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Optimized Allele-Specific Real-Time PCR Assays for the Detection of Common Mutations in *KRAS* and *BRAF*

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Mutations in the oncogenes *KRAS* and *BRAF* have been identified as prognostic factors in patients with colorectal diseases and as predictors of negative outcome in epidermal growth factor receptor–targeted therapies. Therefore, accurate mutation detection in both genes, *KRAS* and *BRAF*, is of increasing clinical relevance. We aimed at optimizing allele-specific real-time PCR assays for the detection of common mutations in *KRAS* and the *BRAF* Val600Glu mutation using allele-specific PCR primers for allelic discrimination and probes (TaqMan) for quantification. Each reaction mix contains a co-amplified internal control to exclude false-negative results.

Allele-specific real-time PCR assays were evaluated on plasmid model systems providing a mutation detection limit of 10 copies of mutant DNA in proportions as low as 1% of the total DNA. Furthermore, we analyzed 125 DNA samples prepared from archived, formalin-fixed, paraffin-embedded colorectal carcinomas and compared results with those obtained from direct-sequence analysis. All mutations determined by sequence analysis could be recovered by allele-specific PCR assays. In addition, allele-specific PCR assays clearly identified three additional samples affected by a mutation. We propose these allele-specific real-time PCR assays as a low-cost and fast diagnostic tool for accurate detection of *KRAS* and *BRAF* mutations that can be applied to clinical samples. ***(J Mol Diagn 2011, 13: 23–28; DOI: 10.1016/j.jmoldx.2010.11.007)***

Activating mutations in the genes encoding *KRAS* (Kirsten rat sarcoma viral oncogene homolog) and *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) are early events in colorectal cancer development. *KRAS* mutations lead to constitutive activation of the RAS/RAF/MAPK/ERK pathway and have been reported to occur in approximately 30% to 40% of colorectal cancer cases.[1,2] Genetic and biochemical evidence indicates that *BRAF* is the principal downstream effector of *KRAS*.[3] Activating mutations in *KRAS* and *BRAF* may be independent risk factors for reduced overall survival in patients with colorectal cancer.[1,4–6] Moreover, the association of *KRAS* mutations and resistance to anti–epidermal growth factor receptor treatment, either cetuximab or panitumumab, was confirmed in large retrospectively evaluated phase III studies.[7,8] Also, a *BRAF* Val600Glu mutation has been associated with resistance to monoclonal antibodies targeting epidermal growth factor receptor.[9,10] Therefore, mutation detection in both genes, *KRAS* and *BRAF*, is of increasing clinical relevance for identifying patient subgroups at high risk and individualizing therapeutic strategies.

Several methods have been described for the detection of common mutations in *KRAS* and *BRAF*, including Sanger sequencing,[6,11] pyrosequencing,[12,13] high-resolution melting analysis,[14,15] and allele-specific PCR.[16] The latter has the advantage of mutant enrichment, resulting in high sensitivity, which is essential for mutation detection in samples with a low tumor cell percentage. Allele-specific PCR, also known as an amplification-refractory mutation system, is based on the principle that extension is efficient when the 3′ terminal base of a primer matches its target, whereas extension is inefficient or nonexistent when the terminal base is mismatched.[17] Combining allele-specific PCR with real-time quantitative PCR techniques allows monitoring template amplification, consequently improving interpretation of PCR results. Real-time PCR protocols for *KRAS* and *BRAF* genotyping have been published, but these protocols showed

