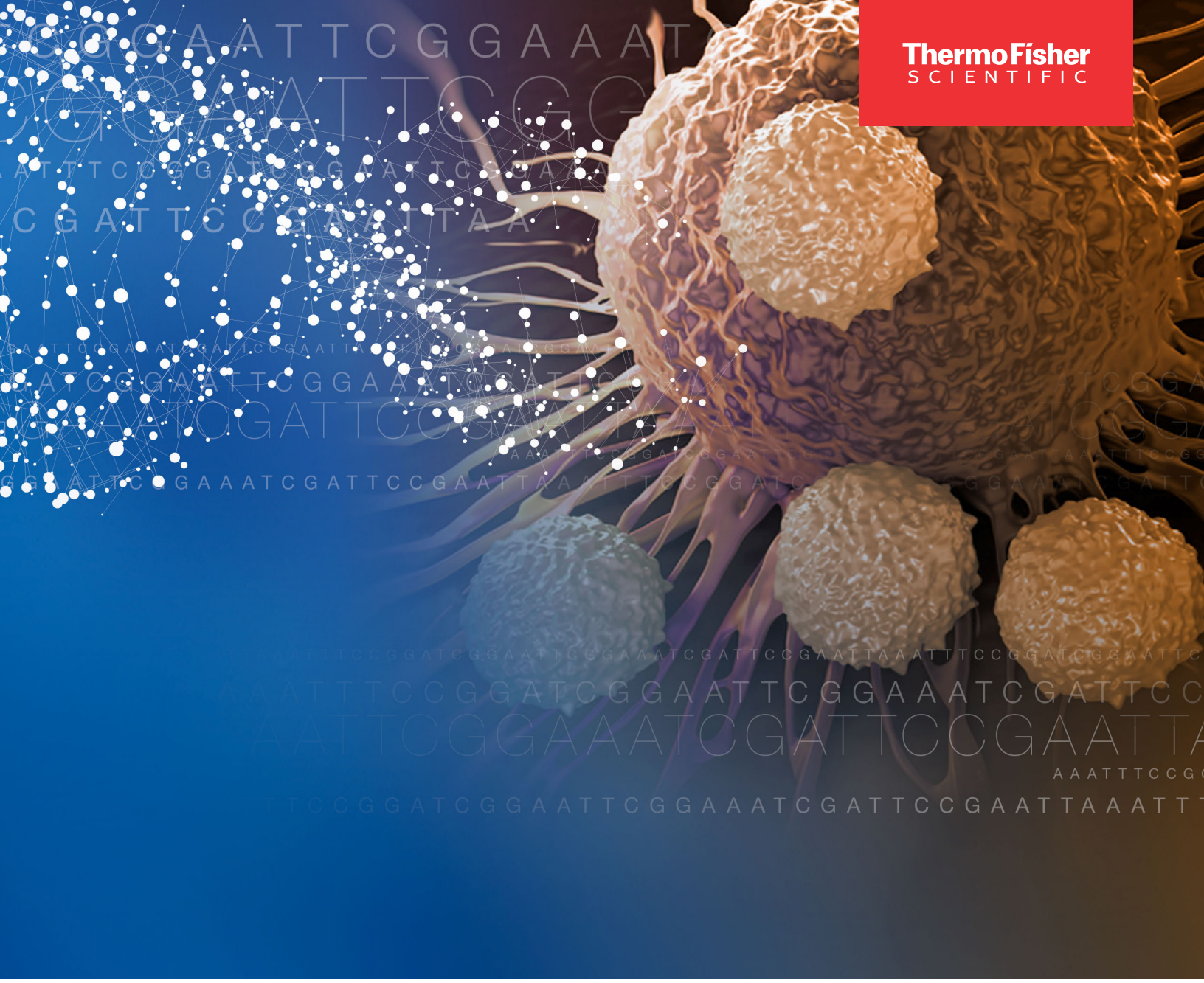

Thermo Fisher SCIENTIFIC

Sanger sequencing

Protocol guide: KRAS variant identification

applied biosystems

Introduction

Kirsten rat sarcoma viral oncogene homologue (KRAS) is a hyper mutated oncogene across all cancer types that is associated with poor prognosis[1]. It is the most frequently mutated of the three RAS isoforms, additionally, 1 in 7 of all human cancers have a KRAS mutation.

