

# Rapid Multiplex Real-time PCR by Molecular Beacons for Different BRAF Allele Detection in Papillary Thyroid Carcinoma

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**Abstract:** BRAF is an oncogene that is commonly mutated in both melanomas and papillary thyroid carcinomas (PTCs). Usually, mutations in the codons 600 or 601 lead to constitutive activity in the Ras-mitogen-activated protein kinase pathway and, recently, the BRAF<sup>VK600-1E</sup> deletion was described as a relevant risk factor for loco-regional PTC lymph node metastasis. For these reasons, BRAF mutations may be considered a key genetic factor for the metastatic progression of PTC and also for other tumors such as melanoma and colon cancer and a new BRAF-specific therapeutic strategy was already suggested. In this report we describe the development of a rapid qualitative fluorescent real-time polymerase chain reaction assay designed for the detection of BRAF<sup>VK600-1E</sup> deletion using 2 specific molecular beacons. The assay is able to detect in a single tube the homozygous as well the heterozygous genotypes. The procedure combines the great sensitivity of the polymerase chain reaction, the specificity provided by allele-specific molecular beacons, and the throughput of a multicolor fluorescence detection procedure. This technique, together with an earlier described real-time test specific for V600E and K601E will be useful for research and molecular diagnostic laboratories involved in the study of BRAF-related neoplasia.

**Key Words:** BRAF, thyroid carcinoma, real-time PCR, molecular beacon

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**B**RAF, the gene for the B-type RAF kinase, is a member of the MAP kinase pathway that, when constitutively activated, upregulates cell division and proliferation, leading to the genesis and progression of different human cancers. In particular, mutations of the BRAF gene are common events in melanoma, colon carcinogenesis, and thyroid cancer, and represent the most important molecular hallmark in papillary thyroid carcinomas (PTCs), the most common malignant tumor of the thyroid gland.<sup>1,2</sup>

Multiple histologic variants of this neoplasia have been described, and their pathologic behaviors are often linked with specific morphologic features and molecular patterns.<sup>1–3</sup>

Recent data suggest that, in thyroid carcinogenesis, the most frequent BRAF mutation is the T1796A, a valine/glutamic acid substitution of the codon 600 in the exon 15 of the BRAF gene (BRAF<sup>V600E</sup>).<sup>3,4</sup> This mutation occurs most frequently in the classic variant and in the Tall-cell variant of PTC, and correlates with distant metastases and a more advanced clinical stage at presentation.<sup>1,5–7</sup>

Another BRAF point mutation, K601E (BRAF<sup>K601E</sup>), was detected in a minor percentage of cases of the follicular-variant PTC,<sup>5,8</sup> and in rare cases of follicular adenoma.<sup>9,10</sup>

Recently, a rare BRAF mutation, the BRAF<sup>VK600-1E</sup> in-frame deletion, was described in an incidental micro-PTC solid variant,<sup>11</sup> and was also exclusively found in lymph node metastasis of 3 distinct cases of classic PTC, which did not bear this mutation in the primary tumor.<sup>12</sup>

All of these 3 mutations, the BRAF<sup>VK600-1E</sup>, the BRAF<sup>V600E</sup>, and the BRAF<sup>K601E</sup> are localized in the 15th exon of the BRAF gene between the 2 major phosphoregulatory residues (T599 and S602), and are able to incorporate a glutamate in this region that constitutively activates the MAP kinase pathway.<sup>3,13</sup> The same BRAF mutations are frequently found in melanoma and in colon cancer.<sup>14</sup>

There are several methods that can be currently used for the molecular identification of the BRAF mutations: the method usually used is the genomic polymerase chain

reaction (PCR) amplification, followed by single-strand conformation polymorphism and validation of the results by DNA sequencing.<sup>8</sup> Alternative methods such as colorimetric mutation detection, restriction fragment length polymorphism, mutant allele-specific PCR amplification, real-time allele-specific amplification, and, more recently, pyrosequencing have been proposed to detect BRAF mutations.<sup>15–18</sup> These methods are not always able to discriminate with sufficient specificity and sensibility the BRAF<sup>VK600-1Edel</sup>, the BRAF<sup>V600E</sup>, and the BRAF<sup>K601E</sup> mutations. In particular, with the exception of the sequencing and pyrosequencing methods, there are no specific molecular tests for VK600-1E deletion, which recently, in 2 separate studies, was reported to be higher than most other BRAF mutations reported, with the noted exception of V600E.<sup>3,8</sup>

