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ARMS-PCR for detection of *BRAF* V600E hotspot mutation in comparison with Real-Time PCR-based techniques

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***BRAF* mutation testing is one of the best examples how modern genetic testing may help to effectively use targeted therapies in cancer patients. Since many different genetic techniques are employed to assess *BRAF* mutation status with no available comparison of their sensitivity and usefulness for different types of samples, we decided to evaluate our own PCR-based assay employing the amplification refractory mutation system (ARMS-PCR) to detect the most common hotspot mutation c. T1799A (p. V600E) by comparing it with two qPCR based assays: a commercially available test with hybridizing probes (TIB MOLBIOL) and high resolution melting (HRM). Positive results were verified with Sanger sequencing. DNA from two cancer cell lines with known mutation status and from tissue samples from melanoma and gastric cancer was used. ARMS-PCR was the most sensitive method with the level of detection of the mutant allele at 2%. Similar sensitivity was observed for the qPCR-based commercial test employing hybridizing probes; however, this test cannot exclude negative results from poor or low quality samples. Another qPCR-based method, HRM, had lower sensitivity with the detection level of approximately 20%. An additional drawback of HRM methodology was the inability to distinguish between wild type and mutant homozygotes in a straightforward assay, probably due to the character of this particular mutation (T>A). Sanger sequencing had the sensitivity of the detection of mutant allele similar to HRM, approx. 20%. In conclusion, simple ARMS-PCR may be considered the method of choice for rapid, cost-effective screening for BRAF p. V600E mutation.**

Key words: *BRAF* mutation screening, molecular diagnostics

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INTRODUCTION

In recent years, the *BRAF* gene encoding a cytoplasmic serine/threonine kinase, has become a highlight of targeted treatment concept in cancer therapy. Initial discovery of *BRAF* mutations in a variety of cancers (Davies *et al.*, 2002), followed by research on the role of *BRAF* in carcinogenesis have led to the successful introduction of its inhibitor, vemurafenib (formerly PLX4032) (Chapman *et al.*, 2011), marking a significant progress in the treatment of advanced melanoma, and opening new therapeutic possibilities in other cancers (Kopetz S, 2010).

BRAF kinase is one of the major components of the RAS-RAF-MEK-ERK mitogen-activated protein kinase signalling pathway, which has a crucial impact on cell proliferation (Robinson & Cobb, 1997), therefore the discovery of *BRAF* activating mutations quickly led to its recognition as an oncogene (Garnett & Marais, 2004). In contrast to wild-type BRAF, physiologically activated by RAS protein, constitutively active BRAF mutants phosphorylate their downstream targets in a RAS-independent manner, which results in mitogen-independent signalling (Wan *et al.*, 2004). While it is currently accepted that oncogenic *BRAF* is incapable of driving tumorigenesis alone (Michaloglou *et al.*, 2005) and mutations are frequently observed in benign skin lesions (Pollock *et al.*, 2003), tumors harbouring *BRAF* mutation are highly dependent on its effects (Hingorani *et al.*, 2003; Solit *et al.*, 2006). This is reflected in the significant prevalence of *BRAF* variants in many types of cancer. Mutations can be found at high frequencies in melanoma (40%–68% of samples) (Kumar *et al.*, 2003; Davison *et al.*, 2005), papillary thyroid carcinoma (36–69%) (Cohen *et al.*, 2003; Trovisco *et al.*, 2004), nervous system tumors (up to 66%) (Schindler *et al.*, 2011) and they are present virtually in every case of hairy-cell leukemia (Tiacci *et al.*, 2011; Arcaini *et al.*, 2012). Furthermore, *BRAF* mutations are detected at lower frequencies in other cancers, such as multiple myeloma (4%) (Chapman *et al.*, 2011), colorectal (7.2–22% of samples) (Wang *et al.*, 2003; Saridaki *et al.*, 2011) and adrenocortical carcinomas (5.7%) (Kotoula *et al.*, 2009) as well as lung adenocarcinomas (1.6–2%) (Naoki *et al.*, 2002; Schmid *et al.*, 2009). Apart from those cancers, *BRAF* seems to be implicated in development of Langerhans' cell histiocytosis, where its mutations are present in 57% of cases (Badalian-Very *et al.*, 2010), and cardiofaciocutaneous syndrome, where 78% of individuals have germline *BRAF* mutations (Rodriguez-Viciana *et al.*, 2006).

The characteristic feature of *BRAF* variants is their restricted diversity. Generally, exons 11 and 15 are most frequently being affected by various substitutions (Davies *et al.*, 2002), but an overwhelming majority of mutations can be found in codon 600 in exon 15 and one of them, the c. T1799A substitution resulting in valine to glutamic

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Abbreviations: HRM, high resolution melting; ARMS, amplification refractory mutation system; qPCR, Real-Time quantitive PCR; PCR, polymerase chain reaction

acid change (p. V600E), accounts for 80–100% of mutations (Kumar *et al.*, 2003; Houben *et al.*, 2004; Libra *et al.*, 2005; Tiacci *et al.*, 2011).