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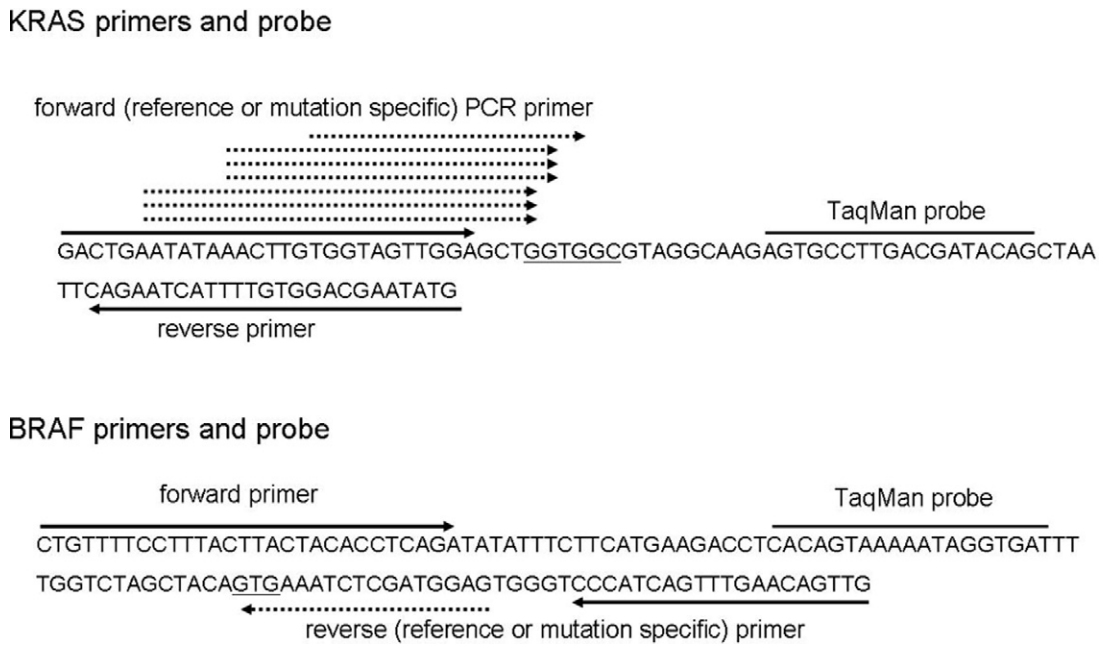

Figure 1. Primer and probes used for *KRAS* and *BRAF* real-time PCR. The figure illustrates positions of primer and probes used for *KRAS* and *BRAF* real-time PCR. Reference and mutation-specific PCRs share the same probe and the opposite PCR primer. Solid arrows display mutation-unspecific primers; and dotted arrows, mutation-specific primers. Codons affected by the mutation are underlined.

heterogeneous amplification detection techniques[18,19] and lacked an internal control reaction.

Therefore, we aimed at establishing allele-specific real-time PCR for the detection of seven common mutations in codons 12 and 13 of the *KRAS* gene (Gly12Ala, Gly12Asp, Gly12Arg, Gly12Cys, Gly12Ser, Gly12Val, and Gly13Asp) and the *BRAF* Val600Glu mutation. The protocol described herein is standardized *KRAS* and *BRAF* allele-specific real-time PCR using probes (TaqMan) for amplification detection and a commercially available PCR master mix. Furthermore, our PCR assays contain an internal control reaction. The sensitivity, selectivity, and specificity of PCR assays were to be evaluated on plasmid model systems. We validated the use of the real-time assays for mutation detection on archived formalin-fixed paraffin-embedded samples of colorectal carcinomas.

Materials and Methods

Primers and Probes

PCR primers for *KRAS* (accession No. NG_007524) and *BRAF* (accession No. NG_007873) were designed against each mutation, and a mutation-unspecific region was used as a reference amplicon. The 3′ terminal base of each allele-specific primer was adapted according to its corresponding mutation. In addition, an artificial mismatch at the penultimate or antepenultimate base was included in the allele-specific primers to improve specificity. Target amplification was detected by probes (TaqMan). Reference and allele-specific PCRs shared the same probe and opposite PCR primer, as illustrated in Figure 1. All unlabeled primers were synthesized by Microsynth, Balgach, Switzerland; and probes (TaqMan) were purchased from Applied Biosystems, Foster City, CA. Probes (TaqMan) for *KRAS* or *BRAF* PCR quantification were labeled with 6-fluorescein at the 5′ end, and a minor grove-binding domain was found at the 3′ end. An exogenous internal control PCR product, a 98-base-long fragment in the *CYP17* promoter region (accession No. NG_007955), was coamplified in each reference and allele-specific PCR. A probe (TaqMan) for internal control PCR detection was labeled with VIC-fluorophore at the 5′ end and a minor grove-binding domain at the 3′ end. All primer and probe sequences are listed in Table 1.