The laboratory protocol described in this study is a fast and specific real-time PCR assay, performed with 2 different molecular beacons<sup>19</sup> (MBs) able to detect in a single tube the presence of the BRAF<sup>VK600-1E</sup> in-frame deletion in selected neoplastic PTC cells. MBs are increasingly being used in many applications involving nucleic acid detection and quantification because they are characterized for high specificity and good selectivity when discrimination among different allelic profiles in 1 multiplex PCR is necessary.<sup>19</sup> In fact, the stem-loop structure of the MBs provides a competing reaction for the probe-target hybridization that serves to increase probe sensitivity and specificity.<sup>20</sup>

Recently, the discovery of a new BRAF-specific therapeutic strategy, used in patients with melanoma, has given more emphasis to this new rapid, sensible, and specific BRAF<sup>VK600-1E</sup> molecular test.<sup>21,22</sup>

**MATERIALS AND METHODS**

**Clinical Samples**

For this study, among 20 PTC clinical cases (15 formalin-fixed samples and 5 fine-needle aspiration biopsies) we have selected 3 tumor samples (from case 1 and case 2) as positive controls, each of which carried, respectively, the BRAF<sup>VK600-1Edel</sup>, the BRAF<sup>V600E</sup> and the BRAF<sup>K601E</sup> mutations. Histologic and molecular details are described in Table 1. In particular, case 1 was characterized by a nodule isolated from the left thyroid lobe and a mediastinal metastasis with typical histologic features of the Tall-cell PTC variant and heterozygosity for BRAF<sup>VK600-1Edel</sup>. Another nodule, isolated from the right lobe and diagnosed as follicular-variant PTC, was characterized by the presence on the BRAF<sup>K601E</sup> mutations in a heterozygous state (Fig. 1).

**Synthetic Oligonucleotide Targets**

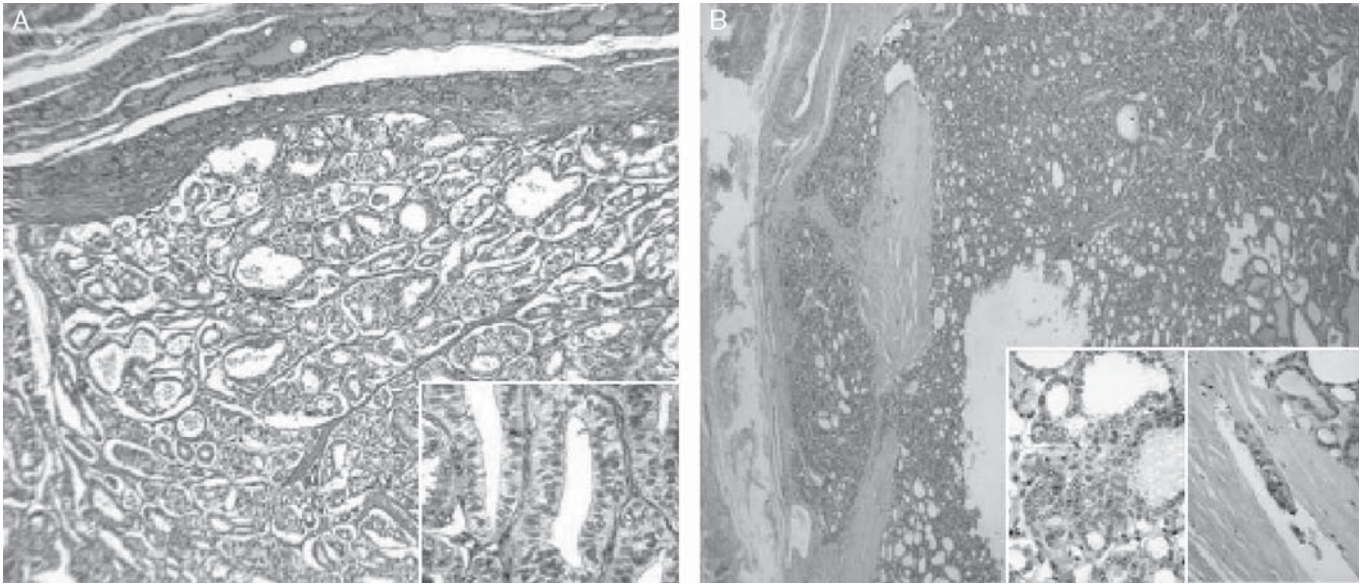
**PCR Amplicons Carried on Plasmids**

PCR amplicons representative of different BRAF allelic profiles (BRAF<sup>VK600-1Edel</sup>, BRAF<sup>V600E</sup>, and BRAF<sup>K601E</sup>) were obtained from DNA isolated from

