

## ORIGINAL RESEARCH ARTICLE

Identifying *KRAS* mutations in non-small cell lung cancer of Iraqi patientsMays Talib Abdallah\*

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## Abstract

Lung cancer remains one of the most common malignancies. It is usually categorized into two types: small-cell lung cancer and non-small-cell lung cancer (NSCLC). Among the types of NSCLC, adenocarcinoma is the most common and is more prevalent in women than in men. The current study aims to identify *KRAS* mutations in a population with metastatic lung cancer. Moreover, the clinical significance of the *KRAS* mutations was also explored. For this purpose, *KRAS* status was evaluated by polymerase chain reaction and reverse hybridization. According to the molecular results, mutations were observed at codons 12, 13, 59, 61, 117, and 146. Interestingly, the number of patients with stage II disease was significantly higher, indicating that the type of *KRAS* alteration may be associated with early disease spread. Thus, these mutations were associated with poor responses to cetuximab and panitumumab, underscoring the important role of *KRAS* profiling in strategizing treatment modifications. We can also conclude that molecular diagnostics plays its role in everyday clinical practice by complementing precision oncology and improving the outlook for patients with NSCLC.

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## 1. Introduction

Lung cancer, being the most common and deadliest cancer worldwide, affects millions of people every year. It is commonly classified into two major types: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), with the latter having more varied biological characteristics, treatment types, and clinical patterns. NSCLC is the most prevalent lung cancer, representing about 85%, and is treated with a combination of surgery and the most advanced targeted therapy.<sup>1</sup> In contrast, SCLC responds more rapidly to chemotherapy, yielding immediate results.<sup>2</sup> Even though studies on cancer and its treatment are ongoing, lung cancer remains a serious issue that needs urgent and concentrated efforts to avert further deterioration.<sup>3</sup> NSCLC has three histologic types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.<sup>2</sup> In non-smokers, the most common NSCLC subtype is adenocarcinoma. It is most common in women, indicating the role of genetics and environment in the development of the disease.<sup>4</sup> On the other hand, SCLC (about 15%) is the most aggressive form, with the fastest growth rate and the most widespread metastases.

*KRAS* is a gene that encodes a GTPase, a protein that regulates the balance of cellular signaling pathways necessary for cell division and differentiation. *KRAS* is an oncogene, but its role in cancer is not as obvious as that of other genes, and its effects are slow, eventually promoting cancer cell growth. In colorectal cancer, about 40% of the patient population has a *KRAS* mutation, leading to the promotion of the cancer cell.<sup>5</sup> *KRAS* mutations also cause resistance to epidermal growth factor receptor (EGFR) inhibitors, such as cetuximab and panitumumab.<sup>6</sup> Interestingly, this situation has recently improved with the development of mutation-specific medications (such as sotorasib and adagrasib), providing patients with new treatment options.<sup>7</sup> In addition, activated Kirsten rat sarcoma viral oncogene homolog (*KRAS*) facilitates the creation of a tumor microenvironment that supports tumor growth by promoting the production of fibrous tissue and immunosuppressive cells, such as myeloid-derived suppressor cells.<sup>8</sup> These are some of the pathways through which *KRAS* mutations alter the tumor microenvironment, increasing its diversity and thereby affecting disease progression.

Because *KRAS* alterations increase programmed death-ligand 1 (PD-L1) expression by sustaining extracellular signal-regulated kinase (ERK) phosphorylation, immunotherapy has become a promising strategy for *KRAS*-mutated NSCLC. High levels of PD-L1 promote the depletion of CD3<sup>+</sup> T cells, an effect that can be reversed by inhibitors of programmed cell death protein 1 (PD-1)/PD-L1 or by drugs targeting ERK. This provides the rationale for PD-1 inhibition's ability to restore T-cell-mediated antitumor immunity in *KRAS* mutation-driven NSCLC.<sup>9</sup> This tumor type tends to harbor a significant mutational burden because it is often linked to exposure to tobacco.<sup>10</sup> Further evidence for the benefit of immunotherapy comes from a meta-analysis of six studies, showing that adding immunotherapy to chemotherapy results in a marked improvement in overall survival.

