

## Original Article

# Short fragment approach for genotyping KRAS and BRAF genes in Tunisian patients with colorectal cancer

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**Abstract:** Colorectal cancer (CRC), of which KRAS and BRAF activating mutations are major contributors, is among the most frequent digestive cancer in Tunisia and in the world. The determination of the mutation status of these two biomarkers has become decisive in patients who are candidates for anti EGFR treatment. Several methods were developed to determine KRAS genotype, but until now, validated methods and standardized testing are lacking. In this study, we have firstly developed a simple approach to genotype KRAS and BRAF in CRC Tunisian specimens. Our method was based exclusively on bidirectional Sanger sequencing of KRAS and BRAF genes by using the ultra short fragments. A total of 34 Formalin-fixed, paraffin-embedded (FFPE) specimens were histologically diagnosed as CRC and were used in the analysis. Positive mutations, which were predominant in KRAS gene, were found in 7 out of 34 samples. The negative KRAS samples were also wild types for BRAF gene. Patients with oncogenic mutations could not respond to anti EGFR treatment. For better diagnosis, it has been recommended to determine KRAS status for the oncologist. Here, we have established a new approach based on one analytical step using short fragment analysis. According to us, this method would be considered as a strong tool in KRAS testing and could be applied in routine diagnosis.

**Keywords:** Bidirectional sequencing, colorectal cancer, oncogenic mutations, PCR touchdown, short fragment

## Introduction

KRAS and BRAF are key factors that play as central effectors in downstream of signaling cellular pathway mainly the mitogen-activated protein kinases (MAPKs), which are involved in controlling embryogenesis, cell differentiation, cell proliferation, cell death and angiogenesis. KRAS and BRAF genes are also considered as major candidates in different developmental disorders, and are predictive biomarkers in tumor genesis process [1].

Oncogenic mutations render KRAS insensitive to GTPase activity and lock it in the constitutive activated state resulting in KRAS amplification of cellular response [2]. This aberrant activation of KRAS, able to trigger downstream signaling pathway via EGFR or VEGF receptor, has been identified in a wide range of human cancers in nearly more than 20% including colorectal cancer (CRC), melanoma, pancreatic cancer

and lung tumor. Actually, developing small molecule inhibitors of activating KRAS or BRAF in early clinical trials was being decisive to predict the prognosis quality of response to target therapy [3]. KRAS wild type status may benefit from anti EGFR drug; cetuximab and panitumumab contrary to KRAS mutated status. Both KRAS and BRAF genes are involved in sporadic and metastatic CRC. In addition to somatic mutations, genetic instability was implemented in colorectal cancer genesis, which is well documented [4].

The KRAS mutation was frequency estimated between 35-40% of cases, [5, 6] whereas BRAF mutation rate was around 5-10% of patients with sporadic and metastatic colorectal defect. The most common oncogenic mutations have occurred in KRAS gene in codon 12 (approximately 82%) and 13 (closed to 17% of all reported KRAS mutations) of exon 2. Other mutations are also found in other residues, mainly in codon 61 and in codon 146 [6, 7].

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It was also documented that a common BRAF (exon15) mutation at 600 of amino acid coding region (p.V600E) caused colorectal cancer and other human cancers [8]. Several methods with varying accuracy and sensitivity have been proposed to attempted target mutant KRAS and/or BRAF genes implemented in routine analysis [9]. There is a wide range of technical approaches, including single strand confirmation polymorphism, allele/specific amplification, automated dideoxy sequencing, ligation based assays, ASO hybridization probes, base extension, pyrosequencing, HRM analysis, and different diagnostic commercial kits such as the DxS K-RAS Mutation Test Kit and the KRAS Strip Assay [10, 11]. So far, the sequencing has been considered as a golden standard method for genotyping of CRC biomarker: KRAS and BRAF in clinical samples although it is costly and time consuming.

The current study has been designed to establish a new approach for the CRC diagnosis of Tunisian patients. It is based exclusively on PCR amplification and direct sequencing, using short fragment of amplicon situated between the target consensus mutation in both KRAS and BRAF genes. This method may be invoked by oncologists to make a decision prior to any therapies with short delay.

### Materials and methods

#### *Population study*

Unselected samples of primary colorectal carcinomas, already embedded on formalin fixed paraffin, were collected from private clinical laboratories of histological and pathological analysis, with written informant consent from 34 patients prior to the testing. The clinical stage was determined according to the Tumor, Node, and Metastasis (TNM) classification of the staging system of the International Union against Cancer (UICC) [12].