Ubiquity of *BRAF* mutations, along with their uniformity and availability of BRAF inhibitors implies a promising perspective for targeted therapy. However, *BRAF* mutation status can also serve as a predictive or prognostic factor, as in colorectal cancer. Clinical response of the anti-EGFR treatment in colorectal cancer with anti-EGFR antibodies (e.g. cetuximab, panitumumab) depends on preserved wild-type status of *BRAF* (as well as *KRAS*) (Di Nicolantonio *et al.*, 2008). In this tumor, *BRAF* alterations were also found to correlate with worse survival, making *BRAF* mutation status a strong prognostic factor (Di Nicolantonio *et al.*, 2008; Yokota *et al.*, 2011).

Therefore, there is a growing need to screen for the status of BRAF mutation in different tumors and although there are several different methods available there are only few reports comparing some of the methods (Yancovitz *et al.*, 2007; Ellison *et al.*, 2010). Moreover, there is a need for a cheap and sensitive PCR-based assay to rapidly and reliably check the status of BRAF.

Here we decided to detect hotspot c. T1799A (p. V600E) mutation employing three different PCR-based screening methods, i.e. Amplification Refractory Mutation System PCR (ARMS) (Newton *et al.*, 1989), High Resolution Melting (HRM) (Wittwer *et al.*, 2003) and commercially available, BRAF T1799A (p. V600E) mutation-targeted qPCR with hybridizing probes. Since Sanger sequencing is commonly used to verify results obtained with different methods, we also applied this technique to confirm the results of PCR-based methods and to compare their sensitivity and specificity.

MATERIALS AND METHODS

Cell lines and patient samples. Optimization and comparison of different detection methods were performed using two human cancer cell lines: A375 melanoma cell line harbouring homozygous *BRAF* c. T1799A (p. V600E) mutation, and MIA PaCa-2 pancreatic cancer cell line homozygous for the wild-type *BRAF* allele and harbouring mutually exclusive homozygous *KRAS* c. G34T (p. G12C) mutation (COSMIC Database, Forbes *et al.*, 2011) as well as gastric cancer samples, which were obtained from fresh tumor tissue, collected intraoperatively or gastroscopically with patients' informed consent and approval from relevant Ethics Committee. Additionally, reference melanoma DNA samples with known mutation status were kindly provided by Dr. Ahmad Jalili (Division of Immunology, Medical University of Vienna). Two of them (MM1 and MM2) had wild-type genotypes and two (MM3 and MM4) were heterozygous for BRAF c. T1799A mutation.

Cell lines were cultured in Dulbecco's Modified Eagle's Medium (D6429, Sigma-Aldrich), supplemented with antibiotic-antimycotic solution (A5955, Sigma-Aldrich) and 10% fetal bovine serum at 37°C in an atmosphere enriched with 5% CO_2 at 95% relative humidity.

For comparison of the sensitivity of detection methods DNA from A375 and MIA PaCa-2 cell lines were mixed to create samples containing 50%, 20% and 2% of the A375 DNA. Furthermore, various DNA samples were diluted 10-, 100- and 1000-fold for the same purpose.

All samples are listed in Table 1.

DNA extraction from cancer samples and cell lines was performed using proteinase K and JETFLEX Genomic DNA Purification Kit (GENOMED). After extraction DNA was dissolved in water and quantified spectrophotometrically with NanoDrop 2000 (Thermo Scientific). Typically, DNA concentration varied between 100–500 ng/μl.

ARMS

The ARMS assay (Newton *et al.*, 1989), a simple PCR-based technique, utilizes a phenomenon of amplification arrest caused by non-complementary nucleotide(s) at 3' end of the primer. In this study we used a mutation-specific primer, resulting in formation of an additional product only in the presence of the c. A1799 allele. Three primers were used in a total concentration of 400 nM: one forward primer 5'-GCTTGCTCTGATAGGAAAATGAG-3' and two reverse primers 5'-ACCCACTCCATCGAGATTTCT-3' (mutation specific) and 5'-CTGTGGATCACACCTGCCTTA-3' (control) at the concentrations of 133 nM, 173 nM and 93 nM, respectively. The 25 μl reaction mixture consisted of 1.25U HotStartTaq polymerase (Qiagen), 160 μM dNTPs (VWR), 1× Coral Load PCR Buffer (Qiagen), 1.5 mM $MgCl_2$ and from 100 ng to 500 ng of genomic DNA (the latter concentration for clinical samples). PCR

Table 1. Samples used for *BRAF* c. T1799A (p. V600E) mutation detection method comparison.