Because *KRAS* G12C-specific inhibitors are being rapidly developed and our understanding of the molecular pathways driving *KRAS*-related lung cancer is increasing, *KRAS* has emerged as a therapeutic target in NSCLC. Sotorasib and adagrasib, *KRAS* G12C inhibitors, demonstrated significant clinical efficacy and tolerability in previously treated patients with advanced *KRAS* G12C-mutated disease, establishing them as second-line standards of care. However, through several mechanisms, such as direct modifications of *KRAS*, activation of alternative pathways, and morphological changes, resistance to these drugs undermines the durability of response when these treatments are given in isolation.

Novel inhibitors and combination therapies are now being explored to overcome this resistance and extend treatment to additional *KRAS* mutant subtypes. Future treatments will be based on personalized strategies, given the significant biological heterogeneity of the disease driven by *KRAS* mutations. Novel approaches, such as oncolytic vaccines and adoptive T-cell treatments, also hold promise for improving the therapeutic landscape.<sup>11</sup> Data regarding *KRAS* mutation patterns in lung cancer patients from Iraq remain limited. Therefore, investigating these mutations in the Iraqi population may help elucidate regional molecular profiles and support the implementation of precision oncology strategies in local clinical settings.

This work investigates the frequency and molecular characteristics of *KRAS* mutations in lung cancer, highlighting their clinical role and impact on therapeutic outcome. By testing mutations within critical codons, namely 12, 13, 59, 61, 117, and 146, this study aims to deeply investigate how these mutations contribute to resistance to therapy (particularly anti-EGFR drugs). This study highlights the need for molecular diagnosis to support tailored therapeutic strategies and improve clinical management in NSCLC.

## 2. Materials and methods

### 2.1. Sample collection

Thirty formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected from 30 patients with NSCLC, including both males and females, who were treated at the Baghdad Medical City and Al-Yarmouk Teaching Hospital in Iraq. Then, they underwent molecular investigation to detect *KRAS* mutations using polymerase chain reaction (PCR) followed by reverse hybridization.

### 2.2. Molecular study

#### 2.2.1. DNA extraction kit

In this work, DNA from FFPE tissues was extracted using a FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). The contents of the extraction kit are presented in [Table 1](#).

#### 2.2.2. DNA extraction reagents

The DNA extraction reagents included:

- Xylene: Xylene was used for the deparaffinization of FFPE tissue samples. For each sample, 500  $\mu$ L of xylene was added to 25 mg of tissue in a 1.5 mL microcentrifuge tube.
- Beta-mercaptoethanol ( $\beta$ -ME):  $\beta$ -ME was used during the extraction process as a reducing agent to facilitate nucleic acid purification.<sup>12</sup> Approximately

200  $\mu$ L of  $\beta$ -ME was added to 25 mg of tissue in a 1.5 mL microcentrifuge tube.

**Table 1. Contents of the DNA extraction kit (from paraffin-embedded tissue)**

Components	K-3032 (100 reactions)
FATG1 buffer	30 mL
FATG2 buffer	30 mL
W1 buffer	44 mL
Wash buffer	20 mL
Elution buffer	30 mL
Proteinase K	11 mg ( $\times 2$ )
Collection tube	200 pcs
Elution tube	100 pcs
FATG mini column	100 pcs
Micropestle	100 pcs

### 2.2.3. DNA extraction

In general, FFPE tissues are widely used for long-term preservation of clinical samples and enable correlation of genetic alterations with disease pathology.<sup>13</sup> However, formalin fixation can cause protein-DNA and DNA-DNA cross-linking, which may reduce DNA quality for downstream molecular analyses.<sup>14</sup>

Genomic DNA was extracted from FFPE tissues using the FavorPrep Tissue Genomic DNA Extraction Kit according to the manufacturer's protocol. Briefly, 25 mg of tissue was deparaffinized with xylene, followed by ethanol washing. The samples were then lysed with FATG1 buffer and Proteinase K, followed by incubation to ensure complete digestion. After lysis, FATG2 buffer and ethanol were added; then, the mixture was transferred to a FATG mini column for purification. The column was washed using W1 and Wash buffers, and purified DNA was eluted with 50  $\mu$ L of elution buffer or nuclease-free water. The extracted DNA was stored at 4 °C for immediate use or at -20 °C for long-term storage.