KRAS is a GTPase binding protein, and several common mutations can result in constitutively active KRAS. KRAS activation promotes cell proliferation, cell survival and tumor progression and have also been found in tumor adjacent cells and may influence the tumor microenvironment.

As previously mentioned, mutations within KRAS are associated with a series of highly fatal cancers, including pancreatic ductal adenocarcinoma (PDAC), non-small-cell lung cancer (NSCLC), and colorectal cancer (CRC). KRAS is part of the epidermal growth factor (EGFR) signaling cascade. These mutations make tumors unresponsive to cancer therapeutics targeting EGFR as well as other tyrosine kinase inhibitors (TKIs). This includes at least 10 FDA approved tyrosine kinase inhibitors TKIs and monoclonal antibodies. Furthermore, tumors that are treated with drugs targeting EGFR frequently become resistant to treatment. KRAS mutations that were not present prior to treatment may be detected after treatment, correlating with drug resistance.[2]

Since tumors with KRAS mutations are resistant to many cancer therapeutics, it is important to screen for them prior to and throughout treatment. Accurate KRAS characterization depends on Sanger sequencing due to its ability to accurately detect single or multiple mutations. Unlike other methods Sanger sequencing also allows identification and detection of novel mutations. It is the gold standard for mutation detection.

Detection of KRAS mutations is useful for diagnosis, monitoring, and potential treatments. Knowledge of specific KRAS mutations can inform potential treatments. FDA approved drugs are on the market that specifically target KRAS G12C mutations. Inhibitors targeting other KRAS mutations or multiple mutations are also in development. KRAS mutations result in EGFR signaling pathway activation in the absence of EGFR itself, making anti-EGFR therapeutic agents. Thus, here we define the sequencing protocol specifically developed to accurately detect KRAS mutations.

