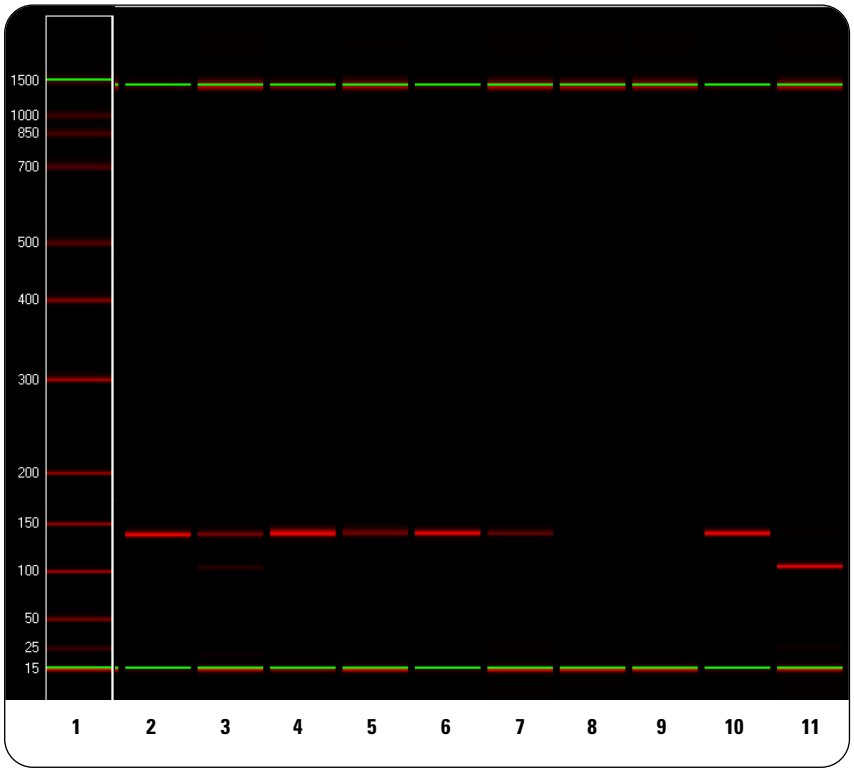

Figure 1
LabChip image of DNA samples PCR'd for the codon 12 region of the K-*ras* gene. Lane 1-Ladder, Lane 2-Heterozygous sample PCR, Lane 3-Heterozygous sample PCR after BstNI digest, Lanes 4 & 6-Mutant sample PCRs, Lanes 5 & 7-Mutant sample PCRs after BstNI digest, Lane 8-Water negative control PCR, Lane 9-Water negative control PCR after BstNI digest, Lane 10-Wild-type sample PCR, Lane 11-Wild-type PCR after BstNI digest.

Figure 2 displays the corresponding DNA sequence chromatograms for these samples. The analysis of the exon 3 region of the P16 gene was researched. At first, the discovery of multiple bands on the chip image was thought to be primer dimer or an annealing stringency problem. After comparing normal and mutated samples from DNA sequencing data to the Agilent data, the correspondence was perfect. The PCR product at size 198 bp was the main band. Only mutated samples showed these extra bands. The mutated samples were found to be heterozygous at either position 316 or 356. The CG mutation at position 316 produced one extra band. However, when a sample displayed the CG mutation at position 316 and a CT mutation at position 356, two extra bands could be seen. The extra bands detected here are due to the slower mobility of the heteroduplex formed by heterozygote mutant of the samples. The detection on the Agilent 2100 bioanalyzer are