### 2.2.4. DNA quantification

It is not possible to use UV spectrophotometry to quantify the amount of DNA present in FFPE tissue samples because the amount of DNA is overestimated when it is measured

at 260 nm. In this study, the Invitrogen Qubit method was used to quantify the DNA content in FFPE tissue samples through a fluorometer (Qubit 4, Thermo Fisher Scientific, US).

### 2.2.5. Polymerase chain reaction

Following DNA quantification, PCR was performed to detect *KRAS* mutations. The PCR reaction mixture (15  $\mu$ L total volume) consisted of Taq DNA polymerase, DNA template, and PCR buffer. Taq DNA polymerase was diluted using Taq dilution buffer to a final concentration of 0.2 U/ $\mu$ L before use. The prepared reaction mixtures were loaded into a thermocycler (Veriti 96-Well, Applied Biosystems, USA), and amplification was carried out under the thermal cycling conditions described in Table 2.

### 2.2.6. Hybridization of amplification

Hybridization of PCR products was performed using a reverse-hybridization strip assay (*KRAS* StripAssay®, ViennaLab Diagnostics GmbH, Austria) according to the manufacturer's instructions:

- **Step 1: Sample preparation**  
Ten microliters (10  $\mu$ L) of DNAT was added to each lane of the typing tray, followed by 10  $\mu$ L of the PCR amplification product. The mixture was gently pipetted to ensure proper mixing.
- **Step 2: Hybridization and incubation**  
One milliliter (1 mL) of hybridization buffer preheated to 45 °C was added to each lane, and the corresponding test strip was inserted. Hybridization was carried out in a shaking water bath at 45  $\pm$  1 °C for 30 min.
- **Step 3: Post-hybridization**  
After incubation, the hybridization solution was removed, and the test strips were immediately processed for subsequent washing and detection.

### 2.2.7. Stringent wash

Following hybridization, a series of washes was conducted to remove nonspecifically bound materials:

- Each lane was washed with 1 mL of Wash Solution A preheated to 45 °C for 10 s, and the solution was removed.
- A fresh 1 mL aliquot of Wash Solution A (at 45 °C) was added to each lane.
- Samples were incubated in a shaking water bath at 45 °C for 15 min, after which the solution was removed.
- 1 mL of prewarmed Wash Solution A (45 °C) was added again.
- Samples were incubated for an additional 15 min at 45 °C, and the washing solution was removed to complete the washing process.

Table 2. Thermal profile of *KRAS* gene expression

Cycle step	Temperature (°C)	Time (min)	Cycles no.
Initial denaturation	94	2	1
Denaturation	94	1	1
Primer annealing	70	0.83	
Elongation	56	0.83	- 30
Extension	60	1	
Final extension	60	3	1

### 2.2.8. Color development

For color development, 1 mL of Conjugate Solution was added to each lane, and the mixture was incubated at room temperature for 15 min. The strips were then rinsed with 1 mL of Wash Solution B for 10 s, followed by two washes with 1 mL of Wash Solution B, each with 5 min incubation at room temperature.

Subsequently, 1 mL of Color Developer was added, and the mixture was incubated at room temperature in the dark for 15 min. A positive reaction appeared as a purple stain on the test strips. After color development, the strips were washed with distilled water and dried. [Figure A1](#) shows the test strip design and representative patient test strip results.

