

KRAS Testing in Clinical Laboratory: Optimizing Targeted Therapy

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Abstract. *Background/Aim:* Activating mutations in the *KRAS* gene are found in more than 30% of colorectal tumors, where they are associated with a poor response to anti-epidermal growth factor receptor therapies. Mutation testing techniques have therefore become an urgent concern. Several methods for *KRAS* mutation detection have been described in the literature. Most of these are laboratory developed tests and only a few commercial assays are currently available. *Materials and Methods:* We studied the performance characteristics of a *KRAS* mutation detection assay on the ABI-3130XL genetic analyzer using a new commercial mutation detection kit based on shifted termination assay technology. Samples were analyzed in parallel by different reference laboratories using alternative methodologies. Various sample types were used including formalin-fixed paraffin-embedded tissue, fine-needle aspirates, and cyst fluid specimens. *Results:* A high level of agreement (100% correlation for formalin-fixed paraffin-embedded tissue and fine-needle aspirate samples and 93% correlation for cyst fluid specimens) was obtained despite the use of different methodologies. *Conclusion:* Shift termination assay is a simple, robust, and sensitive method for the identification of *KRAS* mutations in a wide variety of specimen types.

Activating mutations in the *KRAS* oncogene are frequently found in human cancer. Somatic *KRAS* mutations are found

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in 75% to 90% of pancreatic adenocarcinomas, 35% to 50% of colorectal carcinomas (CRC), and approximately 30% of lung adenocarcinomas (1). *KRAS* mutations are almost exclusively found in codons 12 and 13 of the *KRAS* gene, with a small number of mutations occurring in codon 61 (1). *KRAS* mutations alter the conformation of the *KRAS* protein, causing impaired GTPase activity that results in epidermal growth factor receptor (EGFR)-independent intracellular signal transduction activation (2).

The discovery that mutations in *KRAS* may abolish the response to anti-EGFR therapy has revolutionized the treatment of cancer such as CRC or non-small cell lung carcinoma (NSCLC) (3, 4). Therefore, there is an urgent demand for *KRAS* mutation testing in clinical laboratories. Several methodologies for *KRAS* mutation detection have been described in the literature. These include automated dideoxy (Sanger) DNA sequencing, pyrosequencing, single stranded conformation polymorphism (SSCP), high resolution melting (HRM) analysis, and a combination of allele-specific polymerase chain reaction (PCR) with real-time PCR (Scorpions) as well as others (5, 6).

A sensitive, accurate, and simple method called shifted termination assay (STA) (TrimGen Corporation, Sparks, MD, USA) has been reported for the detection of genetic mutations (7). STA is a multiple-base and multiple-cycle primer extension based method that can identify low-level somatic mutations. The STA reaction recognizes wild-type or mutant target sequences and selectively extends detection primers with 1 to 20 labeled nucleotides. The fragments are then separated by capillary electrophoresis. The *KRAS* Mutation Analysis Reagents (TrimGen Corporation) is a new commercially available assay for the detection of mutations in codons 12 and 13 of the *KRAS* gene. This assay was designed using the STA technology. Herein, we report the performance evaluation of the *KRAS* Mutation Analysis Reagents using a wide variety of specimen types.

Materials and Methods

Samples. We analyzed 93 human specimens for *KRAS* mutations. These include formalin-fixed paraffin-embedded (FFPE) tissue (62 samples), pancreatic fine-needle aspirates (FNA) (17 samples), and pancreatic cyst fluid specimens (14 samples). FFPE samples represented primary or metastatic tumor samples from colon, rectum, liver, pancreas, lung, brain, skin, and ovary. Tumor burden varied depending on the case, with cases containing as few as 10% tumor cells present on the sections. FNA and cyst fluid specimens represented specimens from malignant and premalignant pancreatic lesions.