The age range of patients was between 34 and 70 years. The histological type of tumor was determined on biopsy section according to the World Health Organization criteria [13]. All patients presented a differentiated adenocarcinoma lieberkuhnien with colloid component infiltrating the colon wall. The clinical diagnosis was assessed by colonoscopy and anatomical pathology examination. A serial of FFPE tissue

section of 10  $\mu\text{m}$  thick was generated and stained in deparafinised slide. Pathological examination was carefully solicited for all samples to ensure tumor cell content (TCC), (with TCC >30%) when possible, and the macro dissection was performed manually.

*DNA extraction:* The areas of interest were marked on the slide and cut out using a new sterile scalpel blade for each area in order to eliminate cross contamination. The tissue samples were scrapped manually from paraffin-embedded tissue sections, and each section was collected in 1.5 ml Eppendorf tubes.

The DNA was then extracted manually using a "BIOstic FFPE tissue DNA isolation kit" by MOBIO laboratories, CA. The extraction steps were carried out in accordance with the manufacturer's recommendations. The DNA was eluted in final volume of 100 $\mu\text{l}$  and was amplified by PCR for KRAS exon 2, 3 and BRAF exon 15. No additional purification step of the DNA was performed.

The quality of the extracted DNA from the FFPE samples was assessed by 1% agarose gel electrophoresis.

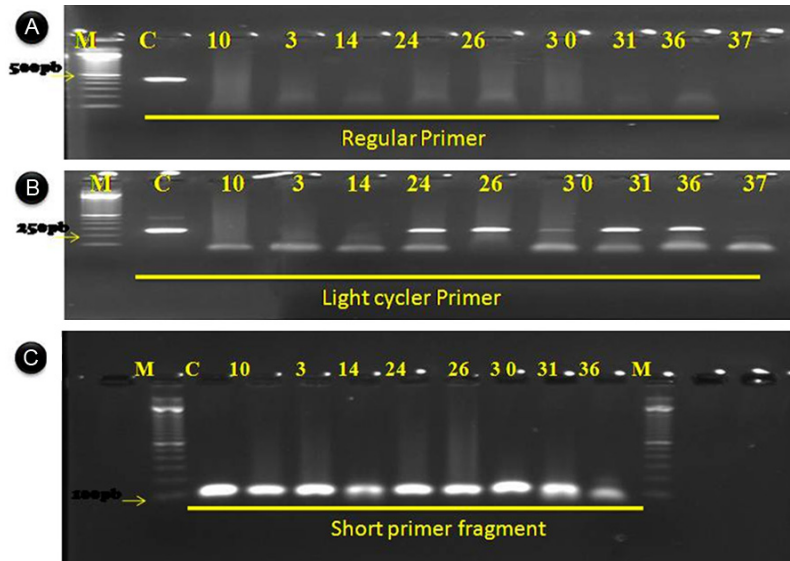
#### *PCR condition*

First, to review the quality of the isolated tumor DNA, a control PCR was done in each sample with a known primer, which has been successfully applied in previous analyses in the laboratory. Then, the PCR reaction to analyze target mutation of both KRAS and BRAF gene was run by using various lengths of primer sets. Three different lengths of primer sets were chosen to amplify targeting exon of KRAS and BRAF gene, and they produced different sizes of amplicons: 400 pb; 250 pb; 80 pb.

For all samples, PCR mixture reaction was done in a total volume of 20  $\mu\text{l}$  as specified in the following: 2.0  $\mu\text{l}$  template DNA (ddH<sub>2</sub>O in case of the negative control); 2.0  $\mu\text{l}$  reaction buffer (10 $\times$  concentrated); 6.2  $\mu\text{l}$  ddH<sub>2</sub>O, 1.0  $\mu\text{l}$  each forward and reverse primer (5  $\mu\text{mol}/\mu\text{l}$ ); 0.6  $\mu\text{l}$  MgCl<sub>2</sub> (50 mmol); 2.0  $\mu\text{l}$  dNTP (10 mM), 1.0  $\mu\text{l}$  DMSO (5%); 4  $\mu\text{l}$  Betaine (5 M) and 0.2 U Taq polymerase (BiolineTaq recombinant Polymerase).