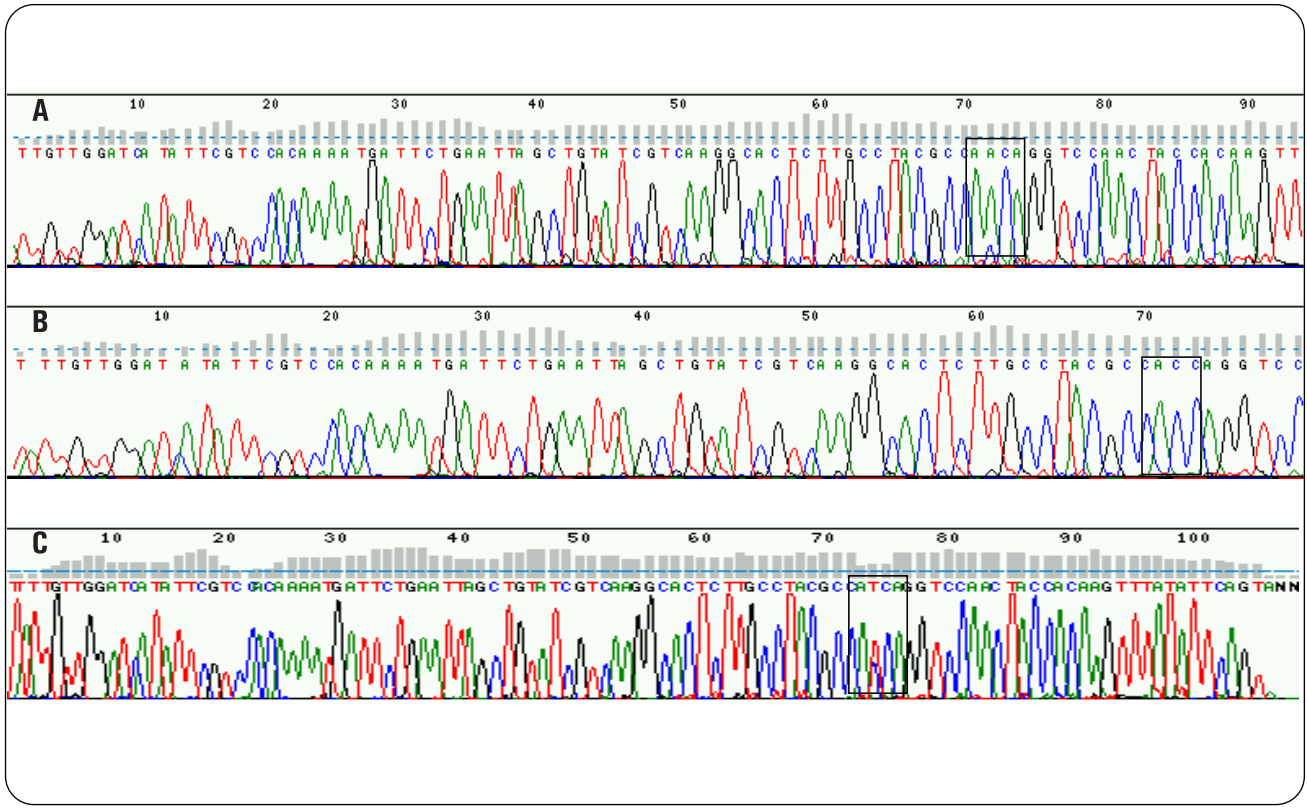

Figure 2
DNA sequence chromatograms from K-*ras* codon 12 PCR's.
A) AAC reverse sequence is GTT forward sequence=valine mutant at codon 12.
B) ACC reverse sequence is GGT forward sequence=glycine normal at codon 12.
C) ATC/ACC reverse sequence is GAT/GGT forward sequence= aspartic acid mutant and glycine normal at codon 12.

completely matched with our genotyping results using ABI's 3700 sequencer. Figure 3 shows hetero-zygous mutations and double homozygous mutations. Figure 4 shows the DNA sequence chromatogram data which corresponds to the Agilent data generated. This size range resolution could not be visualized on a slab gel.

Sample	1	2	3	4	5	6	7	8	9	10	11
Genotype316	C	C	CG	CG	CG	CG	CG	C	C	C	G
Genotype356	C	C	CT	CT	CT	C	C	CT	CT	T	C
Agilent Gel											
Main Band	198	198	197	198	198	198	198	198	199	198	195
Extra Band 1			204	204	204						
Extra Band 2			216	215	215	214	214				

Figure 3
Chip image of P16 gene exon 3 PCRs displaying the presence of extra bands in mutated samples.

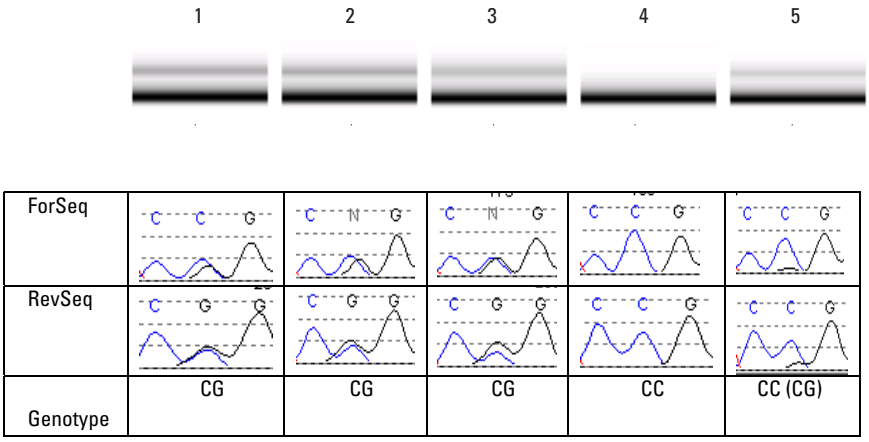

Figure 4
DNA sequence chromatogram data corresponding to Agilent chip data. Heterozygous mutations display an extra upper band.

Conclusion

The data obtained from our studies show how the Agilent 2100 bioanalyzer can distinguish between mutated and normal DNA samples in particular genes. This is extremely important in making a genetic diagnosis in cancer patients. This makes for a rapid and accurate screening assay, which can be employed by any molecular biology laboratory. These findings suggest that the Agilent 2100 bioanalyzer could be used for some SNP detection assays.

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References

1.
Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ and Der CJ., *Oncogene*, *17*, *1395-1413*, **1998**.

2.
Bos JL, *Cancer Res.*, *49*, *4682-4689.*, **1989**.

3.
Khleif SN, Abrams SI, et. al., *Journal of Immunotherapy*, *22*, *155-165*, **1999**.

4.
Pollack PM, et. al., *Genes, Chromosomes & Cancer*, *32*, *89-94*, **2001**.

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