Sample type	Sample name	Number of samples studied	*BRAF* c.T1799A mutation status	Mutation status by			
				ARMS	SEQ	HP	HRM
Malignant melanoma cell line	A375	NA	+/+	+	+/+	+	–*
Pancreatic cancer cell line	MIA PaCa-2	NA	–/–	–	–/–	–	–*
A375 and MIA PaCa-2 cell line mix	50% of A375 content	NA	mix 50:50	+	+/–	nt	+*
	20% of A375 content	NA	mix 80:20	+	+/–	nt	+*
	2% of A375 content	NA	mix 98:2	+	–/–	nt	–*
Malignant melanoma	MM1–2	2	–/–	–	–/–	nt	–*
	MM3–4	2	+/–	+	+/–	nt	+*
Gastric cancer	GC4	1	+/–	+	–/–	+	–*
	GC1–3, 5–69	68	–/–	–	–/–	–	–*

Abbreviations and markings: NA, not applicable; SEQ, sequencing; HP, hybridizing probes; +/+, homozygous mutated; +/–, heterozygous; –/– homozygous wild-type; mix, mixed DNA from MIA PaCa-2 and A375 cell lines; +, mutated; –, wild-type; nt, not tested; +*, or –*, sample clustered to heterozygote or homozygote group, respectively.

was carried out for 40 cycles (40 s at 94°C, 40 s at 61°C, 40 s at 72°C), with initial denaturation for 15 min at 95°C and final extension for 5 min at 72°C, using Mastercycler Epigradient (Eppendorf), resulting in formation of one or two amplicons: 623 bp (control) and 134 bp (mutation). Results for all DNA samples were obtained in at least two independent experiments.

DNA Electrophoresis. All PCR products were separated on 1–2% agarose gels (Sigma-Aldrich) stained with ethidium bromide (Fluka Analytical, Sigma Aldrich). Gel images were acquired with Alphaimager Mini (Protein Simple).

DNA Sequencing. DNA sequences were obtained in two PCR reactions, followed by sequencing in both directions on ABI 3730 automatic sequencer with capillary electrophoresis (Applied Biosystems). Two primers (Di Nicolantonio *et al.*, 2008), forward 5'-TGCTTGCTCTGATAGGAAAATG-3' and reverse 5'-AGCATCTCAGGGCCAAAAAT-3', were used for both amplifications.

Preparative PCR was carried out for 35 cycles (40 s at 94°C, 40 s at 58°C, 40 s at 72°C), with initial denaturation for 15 min at 95°C and final extension for 5 min at 72°C, resulting in formation of 228 bp product, using a 25 µl mixture of 1.25U HotStartTaq polymerase, 2 × 200 nM primers, 160 µM dNTPs, 1× PCR Buffer (Qiagen), 1.5 mM $MgCl_2$ and 100–500 ng of genomic DNA.

Subsequently, 10 µl of product was purified with Shrimp Alkaline Phosphatase (1U) and Exonuclease I (10U) (Fermentas) and reamplified with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) for 55 cycles (10 s at 95°C, 15 s at 50°C, 90 s at 60°C) with initial denaturation for 5 min at 95°C and final extension for 5 min at 60°C in a 10 µl mixture of 0.4 µl BigDye 3.1, 3.6 µl Sequencing Buffer, 0.002 nM primer (forward or reverse) and 2 µl product from the previous step.

Finally, products were purified on Centri-Sep CS-901 Columns with Sephadex (Princeton Separations) and processed for sequencing. All DNA sequences were obtained in at least two independent experiments.

Real-time PCR-based methods. Two detection methods, namely qPCR with hybridizing probes and High Resolution Melting (HRM) were carried out in LightCycler™ 480 instrument (Roche). All samples were amplified in a volume of 20 µl in duplicates in 96-well plates. Acquired data was analysed with LightCycler 480 Software (release 1.5.0.39 SP4). Real-time PCR with fluorescent hybridizing probes was performed with application of LightMix™ Kit BRAF V600E (TIB MOLBIOL), according to the manufacturer›s manual. Reaction components and cycling and melting parameters are provided in supplementary Table 1 and 2 (at www.actabp.pl). Sample mutation status was determined using the melting temperature analysis (*Tm calling* mode). For the HRM analysis LightCycler™ 480 High Resolution Melting Master Real-Time (Roche) was used in accordance with the manufacturer's manual. This method allows to detect point mutations by measuring the change of amplicon melting behaviour versus controls of known genotype. Since amplicon length has a crucial impact on the melting process, three different pairs of primers flanking the V600 codon were designed: forward 5'-TTCATGAAGACCTCACAGTAAAAA-3' and reverse 5'-CTGATGGGACCCACTCCAT-3' for 77 bp amplicon, forward 5'-TCACAGTAAAAATAGGTGATTTTGG-3' and reverse 5'-CCACAAAATGGATCCAGACA-3' for 95 bp amplicon, forward 5'-GCACAGGGCATGGATTACTT-3' and reverse 5'-GATGACTTCTGGTGCCATCC-3' for 195 bp amplicon. Reaction components as well as cycling and melting parameters are provided in supplementary Table 3 and 4 (at www.actabp.pl). Initial experiments allowed to establish the optimal $MgCl_2$ and DNA concentrations at 3 mM and 30 ng/reaction, respectively. Sample mutation status was determined using *Gene scanning* mode, with fluorescence level and temperature normalizations (normalization and temperature shift options) applied when necessary. Samples were clustered automatically and visual analysis was applied only to corroborate the results.