Real-Time PCR

Reference PCR was performed in a 25-μl reaction volume with 1 × TaqMan Genotyping Master Mix (Applied Biosystems), 900 nmol/L of each *KRAS*/*BRAF* mutation-unspecific primer, 100 nmol/L of the *KRAS*/*BRAF* probe, 112.5 nmol/L of each internal control primer, 25 nmol/L of internal control probe, and 5 μl of DNA of varying concentration. Allele-specific PCRs were performed according to the same protocol but using a concentration of 450 nmol/L of allele-specific primer. According to the number of mutations to be detected, eight PCR assays were necessary for *KRAS* genotyping (one reference and seven allele-specific assays) and two for *BRAF* genotyping (one reference and one allele-specific assay). All real-time PCRs were performed on a system (LightCycler 480 Real-Time PCR System; Roche Diagnostics, Vienna, Austria) under the following thermocycling conditions: 95°C for 10 minutes, followed by 50 cycles of 90°C for 15 seconds and 60°C for 1 minute. Cycle threshold (Ct) values were recorded for reference PCR and for each allele-specific PCR, and corresponding ΔCt values (ie,

Table 1. Primer and Probe Sequences*

Target	Primer	Sequence
KRAS	12Ser	5′-AATATAAACTTGTGGTAGTTGGAGCgA-3′
	12Arg	5′-AATATAAACTTGTGGTAGTTGGAGCTC-3′
	12Cys	5′-AATATAAACTTGTGGTAGTTGGAGCcT-3′
	12Asp	5′-AAACTTGTGGTAGTTGGAGCgGA-3′
	12Ala	5′-AACTTGTGGTAGTTGGAGCTtC-3′
	12Val	5′-AAACTTGTGGTAGTTGGAGCaGT-3′
	13Asp	5′-GTGGTAGTTGGAGCTGGaGA-3′
	Reference primer	5′-GACTGAATATAAACTTGTGGTAGTTGGA-3′
	Reverse primer	5′-CATATTCGTCCACAAAATGATTCTG-3′
	Probe	5′-FAM-CTGTATCGTCAAGGCACT-MGB-3′
BRAF	600Glu	5′-CCCACTCCATCGAGATTTCT-3′
	Reference primer	5′-CAACTGTTCAAACTGATGGG-3′
	Forward primer	5′-CTGTTTTCCTTTACTTACTACACCTCAGAT-3′
	Probe	5′-FAM-CACAGTAAAAATAGGTGAT-MGB-3′
Internal control	Forward primer	5′-CCCTAGAGTTGCCACAGC-3′
	Reverse primer	5′-GGTAAGCAGCAAGAGAGC-3′
	Probe	5′-VIC-CTGTCTATCTTGCCTGCC-MGB-3′

FAM, 6-fluorescein; MGB, minor grove-binding domain.
*Probes for *KRAS* and *BRAF* were labeled with FAM at the 5′ end and an MGB at the 3′ end. A probe (TaqMan) for internal control PCR detection was labeled with VIC-fluorophore at the 5′ end and an MGB at the 3′ end. Artificial mismatches are indicated as lowercases.

allele-specific Ct minus reference Ct) were calculated. If both the internal control reaction and the target gene reaction failed, data had to be discarded. However, if the internal control reaction failed but the target gene reaction worked, the positive mutation result was accepted because the target gene reaction had probably outcompeted the internal control reaction.