DNA extraction. DNA was extracted from FFPE tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA). A FFPE-*KRAS* negative control patient sample was included in each extraction. A total of four precut unstained 7-micron-thick tissue slides were used for each extraction. Tissue sections stained with hematoxylin and eosin (H&E) were evaluated by a certified pathologist prior to extraction to ensure sufficient tumor material in the section analyzed. The sections selected for DNA extraction were manually dissected to obtain approximately 100% tumor material in each case. For FNA and cyst fluid specimens, DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen). At least 0.5 ml of FNA tissue material transported in RPMI-1640 medium and at least 0.2 ml of cyst fluid material collected in sterile 4 ml DNase-free tubes were received in the laboratory and were used for DNA extraction. All extractions were performed following the manufacturer's recommendations. DNA concentration/purity was determined by measuring the absorbance at 260/280 nm on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

***KRAS* amplification and detection.** All DNA samples were analyzed using the *KRAS* Mutation Analysis Reagents assay, which identifies 12 different mutations located in codons 12 (six mutations) and codon 13 (six mutations) of the *KRAS* gene (Figure 1). A first step of PCR amplification was performed following the manufacturer's recommendations. The PCR products were cleaned and a second PCR was performed applying STA technology. After removal of free fluorescent dyes, the PCR fragments obtained were separated on an ABI-3130XL genetic analyzer (Applied Biosystems-Life Technologies Corporation, Carlsbad, California) and were analyzed using the GeneMapper software (Applied Biosystems-Life Technologies Corporation). Control DNA for *KRAS* codon 12, containing six different mutations (G12C, G12V, G12S, G12D, G12R and G12A), and control DNA for *KRAS* codon 13, containing six different mutations (G13V, G13C, G13D, G13S, G13A and G13R), were run in parallel with the samples. These controls consisted of DNA extracted from plasmids and were provided in the kit. In addition, a no-template control (H₂O) and a *KRAS*-negative control patient sample were included in each run. The approximate time required to run the assay was estimated to be between 6-8 hours.

Evaluation of analytic performance. The accuracy of the assay was evaluated by comparing the results obtained at our laboratory with those obtained at reference laboratories. A total of 93 patient specimens, including 62 FFPE tissue sections, 17 FNA samples, and 14 cyst fluid samples, were analyzed in parallel at four different laboratories (LabCorp, Burlington, NC, USA; Genzyme Cambridge,

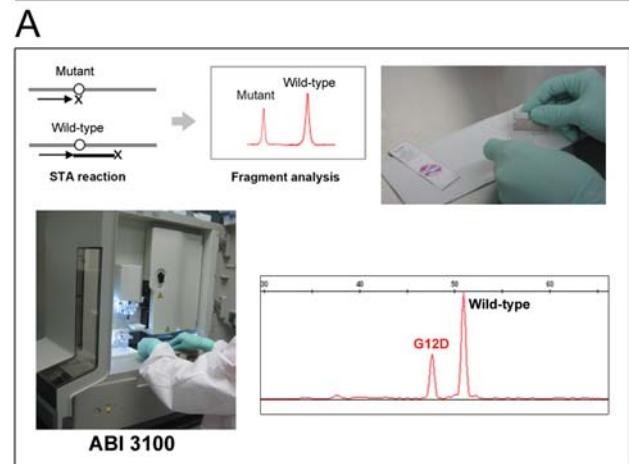
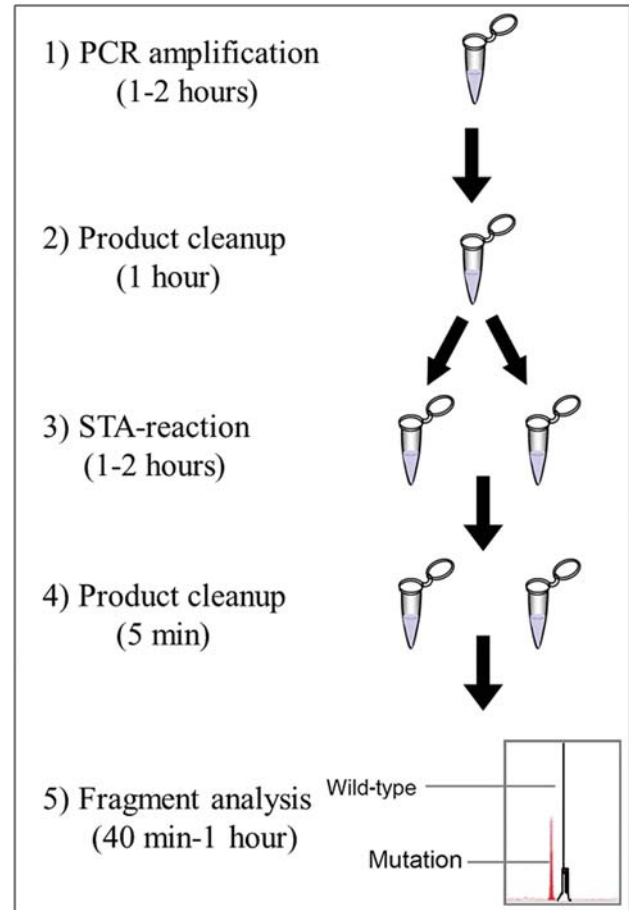