A positive control human DNA was included. The amplification steps of touch down PCR

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**Figure 1.** Result of PCR of KRAS exon 2 by 3 type of primer. A: regular primer (400 pb); B: light cycler primer (250 pb); C: short fragment primer (80 pb); Lanes from (left to right): M-Marker, C- positive control, samples 10, 3, 14, 24, 26, 30, 31, 36, 37.

reactions were as follows: an initial denaturation cycle of 94°C for 3 min; followed by another denaturation step (94°C for 30 s), 2 cycles of annealing (60°C, 63°C, 61°C, 59°C, 57°C, for 30 sec for each and elongation at 45 sec for 72°C, 34 cycle of denaturation (94°C for 30 sec); annealing (55°C for 45 sec) elongation (72°C for 45 sec) and a final extension cycle at 72°C for 10 min. The PCR products were analyzed by horizontal 2% agarose gel electrophoresis followed by purification with magnetic beads (AgencourtAMPure XP beads, Beckman Coulter, Krefeld, Germany), using the Biomek® NX<sup>P</sup> Laboratory Automation Work station for removal of any primer dimmers.

### Sequencing reaction

PCR products were sequenced using the Big Dye Terminator Cycle sequencing ready Reaction Kit (Applied Biosystem). The sequencing reaction was run in reaction volume of 10 µl in 96 well plates as follows: 5.9 µl of ddH<sub>2</sub>O, 2µl of 5× buffer, 0.5 µl of Big Dye, 1 µl of PCR product, 0.6 µl of Primer (5 pmol/µl, forward or reverse)

The sequencing plate was placed in a PCR cycler (the iCycler, Bio Rad Laboratories) for the PCR sequencing reaction. Then, the reaction products were purified, using the Biomek®

NX<sup>P</sup> Laboratory Automation Workstation. The reaction products were analyzed on an automated capillary sequencer (ABI 3500 XL Genetic Analyser, Applied Biosystems). Sequences were analyzed using Chromas lite software.

### Results

#### *Evaluation of isolated tumor DNA by PCR and sequencing reactions*

The quality of tumor DNA was tested in terms of its success in PCR amplification of single copy of fragment with different sizes and by sequencing procedure.

The optimization of PCR conditions was obtained from control slide. KRAS (exon 2 and exon 3) gene and BRAF gene (exon 15) were amplified with varying lengths: 400 pb (regular primer); 250 pb (light cycler primer (LC)) and 80 pb (short primer).

The amplification results through long fragment (400 pb) did not provide a visual band. The LC amplicon gave a visual band in 20/34 of samples. On the contrary, the PCR with ultra short was quite successful as it provided a clear band in almost all the samples. Moreover, these successful PCR products of small amplicon were exclusively selected for sequencing reaction assay. Due to the poor quality of FFPE, some samples provided a very faint product. In this case, we conceded that the increase of cycle number of PCR and the concentration of tumor DNA would allow ameliorating the visibility of the band (**Figure 1**).

### Molecular analyses

The clinical and molecular results were summarized in **Table 1**.

This study includes 13 males and 21 females with a median age of 52 years (range 34-70 years). All patients had the colon as the tumor common location. The tumor risk rate was higher in females than in males. All patients pos-

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**Table 1.** Summary of clinic and molecular results screened for mutation in KRAS and BRAF genes

Patients	Sex	Histology	PCR (RP)	PCR (LC)	PCR (SF)	Sequencing	AA change	Genes	Genotype
P1	M	Lieberkuhnian adenocarcinoma	0	-	+	+	0	Kras Braf	wt wt
P3	F	Lieberkuhnian adenocarcinoma	0	-	+	+	0	Kras Braf	wt wt
P5	F	Lieberkuhnian adenocarcinoma	0	-	+	c.35G>A	G12D	Kras	heterozygous
P10	F	Lieberkuhnia adenocarcinoma	0	+	+	c.34G > A	G12S	Kras	Heterozygous
P14	F	Lieberkuhnian adenocarcinoma	0	0	+	c.35G>A	G12D	Kras	Heterozygous
P17	F	Lieberkuhnian adenocarcinoma	0	+	+	c.38G>A	G13D	Kras	Heterozygous
P19	F	Lieberkuhnian adenocarcinoma	0	0	+	c.35G>T	G12V	Kras	Heterozygous
P23	M	Lieberkuhnian adenocarcinoma	0	+	+	c.35G>A	G12D	Kras	Heterozygous
P29	M	Lieberkuhnian adenocarcinoma	0	+	+	c.35G>T	G12V	Kras	Heterozygous
P30	M	Lieberkuhnian adenocarcinoma	0	-	+	+	0	Kras Braf	wt wt
P32	F	Lieberkuhnian adenocarcinoma	0	-	+	+	0	Kras Braf	wt wt

RP: regular Primer; SF: short fragment; LC: light cycler primer; wt: wild type.

sessed a single mutation site. A total of 7 specimens with hot spot mutation were found in this study. Six mutations were located exclusively in exon 2 of KRAS gene: in codon 12 G12D; (3/34); G12S (1/34); G12V (2/34) and one was in codon 13 G13D (1/34). No BRAF mutation in exon 15 was detected.