### 2.2.9. Quality control and assay validation

*KRAS* mutations were detected using a commercially available reverse-hybridization strip assay that identifies hotspot mutations at codons 12, 13, 59, 61, 117, and 146. The assay includes built-in controls to ensure it has worked and that the result is accurately represented. Positive controls ensure that amplification and hybridization were successful, while negative controls ensure that there is no contamination. Although no confirmation was obtained by sequencing (either Sanger or next-generation), this assay is useful for detecting mutations in FFPE samples and is highly analytically sensitive for detecting mutant alleles.

### 2.3. Statistical analysis

The study was designed as a descriptive molecular analysis. Therefore, results were summarized as frequencies and percentages using SPSS (Version 27.0, IBM Corp, USA). Inferential statistical tests, such as the chi-square or Fisher's exact test, were not performed because the cohort consisted of only *KRAS* mutation-positive patients, and the sample size was insufficient for meaningful comparative

analysis.

## 3. Results and discussion

### 3.1. Clinical characteristics

#### 3.1.1. Age distribution

In the present study, NSCLC was diagnosed in patients aged 30 to 85 years, indicating that the disease is more common among middle-aged and older persons. These patients were categorized into age groups, with their distribution summarized in [Table 3](#).

Table 3. Age distribution of non-small-cell lung cancer patients

Age group (years)	Number of patients
30–39	6
40–49	4
50–59	9
60–69	2
70–79	6
80–89	3

These findings indicate that NSCLC occurs mostly in the age range of 50 to 70 years, aligning with the epidemiological evidence of higher chances of developing NSCLC within this age range. Many studies have established that the incidence of NSCLC rises dramatically after age 50 and continues to increase with age.<sup>15</sup> Of late,

there have been reports of a trend toward early-onset NSCLC (diagnosed before age 50), potentially due to genetic susceptibility, lifestyle factors, and environmental exposures.<sup>16</sup>

### 3.1.2. Interpretation of results

The genotype for each sample was determined using the Collector™ sheet. The processed Teststrip was placed in a specified region on the Teststrip Design and meticulously aligned using the red line at the top and the green line at the bottom as references. The strip was subsequently affixed with adhesive tape.<sup>17</sup>

The appearance of a positive signal on the top control line verifies the correct performance of both the conjugate solution and the color developer and should always be present. A positive result of the PCR positive control confirms that the PCR reagents and DNA template were of sufficient quality to detect *KRAS* mutations. If this control showed a negative result, the procedure was repeated from the beginning, starting with DNA extraction. On the other hand, a negative result of the PCR negative control indicates that the amplification of wild-type *KRAS* codons (12/13, 59/60/61, 117, and 146) was effectively suppressed. A positive result in this control may be due to an overly concentrated DNA template in the PCR reaction.<sup>18</sup>

### 3.1.3. Distribution of lung cancer patients according to disease stages

Table 4 shows that lung cancer patients were diagnosed as stage II (61%) in a majority of cases. In this cohort, the breakdown was 30% for stage IIA, 33% for stage IIB, and 20% for stage IIC. As expected, the prevalence of stage III disease was lower (17% of patients), but there was considerable heterogeneity in the subtypes. These findings align with previous studies, highlighting the role of cancer staging in understanding disease progression and clinical prognosis.<sup>19,20</sup>

### 3.2. *KRAS* mutation detection

This study included 30 patients with lung cancer with a *KRAS* mutation (Table 5).

The distribution of *KRAS* mutations across hotspot codons showed that the most frequent alterations occurred in codons 60/61(50%), followed by codon 12 (36.7%). In contrast, mutations at codons 13, 59, and 117/146 were less common.