**TABLE 1. Clinical Cases: Real-time Molecular Beacons Results**

Clinical Cases	Histology	Sequencing Result	Melting Curve		Melting Curve – df/dt
			MB1 (FAM) Signal	MB2 (ROX) Signal	MB1 (FAM) T <sub>m</sub> Peak (°C)
Case 1	Surrounding	WT	+	–	73
Case 1	PTC follicular variant	V601E	+	–	63
Case 1	Tall-cell PTC	VK600-1E	–	+	None
Case 2	Classic PTC	V600E	+	–	63
Case 3	Classic PTC	WT	+	–	73
Case 4	PTC follicular variant	WT	+	–	73
Case 5	PTC with focal insular component	V601E	+	–	63
Case 6	Classic PTC	WT	+	–	73
Case 7	Classic PTC	V600E	+	–	63
Case 8	Classic PTC	V601E	+	–	63
Case 9	PTC columnar cell variant	V601E	+	–	63
Case 10	Nodalmetastasis of PTC (poorly differentiated)	WT	+	–	73
Case 11	PTC multicentric solid variant	WT	+	–	73
Case 12	Classic PTC	V601E	+	–	63
Case 13	Classic PTC	V600E	+	–	63
Case 14	Classic PTC	V600E	+	–	63
Case 15	PTC follicular variant	WT	+	–	73
Case 16	FNAB classic PTC	V600E	+	–	63
Case 17	FNB classic PTC	V600E	+	–	63
Case 18	FNAB: nodule with moderate atypias	WT	+	–	73
Case 19	FNAB: Hurtle cell follicular neoplasia	WT	+	–	73
Case 20	FNB: classic PTC	600E	+	–	63

Real-time molecular beacons results obtained by using DNA extracted by paraffin sections of 15 different papillary thyroid carcinomas (PTCs) (from case 1 to case 15) and from 5 fine-needle aspiration biopsy (FNAB) fixed in 95% alcohol.  
 – df/dt indicates first derivate; MB1 (FAM), molecular beacon 1 normal sequence; MB2 (ROX), molecular beacon 2 mutated sequence; WT, wild type.



**FIGURE 1.** Hematoxylin and eosin histologic sections of case 1. A, Tall-cell PTC in the left lobe ( $BRAF^{VK600-1E \text{ del}}$ ). B, Follicular-variant PTC of the right lobe ( $BRAF^{K601E}$  mutation). PTC indicates papillary thyroid carcinoma.

microdissected neoplastic cells from cases 1 and 2. These amplicons were cloned, obtained by the PCR procedure described successively, into plasmid (pCR 2.1-TOPO Vector) using the Invitrogen TOPO PCR Cloning system following the manufacturer's instructions ([www.invitrogen.com](http://www.invitrogen.com)). To determine the PCR detection limit, plasmids were extracted from *Escherichia coli* with the Invitrogen Quick Plasmid mini-prep Kit, according to the manufacturer's instructions ([www.invitrogen.com](http://www.invitrogen.com)). Plasmid DNA was quantified by a spectrophotometer using 10-fold serial dilutions, and a good real-time fluorescence signal was detected with less than 0.2 ng of DNA plasmid.

### Synthetic DNA Fragments

Complementary single-stranded synthetic oligonucleotides (Fig. 1), representative of the four BRAF allelic variations within the MB probe target region, were used as qualitative standards to perform a melting curve analysis. These DNAs were custom-synthesized by the Eurofins MWG Operon, <http://www.eurofinsdna.com/home.html>. Synthetic targets (T1, T2, T3, T4), were suspended in tubes containing 40 pmol/ $\mu$ L in ultra-pure RNase and DNase-free distilled water, and were stored at  $-20^{\circ}\text{C}$  until use.