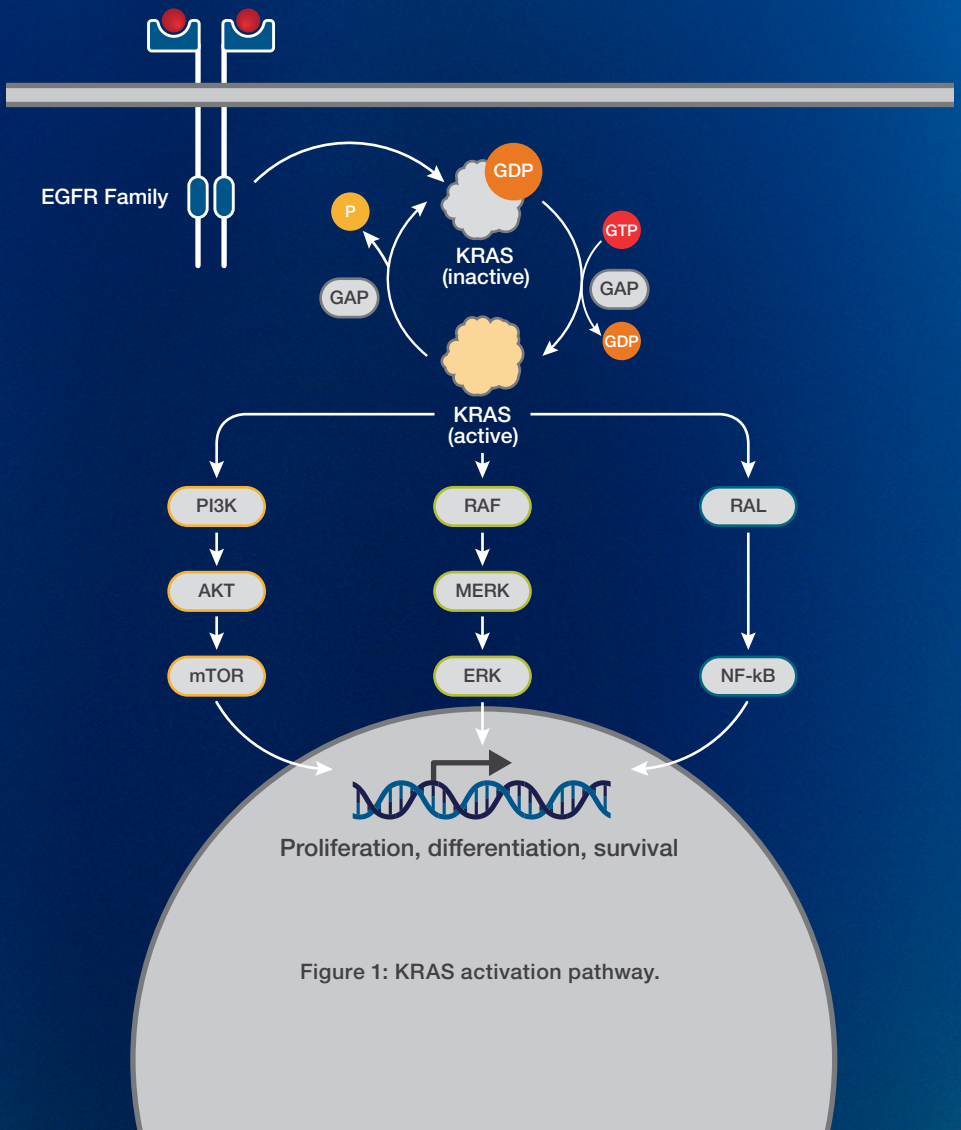

Figure 1: KRAS activation pathway.

KRAS mutation detection using Sanger sequencing

Ninety-eight percent (98%) of KRAS mutations occur within codons 12 or 13 (exon 2) and codon 61 (exon 3). A large variety of mutations within these codons exist and their frequency varies by cancer type. Thus, a robust KRAS assay offers the ability to identify known or previously unknown mutations across exon 2 and 3.