Plasmid Standards

PCR products containing wild-type and variant *KRAS* and *BRAF* sequences were generated from genomic DNA standards with known mutation status, previously determined by sequencing analysis on an analyzer (ABI 3130 DNA Analyzer; Applied Biosystems). Primers for *KRAS* target sequence amplification were previously described.[6] Primers for *BRAF* PCR were the same as used for reference *BRAF* PCR (Table 1). PCR products were cloned into a pCR 2.1 vector using a TA Cloning Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Plasmids were extracted using a kit (QuickLyse Miniprep Kit; Qiagen, Hilden, Germany) and quantified spectrophotometrically. Sequencing was performed to validate recombinant plasmids. Plasmids were linearized with the HindIII restriction enzyme (New England Biolabs, Ipswich, MA) for 3 hours at 37°C. The copy number was estimated according to the molecular weight, amount, and length of plasmids. Various concentrations between 100 and 4000 copies/μl were prepared by diluting linearized plasmids in a solution of Tris, 10 mmol/L, and EDTA, 1 mmol/L (pH 8) containing 20- ng/μl *Escherichia coli* 16S and 23S ribosomal RNA (Roche Diagnostics). Concentrations were verified by absolute quantitative real-time PCR using standard curves generated from human genomic DNA of known concentrations on a system (LightCycler 480 Real-Time PCR System).

Clinical Samples

Formalin-fixed paraffin-embedded tissue blocks from patients with histologically proven colorectal cancer were obtained from the Department of Pathology, Academic

Table 2. Sensitivity and Selectivity of *KRAS* and *BRAF* Assays

Assay	Quantity of MUT and WT copies (% MUT copies)/reaction*			Predetermined cutoff ΔCt
	100 MUT + 900 WT (10%)	25 MUT + 975 WT (2.5%)	10 MUT + 990 WT (1%)	
KRAS				
12Ser	5.1 ± 0.46	6.3 ± 0.36	7.7 ± 0.62	10
12Arg	2.0 ± 0.20	3.7 ± 0.30	4.3 ± 0.26	9
12Cys	3.3 ± 0.32	5.7 ± 0.87	6.4 ± 0.74	9
12Asp	3.5 ± 0.19	5.5 ± 0.21	6.2 ± 0.50	9
12Ala	3.3 ± 0.35	5.0 ± 0.42	6.4 ± 0.80	9
12Val	3.3 ± 0.32	5.3 ± 0.84	6.8 ± 0.57	10
13Asp	2.9 ± 0.14	4.7 ± 0.61	5.5 ± 0.49	9
BRAF				
600Glu	3.6 ± 0.11	5.7 ± 0.28	7.1 ± 0.40	9

MUT, mutant; WT, wild-type; Ct, cycle threshold.
*Data are given as mean ± SD ΔCt values of different admixtures of MUT and WT plasmids. Based on a total DNA amount of 1000 copies per reaction proportion of MUT, plasmid DNA was gradually reduced to obtain decreasing ratios of MUT to WT DNA.

Teaching Hospital Feldkirch, Austria, as previously described.[6] Sections were cut from regions of the tumor with the most tumor cells (typically ≥50%). Genomic DNA was extracted using a kit (peqGOLD Tissue DNA Mini Kit; PEQLAB, Erlangen, Germany), according to the manufacturer's instructions. The mutation status of *KRAS* and *BRAF* was determined by sequencing analysis.