These types of mutations are accounted for the major cause of malignant phenotype of colorectal alteration (**Table 1**). Mutations in codon 12 were the frequent ones. A predominance of mutation was observed more in females (5/34) than in males (2/34).

Representative electropherograms of KRAS mutant phenotype to be found in all patient are shown in **Figure 2**.

### Discussion

The determination of mutational status of KRAS and BRAF biomarker is highly requested in patients with CRC. Their genotype is not only decisive for patients to benefit from drug administration, either cetuximab or panitumumab,

but it is also a prognostic tool of survival rate in patients [14]. The search for best investigation methods for KRAS and BRAF genotyping is recommended to better classify the mutant phenotype.

This study is designed to optimize a new approach for KRAS mutation testing based only on touchdown PCR that is followed by bidirectional sequencing analyses despite the lower sensitivity of detection in the latter when compared to other methods such as high melting curve (HRM) and pyro sequencing [11-15].

The different sets of chosen primers with varying lengths have shown a discrepancy of amplification in band visualization of amplified product with the long and median fragments. The genotyping of KRAS exon 2 and 3 by small amplicon (80 pb) was performed successfully contrary to other types of amplicon in all samples. This result indicates that the size of PCR product influenced the rate of amplification success positively [16] (**Figure 1**). The difference in amplification pattern is eventually due to the DNA fragmentation within speci-

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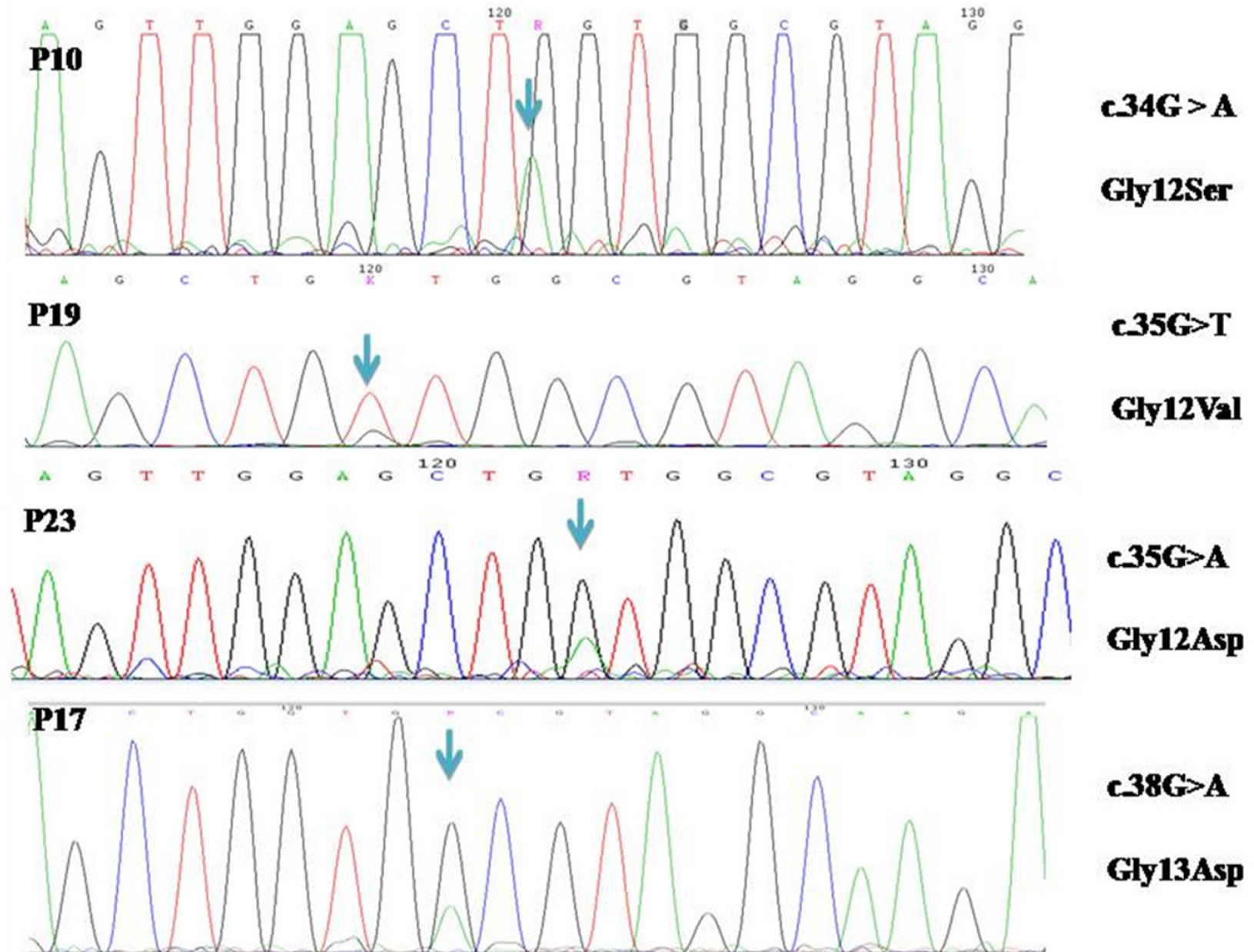