Figure 1. Workflow steps involved in the *KRAS* mutation assay performed using the *KRAS* Mutation Analysis Reagents (A and B). STA, shift termination assay.

MA, USA; RedPath, Pittsburgh, PA, USA; Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA) The methodologies used by the reference laboratories were ARMS/real-time PCR,

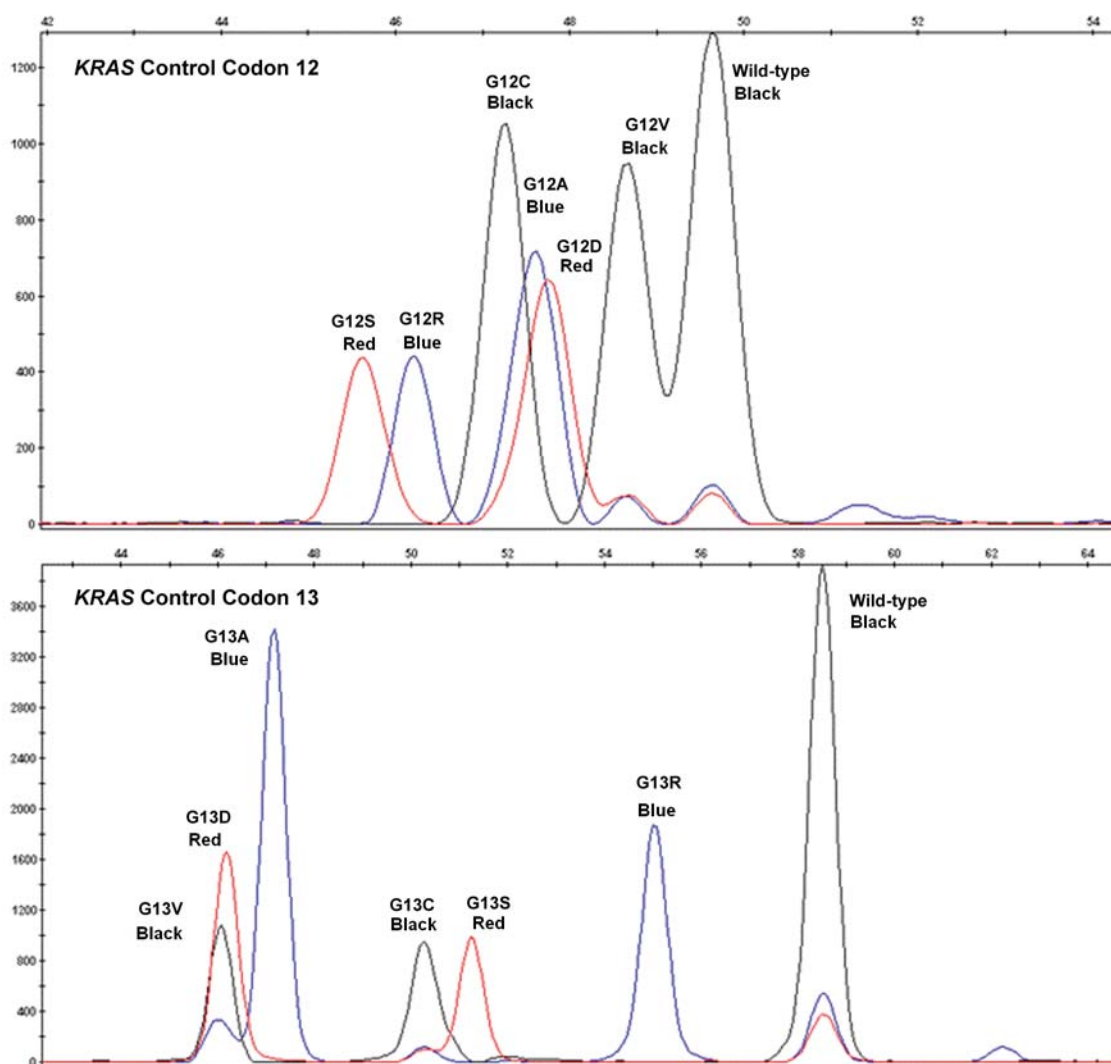


Figure 2. Electropherogram of control DNA samples containing the wild-type and six different mutations in *KRAS* codon 12 (top panel) and in *KRAS* codon 13 (bottom panel). Note that the wild-type peak is a black peak with the largest peak size on the right. The mutations will show as additional peaks.

SNaPshot/Capillary Electrophoresis, TaqMan real-time PCR, pyrosequencing, and Sanger sequencing. To evaluate the analytical sensitivity of the assay, DNA samples from each of the two *KRAS*-mutant plasmid controls, which represent 100% mutant DNA, were mixed with DNA obtained from normal tissue (*KRAS*-negative control DNA) in various fractions (10%, 5%, and 1% mutant DNA in control DNA). We also performed serial dilutions of DNA extracted from two different *KRAS*-positive patient specimens in *KRAS*-negative control DNA. The sections selected for DNA extraction in the *KRAS*-positive patients were manually dissected in order to obtain approximately 100% tumor material in each case. All dilutions were prepared in duplicate. In addition, we estimated the minimum amount of tissue required for successful DNA extraction and amplification. To this end, we dissected FFPE sections into areas of approximately 0.5 cm² or 2 cm², containing approximately 100% tumor cells in those areas. Analytical

specificity was determined by running samples known to be positive for *KRAS* codon 12 mutation with primers/probes for the detection of codon 13 mutations, and by running samples known to be positive for *KRAS* codon 13 mutation with primers/probes for the detection of codon 12 mutations. Precision was evaluated by running duplicates of five patient specimens and two control samples (one control for codon 12 mutations and one control for codon 13 mutations) on three different days.

Results