*KRAS* mutations are important markers for lung cancer, influencing treatment strategies, especially in predicting resistance to EGFR-targeted therapies. A lot of emphasis has been placed on codons 12, 13, 59, 60, 61, 117, and 146 to ascertain the frequency and clinical importance of their mutations.<sup>21-24</sup>

Mutations at codon 12 are one of the most common genetic alterations in *KRAS*-driven lung cancers. The current study also reported various point mutations and insertions/deletions that took place at this locus, including c.35G > C, c.34G > C, c.35G > A, c.34G > T, c.35G > T, and complex variants such as c.34\_35delGGinsAT and c.34\_35delGGinsCT. Amino acid alterations due to these genetic changes include a wide range of missense substitutions, often glycine replaced by valine, aspartate, cysteine, or serine, resulting from changes in the nucleotide sequence; this constitutively activates the *KRAS* protein.<sup>25-27</sup> In this case, the protein becomes resistant to GTPase-activating proteins (GAPs) because it remains permanently active. The continuous signaling persists through the activation of downstream pathways that support proliferation and survival, promoting tumor development. In a clinical context, codon 12 mutations are associated with poor patient prognosis, increased propensity for metastasis, and reduced responses to EGFR-targeted therapy.

The second important spot for *KRAS* mutations occurs

**Table 4. Distribution of the study sample based on disease stages**

TNM stages	Staging	No. of patients
IIA	T2N0	9 (30%)
IIB	T2N1	10 (33%)
IIC	T3N2	6 (20%)
III	T3N3	5 (17%)

Abbreviation: TNM: Tumor, node, and metastasis.

Table 5. Distribution of *KRAS* mutations in the studied patients

No. of patients	Codon	Mutation	Mutation detected	Percentage (%)
11	12	c.35G > C, c.34G > C, c.35G > A, c.34G > T, c.34_35delGGinsAT, c.34_35delGGinsCT, c.34G > A, c.35G > T	+	36.7
1	13	c.38G > C, c.37G > C, c.38G > A, c.37G > T, c.37G > A, c.38G > T	+	3.3
2	59	c. 176C > A, c. 176C > G, c.175G > A	+	6.7
15	60 and 61	c. 179G > T, c. 182A > G, c. 183A > C, c.183A > T, c. 182A > T, c. 181C > A	+	50.0
1	117 and 146	c.351A > C, c.351A > T, c.349A > G, c.436G > C, c.436G > A, c.437C > T	+	3.3

at codon 13, where mutations include c.38G > C, c.37G > C, c.38G > A, c.38G > T, c.37G > A, and c.37G > T. Of all the mutations at this codon, the most studied is the c.38G > A mutation, which changes the amino acid from glycine to aspartate (i.e., G13D).<sup>28</sup> As with mutations at codon 12, mutations at codon 13 also activate *KRAS* by inhibiting GTP hydrolysis, thereby maintaining *KRAS* in the active GTP-bound form. Although it has been suggested that patients with the G13D mutation may have a slightly better response to anti-EGFR therapy compared to those with mutations at codon 12, this has not been conclusively determined. Interestingly, mutations at codon 13 are associated with a milder clinical outcome than those at codon 12, despite both being resistant to EGFR-targeted therapy.<sup>20</sup>

Regarding mutations at codon 59, mutations such as c.176C > A, c.176C > G, and c.175G > A have been noted. These mutations alter the normal functioning of *KRAS*. Although it is less common than mutations at codons 12 and 13, mutations at codon 59 increase GTP affinity and reduce GTPase activity. As a result, it continuously activates oncogenic pathways such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)–AKT pathways.<sup>29</sup> For example, for patient 15, mutations at codons 60 and 61, including c.179G > T, c.182A > G, c.183A > C, c.183A > T, and c.182A > T, occurred at exon 3, which is crucial for the intrinsic GTPase activity of *KRAS*. Among all mutations at codon 61, Q61H, Q61L, and Q61R have been noted to dramatically inhibit GTP

hydrolysis, thereby maintaining *KRAS* in the active form.<sup>30</sup> Furthermore, mutations at codon 61 have been observed in many cancers and are associated with a high propensity for transformation. These mutations have a remarkable impact on tumor development and are often associated with poor responses to traditional targeted therapies.