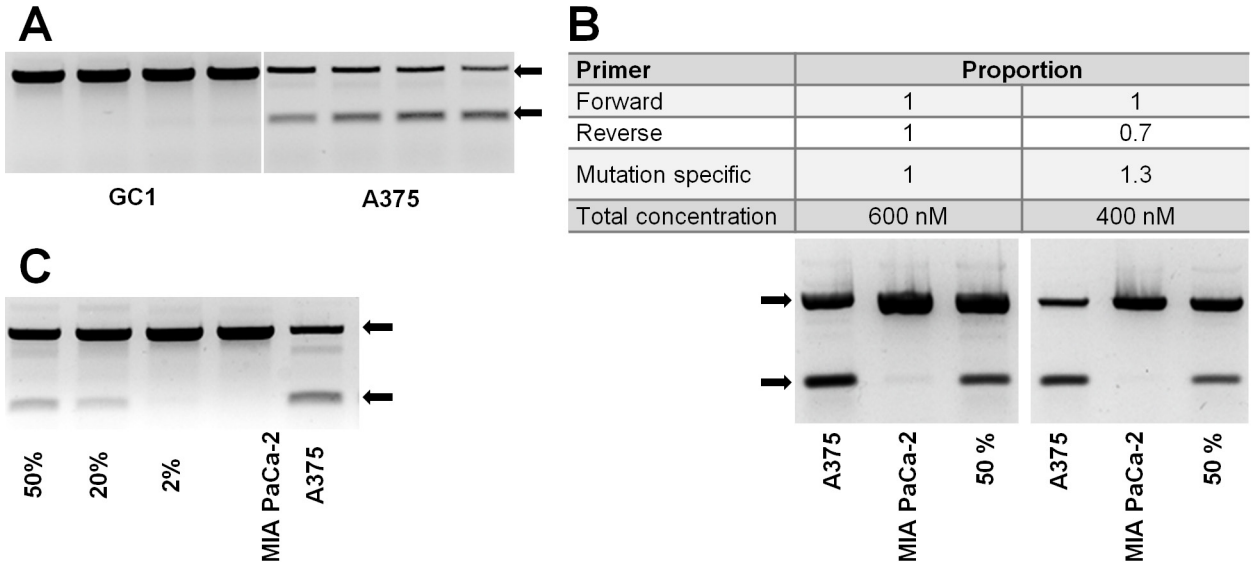

Figure 1. Optimization of the ARMS assay for *BRAF* c. T1799A (p. V600E) detection.
(A) Comparison of product specificity at various annealing temperatures. Four temperatures were tested (63.6°C, 61.4°C, 58.0°C and 56.2°C, from left to right). 61°C was chosen as resulting in best specificity without loss of amplification efficiency. The GC1 sample harbours a wild type BRAF allele and mutually exclusive KRAS mutation. **(B)** Increase in ARMS products' specificity (left to right) after changing proportions of the primers and their total concentration. **(C)** Detection sensitivity of c. A1799 allele after 35 cycles of amplification (no detection at 2% content). Arrows indicate positions of the expected products: control (upper — 623 bp) and mutation specific (lower — 134 bp).

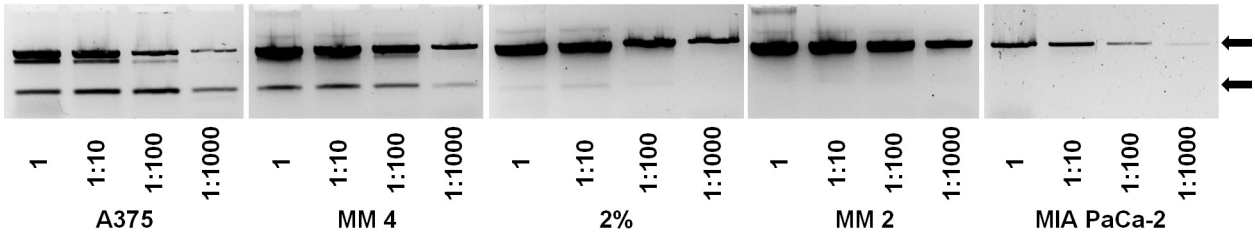

Figure 2. Sensitivity of *BRAF* c. T1799A (p. V600E) detection with optimized ARMS assay.
ARMS detects c. A1799 allele in homozygous A375 cell line and heterozygous melanoma sample even at 1000-fold dilution. Conversely, it does not generate false positive results from homozygous wild-type melanoma and MIA PaCa-2 cell line samples (only control bands are visible). Simultaneously, ARMS discriminates samples containing at least 2% c. A1799 allele from wild-type samples.