### DNA Extraction

Genomic DNA was extracted using commercial kits (Charge Switch DNA Tissue Kits, Invitrogen Life Technologies) from formalin-fixed samples and from cells obtained by fine-needle aspiration biopsy and fixed in absolute alcohol. Neoplastic and non-neoplastic (surrounding) formalin-fixed tissues were microdissected from hematoxylin-stained thyroid tissue.

### PCR Primers and MB Design

The PCR primers and the 2 MBs matching WT and VK 600-1E deletion were used to perform the real-time PCR. These oligos were designed from the BRAF WT sequence available in GenBank accession no. DQ049769.

The theoretical melting temperatures ( $T_m$ s) of the oligos, the formation of oligonucleotide dimers, and self-complementarity were evaluated using Oligo Program version 6 (MedProbe, Oslo, Norway). The MBs' fluorescent probe arm sequences formed a stable stem hybrid at the PCR annealing temperature calculated with the aid of the *mfold* program (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna>).<sup>23,24</sup> The PCR primers, BRAF 1 and BRAF 2, were designed to flank a region of 97 bp of the BRAF gene sequence from positions 1613 to 1688 (Fig. 1). Of the many MB probes examined, the 2 named MB1 WT-FAM (MB1) and MB2 VK600/1-Mut-ROX (MB2) showed the most thermodynamically stable target-loop sequences.<sup>25</sup> MB1 WT-FAM and MB2 VK600/1-Mut-ROX sequences are perfectly complementary, respectively, to a wild-type profile and to VK 600-1E deletion (Fig. 1). The  $T_m$  folding conditions were  $\text{Na}^+$  (1 mol/L) and  $\text{Mg}^{2+}$  (2.75 mol/L) at the hybridization temperature of  $50^{\circ}\text{C}$ . Fluorochromes FAM and ROX were used at position 5' to label MB1 WT-FAM and VK600/1-Mut-ROX, respectively, whereas DABCYL was used as a quencher at position 3'.

### BRAF Profile Detection by Conventional Sequencing

The BRAF gene exon 15 was PCR-amplified with 2 specific primers described earlier<sup>16</sup> and the presence of

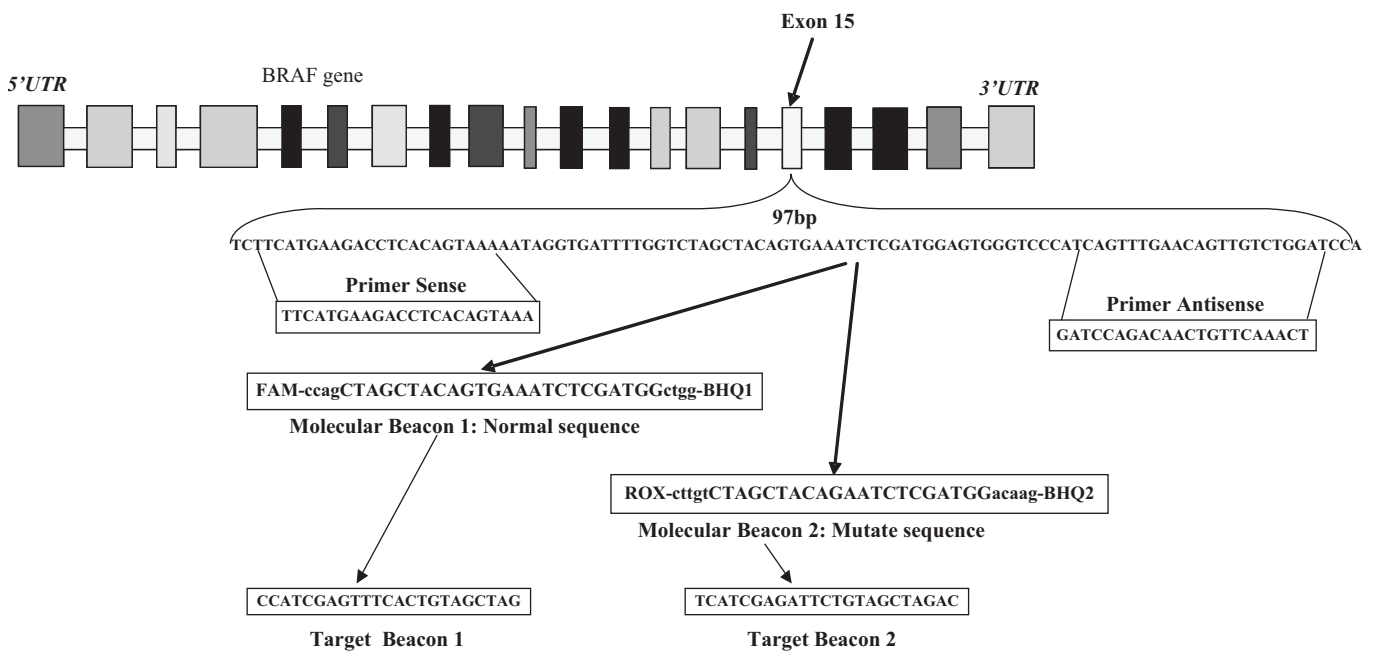
possible mutations was then tested by PCR direct sequencing using a CEQ 8000 genetic analysis system (Beckman Coulter, Inc).