From the 62 FFPE samples tested, 36 samples were found to be positive for *KRAS* mutations (34 samples were positive for *KRAS* codon 12 mutations and 2 samples were positive for *KRAS* codon 13 mutations). Twenty-six samples were found

to be negative for *KRAS* mutations. From the 17 pancreatic FNA specimens evaluated, 13 were positive for *KRAS* codon 12 mutations and four were negative. From the 14 cyst fluid samples evaluated, five were found to be positive for *KRAS* codon 12 mutations and nine were found to be negative. The accuracy between the different laboratories for the detection of *KRAS* mutations was 100% for FFPE and FNA specimens and 93% for the cyst fluid specimens. Consensus for mutation type was observed for all the 36 positive FFPE and for four positive cyst fluid specimens. When analyzing FNA samples, 12 out of 13 (92%) positive specimens exhibited the same mutation; however, in one FNA specimen, a G12R mutation was detected using the *KRAS* Mutation Analysis Reagents and a G12V mutation was observed on the same sample when using pyrosequencing technology. Mutations in *KRAS* were detected in tissue sections of approximately 0.5 cm² (four slides dissected) and in positive control DNA samples diluted up to 1% in *KRAS* negative control DNA. We were able to amplify samples with a DNA concentration as low as 1 ng/μl. We observed that the presence of a significant amount of necrotic tissue inhibited PCR amplification. No mutation was detected when running codon 12-positive specimens with codon 13 primers/probes set nor *vice versa*. A precision of 100% was observed between different runs of the same patient samples (Table I).

After fragments were separated by capillary electrophoresis, the peaks were analyzed by comparing the peak sizes with the ones obtained for the positive control samples. This assay is designed so that a peak corresponding to the *KRAS* codon 12- or codon 13-negative fragments always appear with a constant peak size, which should be determined during the validation step of the assay. These wild-type or *KRAS*-negative peaks serve as internal controls for target amplification. Any mutation in *KRAS* codon 12 or codon 13 will appear as an additional peak that is consistently smaller than the *KRAS*-negative peak (Figure 2).