Bioinformatic tools. Genomic sequence of *BRAF* was derived from NCBI database, accession number NG_007873.1. Primers were designed using primer3 (Rozen & Skaletsky, 2000), primerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and NetPrimer (http://www.premierbiosoft.com/netprimer/index.html). Collected DNA sequences were analysed with FinchTV 1.4.0 software (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and aligned in Jalview 2.6.1 (Clamp *et al.*, 2004; Waterhouse *et al.*, 2009) with MAFFT6 algorithm (Katoh *et al.*, 2002; Katoh & Toh, 2008). Sequences were processed with JustBio online tools (http://www.justbio.com) when needed.

RESULTS

ARMS

Initial experiments were designed to establish optimal conditions for ARMS. To ensure the formation of specific products, annealing temperature was set as high as possible, i.e. 61°C (Fig. 1A), and total concentration of primers was lowered from initial 600 nM to 400 nM, in proportions of 1:0.7:1.3 (forward:reverse:mutation specific, respectively; initially 1:1:1) (Fig. 1B), while the number of cycles was increased from initial 35 to 40, allowing for better sensitivity (Fig. 1C).

After optimization of the ARMS assay we performed sensitivity tests using DNA isolated from cell lines and clinical melanoma samples with various c. A1799 allele content at 1–1000-fold dilutions. ARMS allowed us to detect the c. T1799A mutation in heterogeneous melanoma samples even at 1000-fold dilution, whereas minimal detectable c. A1799 allele content was at least 2%. Simultaneously, the results obtained from negative control samples excluded the possibility of false positive results (Fig. 2). To evaluate ARMS usefulness as a diagnostic tool we performed mutation screening in a small group of gastric cancer samples, resulting in detection of the c. T1799A mutation in one of them (Fig. 3). Arrows indicate positions of the expected products: control (upper — 623 bp), mutation (lower — 134 bp). Dilutions are specified below the gel images. Highest dilutions (1:1000) resulted in 0.1–0.5 ng of DNA per reaction.

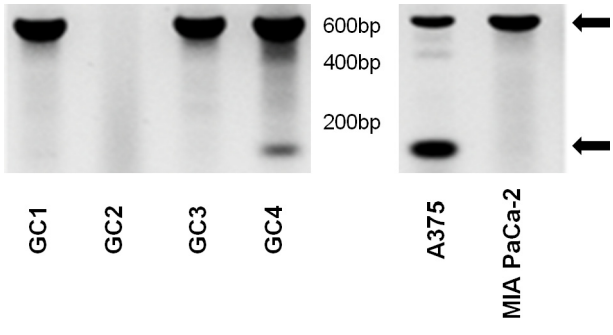

Figure 3. Evaluation of ARMS on gastric cancer samples.
ARMS assay allows to detect c. T1799A (p. V600E) mutation in GC4 positive gastric cancer sample. In GC2 no band is visible, suggesting low quality of the used DNA (upper band serves as an internal control of amplification). Arrows indicate positions of the expected products: control (upper — 623 bp), mutation (lower — 134 bp).

DNA Sequencing

To determine the reliability of ARMS we sequenced DNA from both control cell lines and two gastric cancer samples, positive and negative for *BRAF* c. T1799A mutation, in both directions. In three cases sequencing data were consistent with ARMS results, while chromatogram of GC4 sample displayed a peak for the wild-type nucleotide only (Fig. 4A).

We investigated this discrepancy by sequencing samples containing 50%, 20% and 2% of A375 DNA. The mutation was not detectable at the 2% level, thus showing lower sensitivity of sequencing compared to ARMS (Fig. 4B).