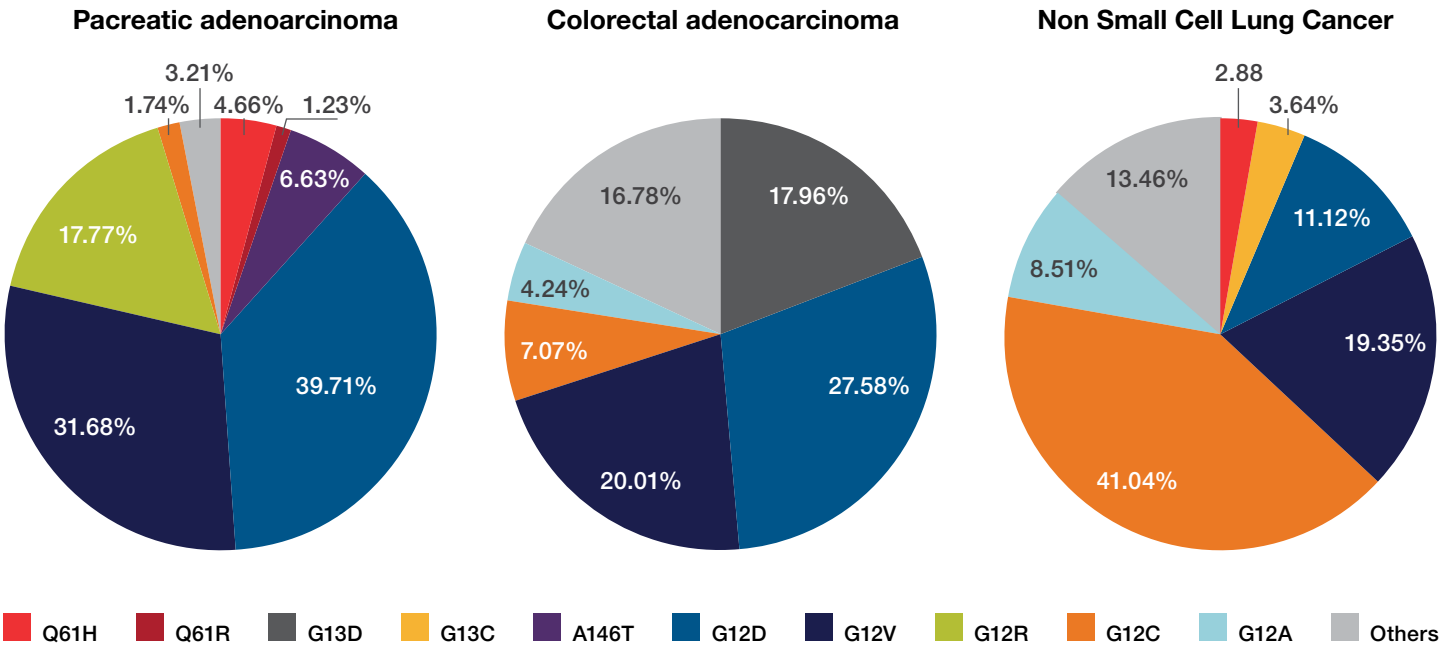

Figure 2. KRAS mutation rate by cancer type and mutation. Adapted from Huang L et al.[3]

Sanger sequencing is a highly accurate method for targeted identification of KRAS mutations with single base resolution. The scalable workflow and simple data analysis allow for rapid, reproducible identification of known and previously unknown variants. Here we present a KRAS sequencing protocol covering both exon 2 and exon 3 that can be performed in <6 hours provide:

- Pre-designed primers covering 8 known hotspots
- Identification of de novo mutations
- Bidirectional sequencing for confirmation
- Simple data analysis and scalable workflow
- Universal sequencing primers for streamlined protocol

Assay coverage

The Applied Biosystems KRAS assay uses a set of four primers: two forward, two reverse to amplify exon 2 and 3. The primers include M13 tails to enable universal sequencing. The PCR reaction produces two amplicons, as shown in figure 3.

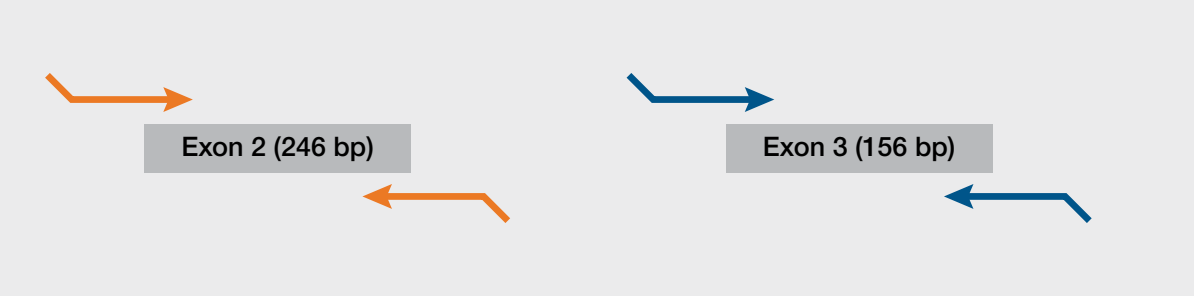

Figure 3. Schematic diagram illustrating the primer design strategy for the interrogation of the KRAS target exons.

Table 1. KRAS mutant nucleotide change, amino acid change and reference standard DNA's product ID for each of the KRAS mutation detections validated in this study.

KRAS genotype	Horizon Discovery DNA Product ID	KRAS mutation	Nucleotide change
G12D	HD272	Gly12Asp	GGT → GAT
G12V	HD289	Gly12Val	GGT → GTT
G12C	HD269	Gly12Cys	GGT → TGT
G12S	HD288	Gly12Ser	GGT → AGT
G12A	HD265	Gly12Ala	GGT → GCT
G12R	HD287	Gly12Arg	GGT → CGT
G13D	HD290	Gly13Asp	GGC → GAC
Q61L	HD140	Gln61Leu	CAA → CTA
WT	HD710		

Table 2. Primer Designer Sequencing assays for each of the KRAS mutation hotspots in four different codons.