Results

The sensitivity, selectivity, and specificity of allele-specific real-time PCR assays were evaluated on plasmid model systems. To determine the specificity of the mutation-specific assays, we ran reactions with up to 20,000 copy numbers of wild-type plasmid to force extension of the mismatched primer and to assess a breakthrough signal for each assay. Reactions were run in triplicate and performed three times on separate occasions. Ct values were recorded for reference PCR and for each allele-specific PCR, and corresponding ΔCt values were calculated. Obtained ΔCt values were used to define a cutoff ΔCt value for each assay. Genotyped samples were declared as positive for carrying a mutation, if they fell under the cutoff ΔCt. The cutoff ΔCt value was defined as 1 Ct less than the lowest obtained ΔCt value. The cutoff ΔCt values of allele-specific PCR assays are provided in Table 2. To further evaluate predefined cutoff ΔCt values, each allele-specific assay was performed with 50 ng of human genomic wild-type DNA three times on separate occasions. The predefined cutoff ΔCt values were not reached in either case by using genomic wild-type DNA (data not shown).

To evaluate the sensitivity and selectivity of the *KRAS* and *BRAF* assays, DNA from mutant plasmids was diluted into wild-type plasmids. Based on a total DNA amount of 1000 copies per reaction, the proportion of mutant plasmid DNA was gradually reduced to obtain decreasing ratios of mutant to wild-type DNA. Reactions were run in duplicate and performed two times on separate occasions. Mean ± SD ΔCt values are shown in Table 2; representative amplification curves from each real-time PCR assay are illustrated in Figure 2.

Considering previously defined cutoff ΔCt mutation-specific assays, we were able to detect 10 copies of mutant DNA in the presence of 990 copies of wild-type DNA, corresponding to a detection limit of one-percent mutant against a background of wild-type DNA.

By using *KRAS* or *BRAF* plasmid DNA as a template in PCR, amplification of the internal control reaction does not occur. Therefore, to evaluate the effects of the internal control PCR on the sensitivity or selectivity of our allele-specific assays, we diluted 10 copies of mutant plasmid DNA in 990 copies of genomic wild-type DNA (corresponding to approximately 3.4 ng) to allow coamplification of the internal control reaction. Reactions were run in triplicate, and mean ± SD ΔCt values are given in the Supplemental Table S1 (*http://jmd.amjpathol.org*). Amplification curves of target-specific PCR and the internal control reaction for *KRAS* 12Ala, 12Val, 12Cys, and *BRAF* Val600Glu assays are illustrated in the Supplemental Figure S1 (*http://jmd.amjpathol.org*). The plateau height of internal control PCR was lower compared with *KRAS*- or *BRAF*-specific PCR, indicating a lower yield of internal control PCR product and minor competition with target PCR. Indeed, determined ΔCt values (see Supplemental Table S1 at *http://jmd.amjpathol.org*) were comparable to those obtained from plasmid dilutions. Therefore, coamplification of the internal control did not affect sensitivity or selectivity of the allele-specific assays.