Hybridizing probes. Application of hybridizing probes allowed for accurate *BRAF* c. T1799A mutation detection; we observed no false positive results among

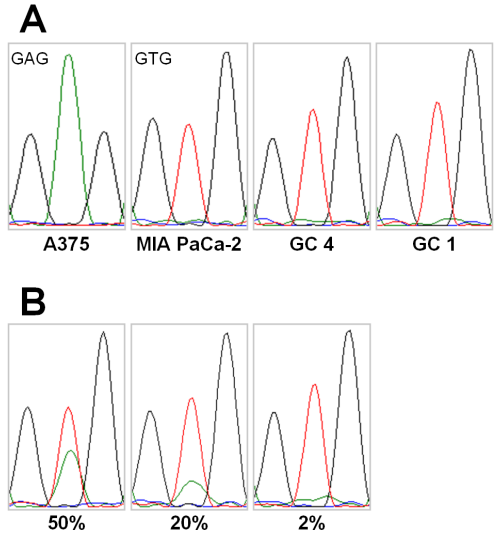

Figure 4. Chromatograms from sequence analysis of BRAF p. 600 codon (c. 1798–1800).
(A) C. T1799A mutation in GC4 sample is not detectable with sequencing — an adenine peak cannot be observed. **(B)** Sensitivity test of sequencing at various c. A1799 allele contents shows no c. T1799A mutation detection at 2% level, and a small adenine peak at 20% level.

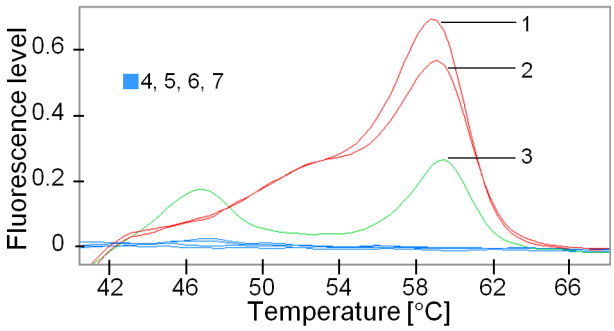

Figure 5. Exemplary melting curves obtained with LightMix Kit BRAF V600E hybridizing probes.
C. T1799A mutation is detected in GC4 sample (3), thus suggesting detection sensitivity comparable to that of the ARMS assay. Samples negative for c. T1799A mutation (4–6) are clustered together with no template control (7). Sample description: 1, A375; 2, positive control DNA provided by manufacturer; 3, GC4, 4, GC3; 5, GC5; 6, GC6; 7, no template control.

the tested samples. The sensitivity of this method was similar to that of ARMS assay since it allowed for c. T1799A mutation detection in the GC4 sample, albeit its detection mechanism does not exclude the possibility of interpretation of low DNA quality samples as negative (there is no internal amplification control according to the manufacturer's information (Fig. 5).

Table 2. Comparison of sensitivity and specificity of ARMS and hybridizing probes.
*LightMix BRAF V600E kit does not allow to discriminate wild type and no template or low quality template results (as verified with the manufacturer).

Sample type	ARMS	hybridizing probes
Number of:		
positive	1	1
negative	68	68*
false positive	0	0
false negative	0	0*

High Resolution Melting. Finally, we made an attempt to optimize and use the HRM method for c. T1799A mutation detection. We designed three pairs of primers flanking the p. 600 codon, amplifying products of different sizes. We could not obtain coherent and reproducible results for the 195 bp amplicon, thus the corresponding primer pair was excluded from further tests. The other two primer pairs allowed only to discriminate between homo- and heterozygous samples,