Discussion

Mutations in the *KRAS* gene negatively predict success of anti-EGFR therapies. Screening patients for *KRAS* mutations before the initiation of therapy may prevent unnecessary toxicity and healthcare expense in patients who are unlikely to respond. Therefore, screening for the presence of *KRAS* mutations has become an urgent matter. A number of alternative methods for the detection of *KRAS* mutations have been described in the literature (5, 6). Many of these methods are laboratory-developed assays and are not commercially available for use in routine diagnostics. Few assays have been developed further and are available as commercial test kits; however, again not directly intended for diagnostic purposes. Some of these commercially available *KRAS* mutation test kits include the *KRAS* RGQ

Table I. Correlation studies using formalin-fixed, paraffin-embedded (FFPE) tissue, pancreatic fine needle aspiration (FNA), and cystic fluid specimens.

Specimen type (n)	Expected result		Obtained result		Total correlation
	Positive	Negative	Positive	Negative	Positive
FFPE (62)	36	26	36	26	100%
FNA (17)	13	4	13	4	100%
Cystic fluid (14)	4	10	5	9	93%

PCR kit (Qiagen), PyroMark *KRAS* Kit (Qiagen), *KRAS* LightMix (TIB MOLBIOL, Berlin, Germany), and Signature *KRAS* Mutations 7 (Asuragen Inc.). None of these assays have been cleared by the Federal Drug Administration for clinical use.

In the present study, we evaluated the assay performance characteristics of a commercially available *KRAS* mutation detection assay distributed by TrimGen Corporation. The main goals of our study were the evaluation of the analytical sensitivity, analytical specificity, and precision of the assay, as well as the accuracy of the results when comparing the data obtained in our laboratory with the results obtained by reference laboratories. In addition, the capability of testing several specimen types, including FFPE tissue, FNA, and cyst fluid samples, was assessed. A high correlation between our results and the results obtained by reference laboratories was observed after testing a total of 93 different specimens (100% correlation for FFPE and FNA samples and 93% correlation for cystic fluid specimens). Only one cystic fluid specimen did not correlate with the results obtained by the reference laboratory. This discrepancy may be attributed to differences in assay sensitivity since the methodology used by the reference laboratory was direct sequencing, a methodology known to lack sufficient sensitivity for detecting mutant alleles in heterogeneous tumor samples (8).

A common drawback for the detection of acquired mutations in clinical samples is sample heterogeneity. Frequently, a variable percentage of cancer cells is included in most tissue blocks with different amounts of normal cells surrounding them, thus reducing the sensitivity of the test. This assay was able to detect *KRAS* mutations from DNA extracted from four sections containing areas as small as 0.5 cm² of tumor material. Tumor burden or section size appeared not to be a limitation in this assay but the presence of necrotic tissue should be avoided in order to obtain successful PCR amplification. Although FFPE material is the most commonly used type of sample for detecting *KRAS*

mutations in patients with CRC, liquid-based cytology samples are very commonly encountered in pathology laboratories. Identifying the presence of *KRAS* mutation in pancreatic cyst fluids has been demonstrated to be an important factor in the preoperative diagnosis of malignant and benign mucinous pancreatic cysts (9-11). This assay successfully amplified DNA from 17 pancreatic FNA and 14 cystic fluid samples and detected *KRAS* mutations with higher sensitivity when comparing with DNA sequencing. The accuracy of results obtained was 100% for FFPE tissue and FNA samples and 93% for cyst fluid specimens. Primers/probes used for the detection of codons 12 and 13 did not cross-react between each other and consensus for mutation type was observed in 53/54 (98.2%) of the total positive samples analyzed. In general, the total time required for *KRAS* testing from DNA extraction to data analysis was between 6-8 hours depending on the number of samples included in each run. The cost of the analysis for running each patient specimen compared with other commercially available kits was significantly lower.

Our data suggested that the STA is an accurate, sensitive, rapid, and reliable assay for the identification of *KRAS* mutations in specimens commonly encountered in surgical pathology and cytopathology practice. The combined performance characteristics make this assay a sensitive and robust method suitable for detection of *KRAS* mutations in a clinical laboratory setting.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

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