Assay product id #	Mutated codon #	Amplicon bp
Hs00679698_CE	12,13	246
Hs00532900_CE	59,61	156
Hs00532827_CE	12,13	172

Assay workflow

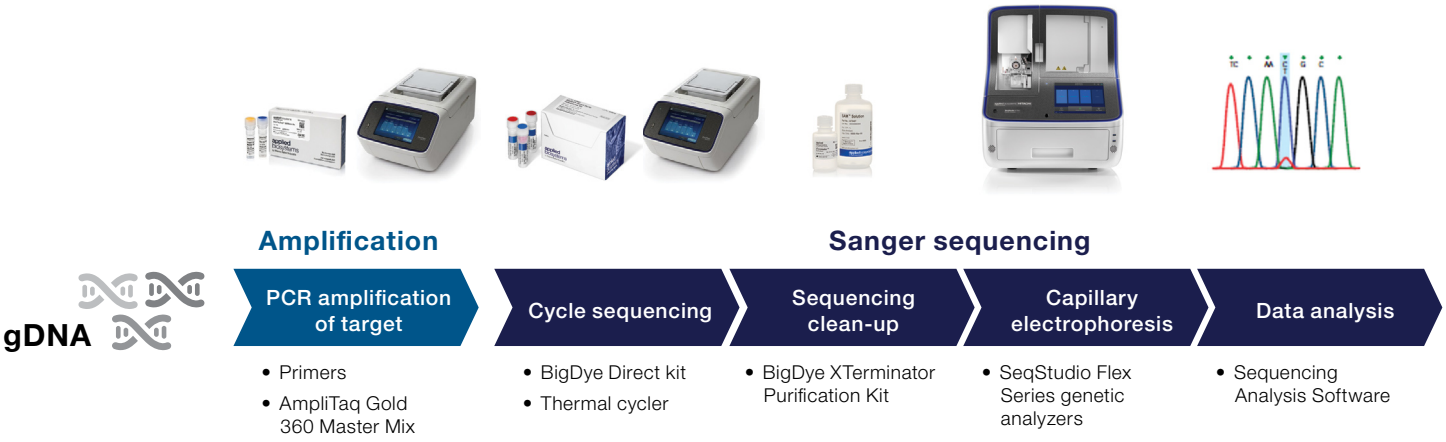

Figure 4. Sanger sequencing workflow to detect KRAS mutations.

Protocol

Important: This protocol is very sensitive; therefore, utmost care must be taken to prepare stock solutions and set up amplification reactions in an amplicon-free environment.

1. Materials needed

1.1 Equipment

Product	Supplier	Cat. No.
Applied Biosystems™ VeritiPro™ Thermal Cycler, 96-well	Thermo Fisher Scientific	A48141
Applied Biosystems™ SeqStudio™ 24 Flex Genetic Analyzer	Thermo Fisher Scientific	A53630
Applied Biosystems™ SeqStudio™ Flex Genetic Analyzer 24-Capillary Array	Thermo Fisher Scientific	A49107
Thermo Scientific™ Basic Vortex Mixers	Thermo Fisher Scientific	88882011
Single-channel and multichannel micropipettes of various sizes capable of pipetting volumes from 1.00µL to 1000.0µL	Major Lab Supplier (MLS)	Any
Cold block or ice	MLS	Any
Microcentrifuge and mini centrifuge	MLS	Any
Plate Centrifuge	MLS	Any

1.2. Reagents, kits, and consumables

Product	Supplier	Cat. No.
AmpliTaq Gold™ 360 Master Mix	Thermo Fisher Scientific	4398881
Invitrogen™ Nuclease-Free Water (not DEPC-Treated)	Thermo Fisher Scientific	AM9932
Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit	Thermo Fisher Scientific	4458689
Applied Biosystems™ BigDye XTerminator™ Purification Kit	Thermo Fisher Scientific	4376487
Applied Biosystems™ POP-7™ Polymer, for 3500/SeqStudio™ Flex	Thermo Fisher Scientific	4393708
Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate	Thermo Fisher Scientific	N8010560
Applied Biosystems™ Anode Buffer Container (ABC), for 3500/SeqStudio™ Flex	Thermo Fisher Scientific	4393927
Applied Biosystems™ Cathode Buffer Container (CBC), for 3500/SeqStudio™ Flex	Thermo Fisher Scientific	4408256
Applied Biosystems™ Hi-Di™ Formamide	Thermo Fisher Scientific	4311320
Applied Biosystems™ MicroAmp™ Clear Adhesive Film	Thermo Fisher Scientific	4306311
5ml Tube, PCR clean	MLS	Any
Sterilized aerosol barrier (filter) pipette tips	MLS	Any