DNA obtained from tall cell variant PTC and the mediastinal metastasis (case 1) showed a sequencing pattern compatible with the BRAF<sup>VK600-1E</sup> deletion in a heterozygous form.

In contrast, the sequence obtained using DNA isolated from the right lobe follicular-variant PTC (case 1) and from the classic PTC variant (case 2) showed heterozygosity, respectively, for the BRAF<sup>K601E</sup> and the mutation BRAF<sup>V600E</sup>. The BRAF 15 exon sequence in the surrounding tissue was normal. Colonies for each of the 3 BRAF mutations were selected by PCR direct sequencing.

### MBs Melting Curve Analysis

To determine the capacity of the 2 MBs to detect different allelic sequences, their thermal denaturation profiles were studied. The resulting melting curves proved reliable, ensuring correct real-time RT-PCR conditions such as maximum specificity of annealing temperature ( $T_a$ ). The reaction was performed with puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Buckinghamshire HP7 9NA, UK) using the Mx3000 Real-time PCR system (Stratagene, www.stratagene.com). We used a final volume of a 25  $\mu$ L solution containing 10  $\mu$ M of each MB only, and subsequently, with the addition of (a) their single-stranded complementary synthetic oligonucleotide targets WT or VK 600-1E (T1 and T2) separately, (ii) the most frequent BRAF