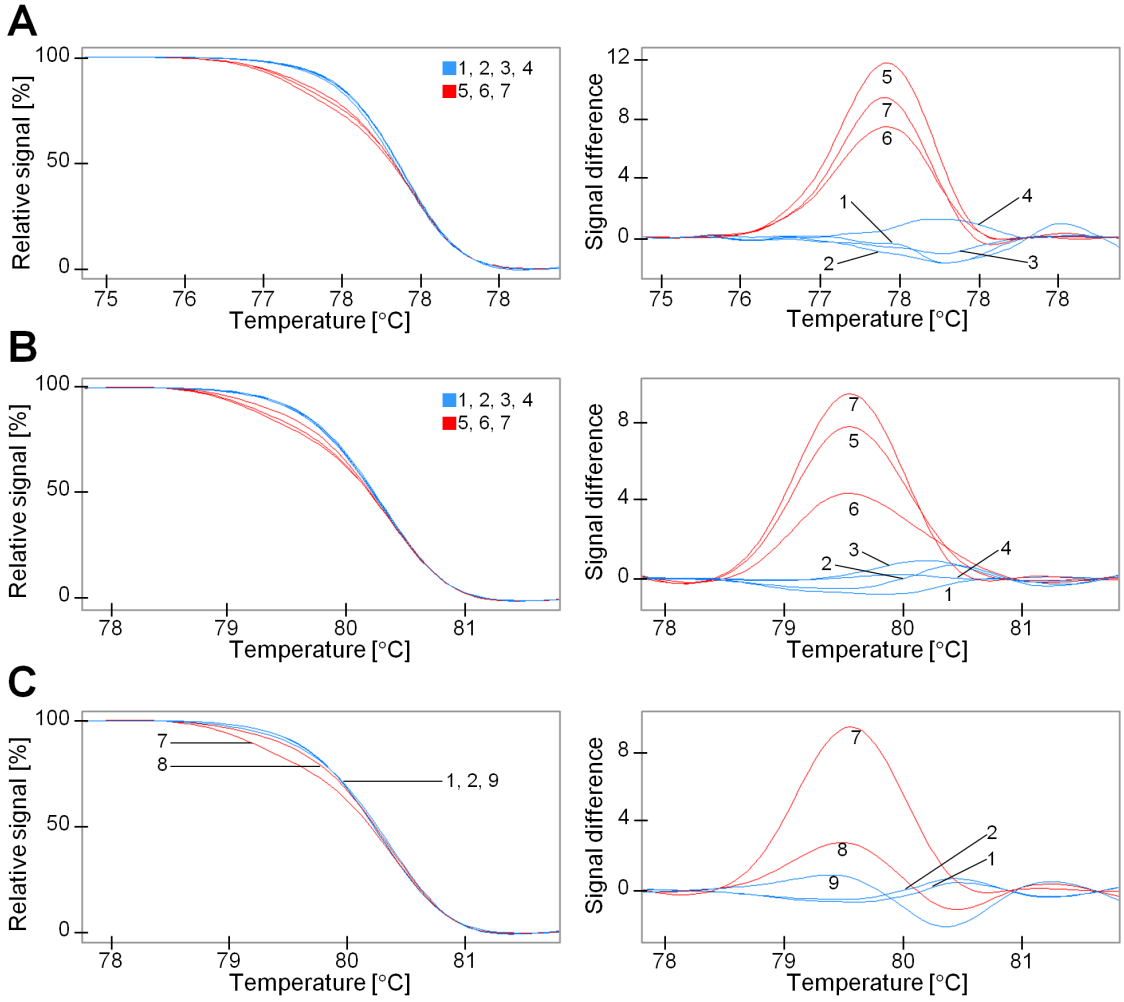

Figure 6. Melting curves and corresponding difference plots of the 77 and 95 bp amplicons generated during HRM.
(A) and **(B)** HRM allows only to discriminate homo- and heterozygote, regardless of amplicon length (A, 77 bp; B, 95 bp). Wild-type MM samples are in the homozygotes group (blue curves), while heterozygous MM and 50% samples create a separate group (red curves). Melting curves were normalized and temperature-shifted. **(C)** Sensitivity test of HRM using the 95 bp amplicon. Samples must contain at least 20% of c. A1799 allele to be classified as heterozygotes (red curves) and therefore as mutated. Melting curves were normalized and temperature-shifted. Sample description: 1, A375; 2, MIA PaCa-2; 3, MM1; 4, MM2; 5, MM3; 6, MM4; 7, 50%; 8, 20%; 9, 2%.

since homozygous samples were clustered together, regardless of their mutation status (Fig. 6A and 6B).

The GC4 sample was classified to the homozygous sample group, despite the mutation detected by ARMS (Fig. 3) and hybridizing probes (Fig. 5), therefore we decided to test the sensitivity of HRM with samples containing 50%, 20% and 2% of c. A1799 allele using the 95 bp amplicon primer pair. As expected, the 2% sample was clustered together with homozygotes, suggesting that HRM sensitivity was similar to that of sequencing (Fig. 6C).

To conclude, HRM allowed only for discrimination of homo- and heterozygous samples, containing at least 20% of the c. A1799 allele. Upper c. A1799 allele content limit was not determined. A summary of the results is shown in Table 1.

Sensitivity and specificity of mutation detection using ARMS and hybridizing probes

As the results of HRM analysis of *BRAF* c. T1799A mutation were unsatisfactory we decided to compare sensitivity and specificity of ARMS and hybridizing probes only and corroborate their results with Sanger sequencing. To achieve that goal we decided to scan 69 clinical samples obtained during gastric cancer resection with the above methods. Both ARMS and hybridizing probes allowed for detection of *BRAF* c. T1799A mutation in a single sample (GC4), (Table 2). We observed no false positive or false negative results.

DISCUSSION

Determining *BRAF* mutation status is becoming a routine genetic procedure in cancer diagnostics. However, there is no widely accepted gold standard assay for *BRAF* mutation detection. The aim of our study was to compare different methods, including ARMS-PCR designed by us, for the detection of the *BRAF* c. T1799A mutation, which is the most common among *BRAF* tumour associated variants. Apart from the functional consequences, this phenomenon allows for application of various detection methods, with different sensitivity, costs, labour intensity or instrument requirements.