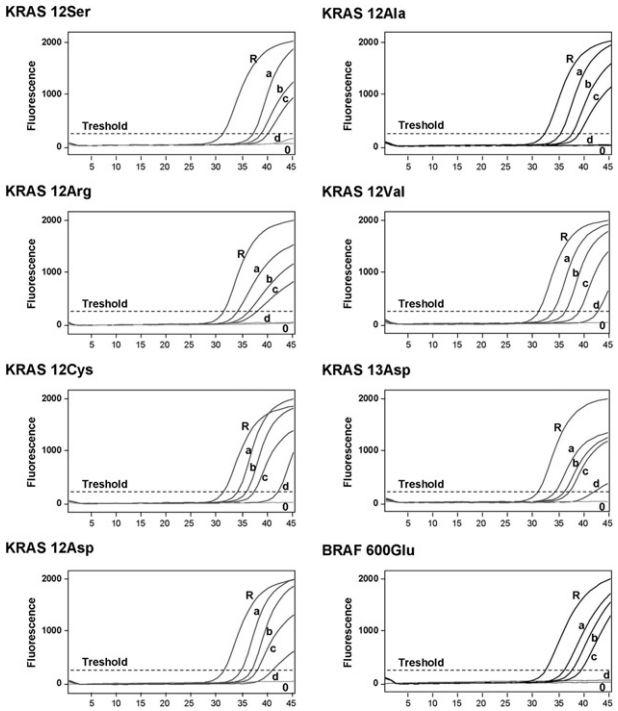

Figure 2. Amplification curves obtained from allele-specific real-time PCR assays. DNA from mutant plasmids was diluted into wild-type plasmids. Based on a total DNA amount of 1000 copies per reaction, the proportion of mutant plasmid DNA was gradually reduced to obtain decreasing ratios of mutant to wild-type DNA. R, reference PCR, representative for all dilutions; a, b, c, and d, 10%, 2.5%, 1%, and 0%, respectively, proportion of mutant DNA; 0, nontemplate control.

By using allele-specific PCR assays, we genotyped 125 DNA samples prepared from formalin-fixed paraffin-embedded colorectal tissues. *KRAS* mutation frequency determined by sequence analysis was 2.4% (n = 3) for Gly12Ser, 0.8% (n = 1) for Gly12Arg, 5.6% (n = 7) for Gly12Cys, 10.4% (n = 13) for Gly12Asp, 1.6% (n = 2) for Gly12Ala, 7.2% (n = 9) for Gly12Val, 7.2% (n = 9) for Gly13Asp, and 11.2% (n = 14) for the *BRAF* Val600Glu mutation. Therefore, every mutation targeted by *KRAS* and *BRAF* allele-specific PCR assays was present in the sample series.

All mutations determined by sequence analysis could also be detected by allele-specific PCR assays. In addition, PCR assays identified three more samples affected by mutations--two samples with the *KRAS* Gly12Val mutation and one sample with the *BRAF* Val600Glu mutation--than sequence analysis. The mean ± SD ΔCt values obtained from three independent runs were as follows: 3.8 ± 0.1, 3.8 ± 1.1, and 5.0 ± 0.9 for the two *KRAS* Gly12Val–positive samples and for the one *BRAF*

Val600Glu–positive sample, respectively; these values were clearly under predefined cutoff ΔCt values (Table 2). On the basis of ΔCt values obtained from the additional positive samples and the ΔCt values received from serial dilutions in the evaluation study (Table 2), a tumor cell proportion of 5% to 10% was estimated in these samples. This indicates that additionally found mutated samples were true positives and not detected because of false extension of the mismatched primer. Furthermore, to verify results, a commercial kit (TheraScreen; Qiagen) was used for *KRAS* genotyping, which confirmed our findings.

Compared with allele-specific real-time PCR, the corresponding sensitivity of sequencing was 95.7% (95% confidence interval, 93.0%-98.6%) and 93.3% (95% confidence interval, 66.0%-99.7%) for *KRAS* and *BRAF* mutation analysis, respectively.

Discussion

We successfully developed highly sensitive allele-specific real-time PCR assays targeting the seven most common mutations in codons 12 and 13 of *KRAS* and the *BRAF* Val600Glu mutation. Other protocols for allele-specific real-time PCR for mutation detection in *KRAS* and *BRAF* have been published.[18,19] However, previously published real-time PCR protocols showed heterogeneous amplification detection techniques (based on molecular beacon[18] and SYBR Green[19] quantification techniques) and lack of internal control reactions. We standardized PCR assays by using probes (TaqMan) and a commercially available PCR master mix simplifying the PCR cocktail and real-time PCR analysis. Furthermore, we integrated an internal control reaction in our PCR assays. Interpretation of allele-specific PCR assays is based on the amplification of a PCR product in case of the presence of the respective mutation. If no amplicon is amplified in the allele-specific reaction, the sample is interpreted as wild-type. However, a lack of amplification can also be due to the presence of PCR inhibitors. Therefore, integration of an internal control reaction is essential to exclude false-negative results. However, coamplification of an internal control reaction may compete with target gene amplification. This may result in a decrease of sensitivity, especially in samples with few tumor cells. To minimize competition of the internal control reaction with the target gene amplification, fewer internal control primers compared with target-specific primers were used in the reaction. Indeed, we could demonstrate that the use of the internal control reaction does not affect sensitivity or selectivity of our assays.