Figure 2. Sequencing results of positive samples in KRAS exon 2.

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mens isolated from formalin-fixed paraffin-embedded.

Indeed, the DNA fragmentation might impose unavoidable limitations on the maximum length of PCR amplicons [17], and might show invisibility or a weakness of the bands in some samples. Therefore, for better amplification, we have increased the amount of DNA three times more than mentioned in Methods Section. Besides, since the concentration of the extracted DNA in some samples derived from the colon sections had very low quality (compared to the positive control used in the reaction), we also increased the cycle number by an additional 15 cycles, and added fresh Polymerase Taq after 35 cycles. This was done because during the PCR reaction with 45 cycles, the polymerase became exhausted and its effect decreased in cycling reaction, which resulted in a visible band.

Our sequencing result revealed a distinctive characteristic molecular pattern in 7 of 34 CRC patients with positive somatic mutation of exon 2 of KRAS gene while the rest were classified as wild type for KRAS. These previously reported findings were described as oncogenically active. They were mainly observed in codon 12 with a predominance G/A transition consistent with previous data [18, 19]. The substitution of glycine by aspartic acid was previously reported as the most common amino acid change in CRC alteration [20]. The mutation rate of KRAS in our study was about 20.6%, which is most common in females than in males.

Our report shows that the mutation rate in Tunisian colorectal patients is less frequent when compared with data from literature (35-45%) [5, 6]. This is probably related to different factors, mainly the limited materials, and our interest was exclusively focused on target mutations that are responsible for CRC. In addition, the poor quality of DNA isolated from FFPE specimen reflects the percentage of tumor cells. However, the manual scrapping directly from the stained sample may preserve a sufficient rate of tumor cells in each slide.

On the other hand, another factor plays a key role in the efficiency of this approach, which was the implication of shortening fragment in sequencing to decrease the false negativity rate.

After KRAS, BRAF gene was found to be associated with poor prognosis or resistance to anti EGFR antibody in metastatic colorectal cancer [21]. Our result did not reveal any mutation in exon 15 of BRAF. This negative result was eventually due to lower mutation rate of BRAF, which is close to 3% [22].

KRAS genotyping was systemically carried out by pre analytical methods based on PCR concept including restriction fragment length polymorphism analysis, single nucleotide extension, HRM analysis and pyro sequencing. These assays were usually followed by analytical methods as bidirectional sequencing to compare the results and evaluate the effect of KRAS status on response to CRC anti-EGFR treatment. This variety of detection methods is reported to be accurate, and to provide reliable results for KRAS genotype testing in clinical samples [15]. Conventionally, several screening strategies of the KRAS status require two analytical steps to obtain a validated genotype. In one previous study, the Smart Amplification Process version 2, which was based exclusively on amplification to detect EGFR or KRAS mutations in DNA extracted from FFPE tissues, was adopted [16]. Several pre analytical methodologies, of which pyro sequencing and HRM analysis, were also regarded as efficient for KRAS genotyping detection and considered to be strong tools for prognostic value estimation [5-23, 24].

Contrary to other studies, we have tried to emphasize relying our experiment on simple amplification assay followed by direct sequencing that uses a short amplicon targeting a consensus region. The molecular findings were suitable for oncologists to make a decision on the use of anti EGFR therapy for patients with sporadic CRC. Therefore, the approach of small amplicon is currently adopted for HRM technique and in gene-disease association.

To sum up, we have successfully developed a simple strategy for the diagnostic validation of DNA from FFPE colorectal sections, which were based exclusively on one analytical step by means of short fragment. The latter was considered as a strong tool for KRAS genotyping in FFPE samples. This approach was so well-validated in CRC patients that we applied it, not only for patients who are sensitive to target therapy in restricted delay, but also in other

diagnostic disease and clinical trials in our institute.

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### Disclosure of conflict of interest

None.

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