For cancers driven by *KRAS*, codons 117 and 146 are new non-hotspot positions with significant functional implications. For patient 1, the following mutations affecting these positions were observed: c.351A > C, c.351A > T, c.349A > G, c.436G > C, c.436G > A, and c.437C > T. The presence of these mutations, particularly those at codon 146 (e.g., A146T), is recognized in the literature as activating the *KRAS* gene by accelerating nucleotide exchange. These are rare occurrences; however, the literature now recognizes these as factors for the development of resistance to therapy. The presence of these mutations may also be a criterion for exclusion from EGFR therapy protocols for these patients.<sup>30</sup>

#### 4. Conclusion

Screening for mutations at codons 12, 13, 59, 61, 117, and 146 in this study revealed a very high rate of *KRAS* mutations among patients with metastatic lung cancer. This fact reinforces the gene's key involvement in driving tumor growth and metastatic spread. Our results further support the need to incorporate molecular diagnostics into clinical evaluation and therapeutic decision-making. PCR



combined with reverse hybridization has proved highly effective for identifying *KRAS* mutations, underscoring the need to incorporate molecular diagnostics into routine clinical practice for personalized therapy. Most patients in the present study were diagnosed as stage II; therefore, there is a possibility that more cases of *KRAS* mutations are present or detectable in an advanced stage of lung cancer. Pre-treatment mutation screening is necessary, as *KRAS* mutations reduce the effectiveness of anti-EGFR therapies, such as cetuximab and panitumumab. An individualized, genetic-profiling-based approach is necessary to achieve better clinical outcomes and avoid exposure to inefficient therapy. The growing importance of genetic analytical tools worldwide further justifies this trend. Genetic profiling will become routine in clinical management as precision oncology is rapidly evolving to optimize treatment and manage complexity in NSCLC.

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## Conflict of interest

The author declares no conflict of interest.

## Author contributions

This is a single-authored article.

## Ethics approval and consent to participate

This study is approved by Al-Nahrain University (approval no: 162/5/7) on March 2, 2025. Patients provided written informed consent prior to their participation in this study.

## Consent for publication

Patients gave consent for the publication of their anonymized data.

## Availability of data

The data are available from the corresponding author upon reasonable request.