We showed that each of our developed PCR assays allows detection of at least 10 copies of mutant DNA in proportions as low as one-percent of total DNA. Sensitive real-time PCR-based assays for *KRAS* and *BRAF* mutation detection are also commercially available (TheraScreen); these assays combine real-time techniques (allele-specific PCR and Scorpions).[20] On the basis of manufacturer's specifications, the assays can detect less than one-percent of mutant in a background of wild-type genomic DNA and have limits of detection of 5 to 10 copies. Therefore, commercial assays show similar selectivity and sensitivity compared with real-time PCR assays provided herein. However, kit prices are high ($3760 and $2150 for *KRAS* and *BRAF* mutation analysis, respectively, in 25 samples), and primer and probe sequences of these assays have not been published. Depending on batch sizes, expenses of reagents used for real-time PCR assays described herein amount to approximately $20 to $30 per sample for both *KRAS* and *BRAF* genotyping, if no replicates are performed. Therefore, our protocol represents a cost-effective alternative compared with commercially available assays for mutation detection. Furthermore, although difficult to compare, estimated reagent costs are comparable to expanses of other genotyping methods, such as capillary sequencing. However, capillary sequencing is of lower sensitivity and is only recommended in samples with a tumor cell percentage of more than 30%.[11] Therefore, the method of choice is more a question of the proportion of tumor cells in the sample than of assay costs.

In our study, although sections were cut from regions of the tumor with the most tumor cells (typically ≥50%), it could not be excluded that some samples showed an amount of tumor cells less than this value. Indeed, allele-specific PCR assays identified three more samples affected by a mutation than sequence analysis. The estimated proportion of tumor cells in these samples was clearly less than the recommended tumor cell percentage necessary for successful genotyping by sequencing analysis. Therefore, our results are well in line with other studies showing that, on the one hand, allele-specific PCR is more sensitive than sequencing analysis[21] and, on the other hand, results obtained from both methods (ie, mutation-specific PCR assays and sequencing analysis) are highly concordant, if tumor cell percentage in samples is sufficient.[11] However, compared with sequencing analysis, post-PCR processes, which always provide a source of contamination, are eliminated and time to result is much lower by using real-time PCR techniques (usually less than 3 hours versus multiple hours).

A limitation of the proposed *KRAS* and *BRAF* PCR assays (and of all other allele-specific PCR assays) is that mutations located outside targeted codons will not be covered by the assays. Sequencing analysis has the potential to detect mutations other than those targeted by the PCR assays. However, in our study, sequence analysis did not detect any other causal mutation than PCR assays were designed to detect.

Real-time PCR assays have the potential to detect low copy numbers of mutated DNA. Depending on the total amount of applied DNA and tumor cell proportion, stochastic variation at low copy numbers may result in failed allele-specific PCR reactions and interpretation of false-negative samples. Therefore, we recommend performing assays in duplicate or triplicate by using the tests in samples with low DNA levels or few tumor cells. This applies to samples that provide a high Ct value or show a ΔCt value close to the cutoff point. All replicates should provide a positive result to class the sample as mutation positive. Furthermore, we recommend the use of ade-

quate positive and negative controls in each run. Appropriate DNA standards can be obtained from the authors on request.

In summary, we describe real-time PCR assays for the detection of the seven most common mutations in codons 12 and 13 of the *KRAS* gene and the *BRAF* Val600Glu mutation. The reported method is rapid and cost-effective and provides the necessary sensitivity, selectivity, and specificity required for analysis of clinical samples, such as formalin-fixed paraffin-embedded tissues. The described protocol is easily reproducible in each laboratory with real-time PCR technology and may, therefore, stimulate further research and assist the clinician in therapeutic decisions.

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