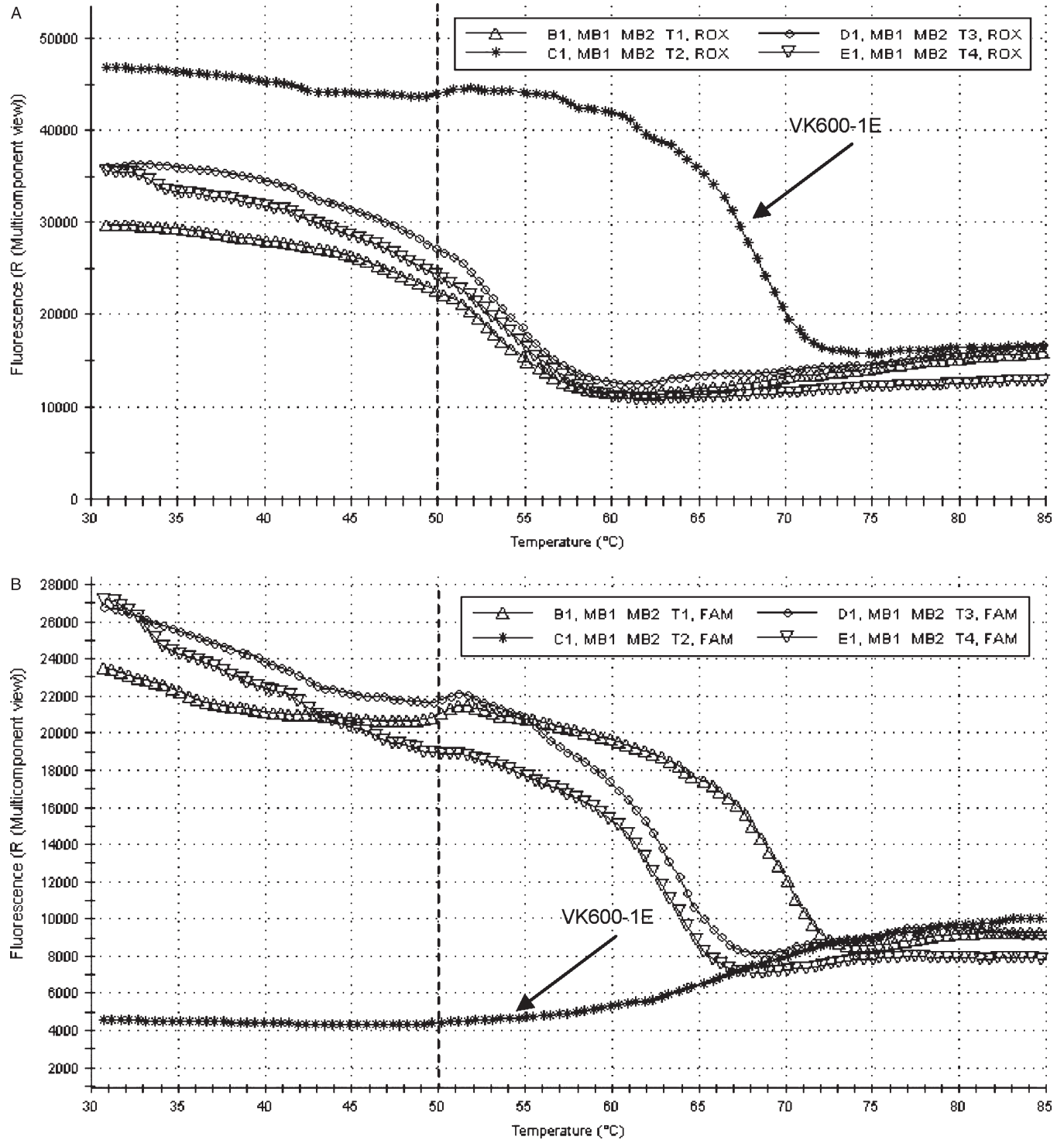


	Sequence 5'-----3'
<b>Primers PCR</b>	
BRAF1 Sense	TTCATGAAGACCTCACAGTAAA
BRAF2 Antisense	GATCCAGACAACCTGTCAAAC
<b>Targets</b>	
T1-WT	CCATCGAGATTTCACTGTAGCTAG
T2-VK600-1E	TCCATCGAGATTCTGTAGCTAGAC
T3-V600E	CCATCGAGATTTCTGTAGCTAG
T4-K601E	CCATCGAGATTTCACTGTAGCTAG
<b>Molecular Beacons</b>	
B1 WT-FAM	FAM-ccagCTAGCTACAGTAAAATCTCGATGGctgg-BHQ1
B2VK600-Mut-ROX	ROX-cttgtCTAGCTACAGAATCTCGATGGacaag-BHQ2

**FIGURE 2.** Schematic representation of BRAF gene fragment containing the mutations analyzed in this study. The table below showed the sequence of polymerase chain reaction (PCR) primer, synthetic targets and the fluorescent probes used in this study.

mutations V600E and K601E (T3 and T4), also at concentrations of 10 μM per reaction. Fluorescence changes during the Mx3000 melting curve protocol (from 45°C to 95°C) were measured using a linear

procedure for WT and VK 600-1E mutations. (Fig. 3); on the contrary, a first derivate procedure was necessary to discriminate the V600E and K601E alleles (Fig. 4).



**FIGURE 3.** Melting curves analysis of molecular beacon fluorescent probes, MB2-ROX (A) and MB1-FAM (B), using 4 synthetic alleles T1, T2, T3, T4 in the targeted region of the BRAF gene.

### Real-time PCR Conditions

To evaluate the applicability of the method described here as a diagnostic tool, plasmids carried fragments representative of different BRAF allelic profiles and the clinical samples were processed with real-time PCR using the two MBs. This procedure was performed by using the Mx3000 instrument (www.stratagene.com) according to the manufacturer's instructions.