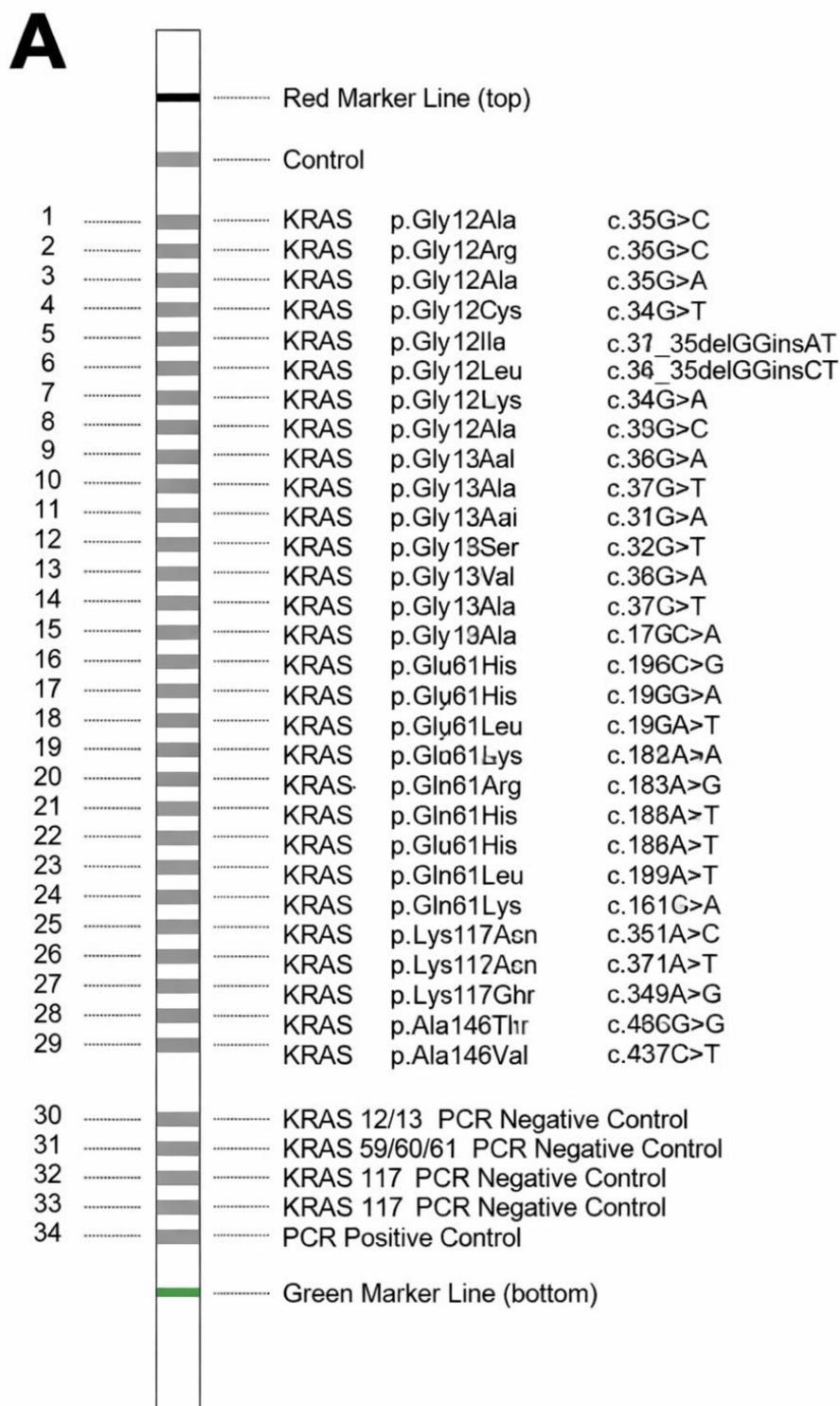
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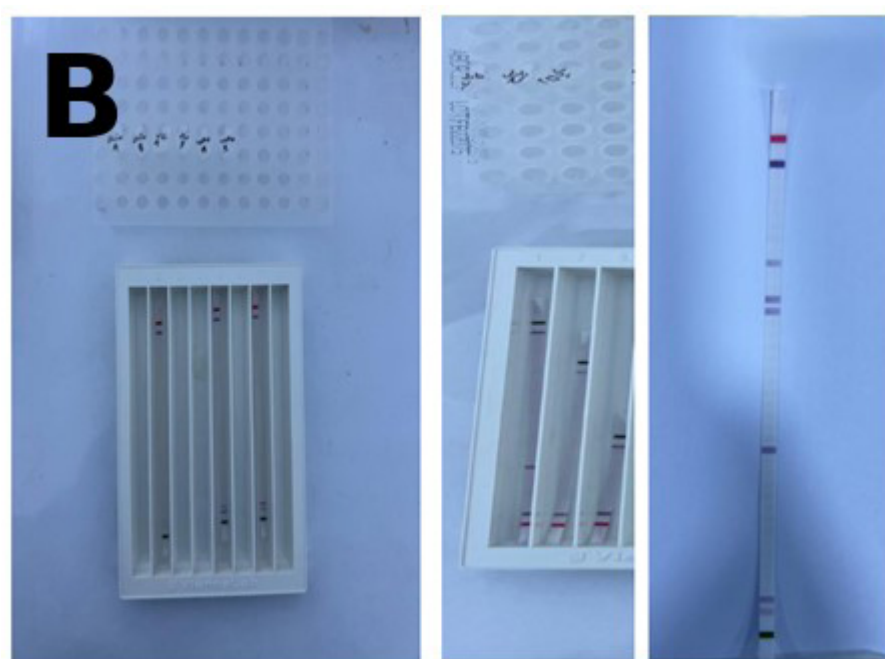
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## Appendix





**Figure A1.** KRAS strip assay results. (A) Test strip design and (B) A representative patient's test strip results.  
Abbreviation: PCR: Polymerase chain reaction.