Our ARMS assay proved to be very sensitive, detecting the c. A1799 allele even at less than 2%. This is not surprising, as various amplification-based methods were shown to be highly sensitive in previous studies. Pyrophosphorolysis-activated polymerization, ARMS with fluorochrome labelling and nested allele-specific PCR allowed to detect the *BRAF* mutation even below the 1% level, although at the cost of more sophisticated reaction mechanisms (Yancovitz *et al.*, 2007; Janssen *et al.*, 2008; Maat *et al.*, 2008; Ellison *et al.*, 2010). This is in contrast with the low sensitivity of Sanger sequencing demonstrated here or in previous studies (Yancovitz *et al.*, 2007), which requires from 20% to 30% of the mutant allele for its detection. Application of more sensitive methods allowed to discover *BRAF* mutations in uveal melanomas, initially described as negative (Janssen *et al.*, 2008; Maat *et al.*, 2008), which raises a question whether some data assessing *BRAF* mutation frequency, obtained with sequencing, could have been underestimated.

While the c. T1799A (p. V600E) is a predominant *BRAF* polymorphism, rare tandem codon 600 mutations were found to have similar functional consequences (Garnett & Marais, 2004; Wan *et al.*, 2004). Our ARMS assay is presumably incapable of detecting most of them, including the p. V600E arising from c. TG1799-1800AA. However, it can be used to detect the c. GT1798-1799AA/p. V600K tandem mutation. Rubinstein *et al.* (2010) suggested that p. V600K prevalence in melanoma was underestimated, reaching approximately 16.3% of all codon 600 mutations, and demonstrated clinical response to vemurafenib in a patient with an advanced, p. V600K-positive tumor.

One of the essential advantages of our ARMS assay is its simplicity. The qPCR-based ARMS method described by Ellison *et al.* (2010) displayed a similar sensitivity of 1% and possessed the ability to detect three different mutations (p. V600E, p. V600K and p. V600D). However, this methodology requires the use of a real-time thermocycler and six different oligonucleotides, including two pairs of primers and two fluorescent probes containing locked nucleic acid nucleotides, thereby considerably increasing the complexity of the reaction. It must be noted, however, that it also eliminates the need for gel electrophoresis, excludes the possibility of accidental contamination after the amplification and facilitates the assay's preparation, but at a considerably higher cost.

Additionally, ARMS versatility was proven by applying it to detect other gene mutations e.g. *JAK2* c. G1849T (Chen *et al.*, 2007) or *NOTCH1* c. 7544-7545delCT (Rossi *et al.*, 2012). Real-time PCR-based ARMS technique has also been successfully implemented to detect *N-RAS* c.A182G or c.C181A mutations in melanoma (Ellison *et al.*, 2010).

We tried to utilize real-time PCR-based methods as an alternative for our ARMS assay. The first one, the LightMix hybridizing probe kit allowed us to confirm ARMS results, thus showing similar sensitivity, though the usefulness of the assay is doubtful because of the lack of an internal amplification control.

The overall performance of the second one, HRM assay was unsatisfactory in our experimental settings. Low sensitivity, combined with the inability to distinguish between homo- and heterozygous or mixed samples, in our opinion disqualifies this method from the *BRAF* mutation status testing. This was quite unexpected, as we have successfully employed HRM for point mutations in other genes (unpublished data), using the same instrument and reagents. A possible explanation is that the T>A transversion alters the homozygous amplicon's melting temperature insufficiently and only when heteroduplexes are formed, a significant change in melting behaviour can be observed. Herrmann *et al.* (2007) obtained similar results for *HBB* c. A20T mutation and demonstrated that discrimination can also be hampered by temperature variation across the plate, which limits the effective resolution of heat block instruments, including LC480 (Herrmann *et al.*, 2007). Although there is a possibility to detect homozygous variants by mixing wild-type and unknown DNA with HRM, so that any homozygous variant in the unknown sample will be detected as a heterozygote in the mixture, we decided not to utilize this method as it is more labor-intensive and may affect reproducibility of the results.

It is tempting to speculate on the rational level of desired detection sensitivity. If we consider testing pure, homo- or heterozygous samples, then every method tested here has sufficient sensitivity, with the exception of HRM for the discrimination of homozygotes. Given that tissue samples are often composed of various cells, including tumor infiltrating leukocytes, and the tumor itself can be highly heterogeneous (Gerlinger *et al.*, 2012), it might be more informative to detect even the smallest amounts of mutated DNA, as it was demonstrated here and in other studies. While this approach may re-

sult in detection of "passenger" mutations, even a low number of cancer cells harbouring *BRAF* mutant alleles can be selected during progression of the disease (Lin *et al.*, 2011).

In summary our results show that simple ARMS is a highly sensitive and cost effective method for *BRAF* mutational screening (Table 1). Comparing real-time PCR based methods hybridizing probe-based detection seems to be more effective than the HRM assay. Limitations of our study include the number of samples tested and detection of only the most common *BRAF* mutation.

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