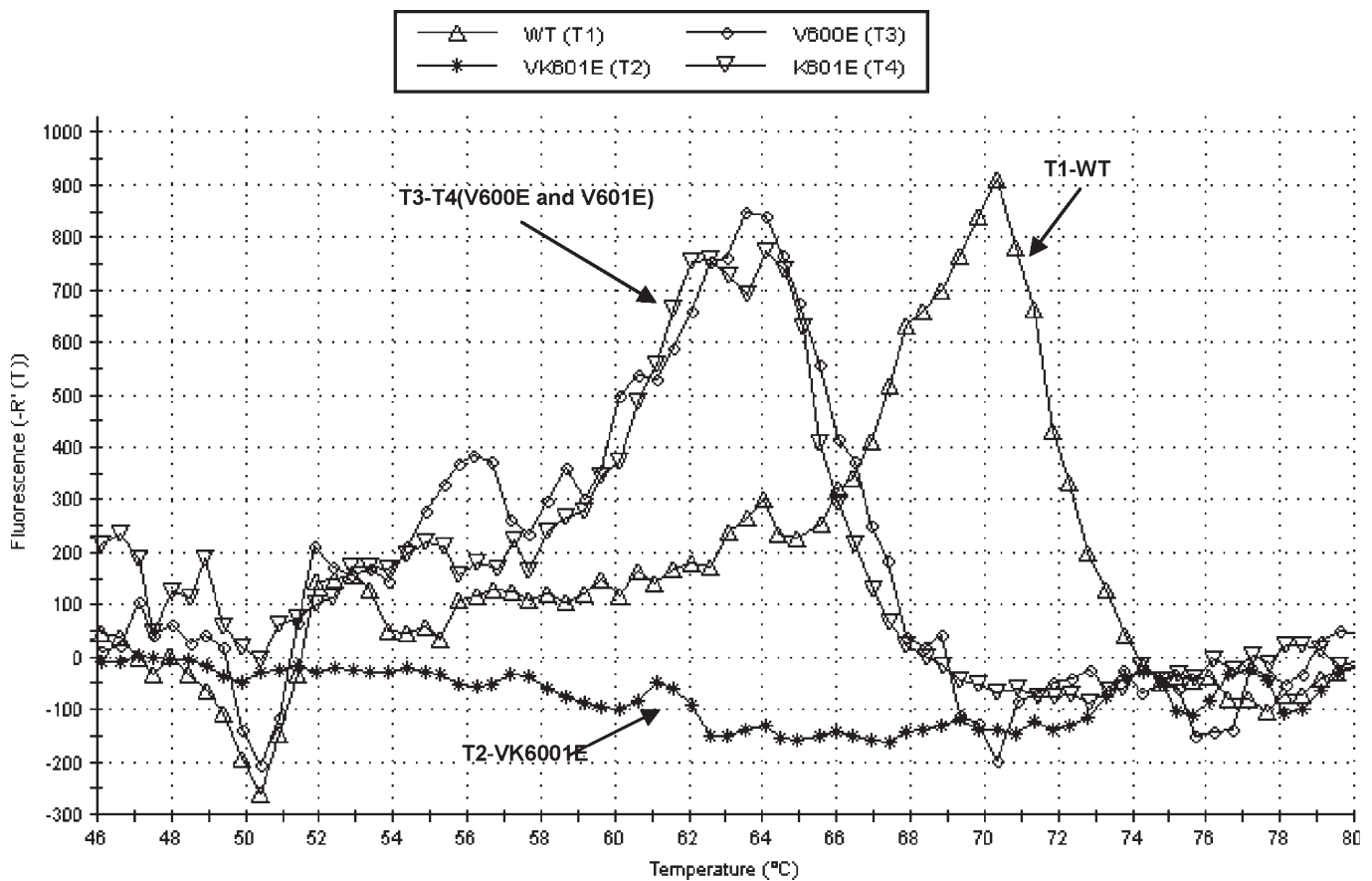
The real-time RT-PCR was performed in puReTaq Ready-To-Go PCR Beads (Amersham Biosciences). The reaction was conducted in a final volume of 25 µL containing 2.75 mM of MgCl<sub>2</sub>, 2.5 µM of each primer, 10 µM of each MB probe and 1 µL of genomic DNA. The PCR program ran as follows: (i) denaturation at 95°C for 3 minutes and (ii) 40 cycles each of 1 minute at 95°C, 1 minute at 50°C, and 1 minute at 72°C.

Fluorescent measurements were recorded during each annealing step and during melting curve with 2 different filter sets (excitation/emission: FAM 492/516 and ROX 595/610).

### RESULTS

#### Melting Curve Analysis

The synthetic oligonucleotides, T1, T2, T3, and T4, represent the four allelic variations of the target sequence corresponding to the MBs' hybridization site (Fig. 2), and were used in a melting curve analysis. This protocol was performed to confirm that, despite the presence of these single nucleotide polymorphisms, the multiplex real-time PCR could be used to detect all allelic forms (Figs. 3, 4). The traditional melting curve analyses revealed that the best hybridization reaction temperature range lay between 45°C and 50°C (Fig. 3). Thus, the annealing temperature of 50°C, selected using an empirical approach, gave the highest specificity and sensitivity in the real-time PCR. At this hybridization temperature, the MBs hybridized with the complementary targets (T1 and T2), whereas no significant fluorescence signal was detected when the reaction was performed without targets or with different T3 and T4 allelic combinations. This indicated the absence of unwanted



**FIGURE 4.** First derivative of melting curve obtained with MB1-FAM (WT) in presence of the 4 synthetic oligonucleotides (T1, T2, T3, T4) showing different fluorescent peak. In particular, with this procedure 3 different results are possible: (i) a 72°C melting peak indicating the complete absence of mutations (WT), (ii) a 63°C melting peak indicating the single nucleotide polymorphisms variations V600E or V601E, and (iii) the absence of a melting peak indicating the VK6001E deletion. WT indicates wild type.

nonspecific MB hybrids and the possibility of revealing WT and VK600IE by the fluorescence curve (Fig. 3). In addition, the first derivative melting curves showed different profiles in the presence of the 4 alleles considered. In fact, with this procedure, it is possible to obtain not only the presence of WT/VK600IE, by fluorescence curve analysis, but also, by using the derivative melting curve, the presence/absence of V600E to V600IE (Fig. 4).

### Clinical Samples Results

As shown in Table 1, a complete laboratory diagnosis is possible using the melting curves analysis (normal and derivative). In particular, the allelic group, V600E/V600IE MB1, peak showed a 10°C  $T_m$  difference from the wild-type peak profile, whereas the deletion VK600-1E, specifically detected by the MB2 (Fig. 3A), was also confirmed with total signal fluorescence absence in the MB1 melting curve (Fig. 4).

All samples used (synthetic targets, plasmids, and clinical samples) showed comparable results (Table 1).

### DISCUSSION

BRAF mutation represents the most common oncogenic event in sporadic PTC and also in malignant melanomas and, in a smaller proportion, also in other types of human cancer.<sup>26</sup> Almost all of the mutations found in the BRAF gene are localized in the 600 to 601 codons and now they are so well characterized that drugs are already being designed to block the action of the defective protein. Therefore, an accurate diagnosis of the altered alleles is becoming critical for a possible preventive treatment.

The PCR has become an essential tool in research and diagnostic laboratories and simpler, faster, and more automated genotyping methods are needed for routine use and population studies, especially when, as for BRAF mutations, only a small number of hot spots are focused.<sup>27</sup> In particular, real-time PCR is now considered the PCR method with the best improved rapidity, sensitivity, and reproducibility, and a reduced risk of carry-over contamination. Most of the popular real-time PCR chemistries depend on the hybridization of a labeled oligoprobe, and, among different types of these probes, MBs are increasingly being used in many applications involving nucleic acid mutation analysis. The stem-loop structure of MBs in fact provides a competing reaction for probe-target hybridization that serves to increase probe sensitivity and specificity.<sup>28,29</sup> There are several real-time PCR tests available for Braf genotyping, but all of these methods are able to detect only the most frequent BRAF<sup>V600E</sup> mutation.<sup>17,30</sup>

Here we report the development of a rapid qualitative fluorescent real-time PCR assay, which, using 2 specific MBs, was designed to witness another BRAF mutation, VK600-1E deletion. The homozygous and heterozygous presence of this deletion may be detected

in real time in the same closed tube at multiple excitation and emission wavelengths. To fully realize the potential of MBs, we have optimized their structure and the binding specificity of a MB target duplex, using melting curves to establish the difference in the fraction of a MB bound to the wild-type target, to colonies carrying different BRAF mutations, and to mutant targets. Optimization of the annealing/hybridization temperature, buffer composition and genomic DNA, primers, and probe concentration were also carefully tested. Clinical data and a comparative sequencing analysis of the samples used in this study showed that this assay is specific, easy to interpret, rapid, and reproducible. Therefore, this real-time PCR assay for BRAF genotyping, together with that already described, may contribute to the detection of a more complete analysis of the BRAF gene in research and molecular diagnostics laboratories involved in the study of BRAF-related neoplasia.

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