1. PCR amplification of targets

1.1. For each sample, a forward and reverse reaction will be used. The initial PCR amplification therefore requires two identical reactions to be set up for each KRAS mutant DNA standard (as labeled). In our example reference DNA standards corresponding to common KRAS mutations were used. An example 96-well plate setup 9 samples and wt control is shown below:

PCR plate layout

	1	2	3	4
A	G12D_98_Rep1	Q61R_00_Rep1	G12D_98_Rep2	Q61R_00_Rep2
B	G12V_98_Rep1	Q61L_00_Rep1	G12V_98_Rep2	Q61L_00_Rep2
C	G12C_98_Rep1	WT_00_Rep1	G12C_98_Rep2	WT_00_Rep2
D	G12S_98_Rep1		G12S_98_Rep2	
E	G12A_98_Rep1		G12A_98_Rep2	
F	G12R_98_Rep1		G12R_98_Rep2	
G	G13D_98_Rep1		G13D_98_Rep2	
H	WT_98_Rep1		WT_98_Rep2	

Note: Positive and negative control (no-template control, NTC) samples can be run on the same plate or on different plates.

1.2 In each well of a 96-well PCR plate, combine:

1.2.1. 1.5 µL 10X M13-tailed sequencing amplification primer mix in duplicate (as suggested in the table above, section 1.1)

1.2.2. 5 µL 2X Applied Biosystems™ BigDye™ Direct PCR Master Mix

1.2.3. 2 µL purified DNA (20 ng total)

1.2.4. Water to 10 µL total volume

1.3. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x g.

1.4. Insert the plate into the thermal cycler and run the following program:

Parameter	Polymerase activation	Cycling (35 cycles)			1 cycle
		Denaturation	Annealing	Extension	
Temperature	95°C	96°C	58°C	70°C	72°C
Time	10 min	30 sec	30 sec	45 sec	7 min

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.
Note: Do not exceed 10 µL total reaction volume. The cycle sequencing and BigDye XTerminator purification steps have been optimized for 10 µL input volumes.

2. Cycle sequencing

2.1. Once the PCR in Protocol 2, step 1.4 is complete, the plate can be used directly for cycle sequencing.

2.2. Remove the seal from the plate.

2.3. To each well of the plate, add:

2.3.1. 3 µL Applied Biosystems™ BigDye™ Direct Sequencing Master Mix (supplied in kit)

2.3.2. 1 µL BigDye™ Direct M13 Fwd or M13 Rev primer (supplied in kit)

Note: It is important to add M13 Fwd primer to one of the duplicate PCR reactions, and M13 Rev primer to the other reaction. An example, based on the plate setup shown in step 1.1, is shown here:

The naming convention is the same as above with repeat replace with sequencing direction

Cycle sequencing plate layout

	1	2	3	4
A	G12D_98_M13_Fwd	Q61R_00_M13_Fwd	G12D_98_M13_Rev	Q61R_00_M13_Rev
B	G12V_98_M13_Fwd	Q61L_00_M13_Fwd	G12V_98_M13_Rev	Q61L_00_M13_Rev
C	G12C_98_M13_Fwd	WT_00_M13_Fwd	G12C_98_M13_Rev	WT_00_M13_Rev
D	G12S_98_M13_Fwd		G12S_98_M13_Rev	
E	G12A_98_M13_Fwd		G12A_98_M13_Rev	
F	G12R_98_M13_Fwd		G12R_98_M13_Rev	
G	G13D_98_M13_Fwd		G13D_98_M13_Rev	
H	WT_98_M13_Fwd		WT_98_M13_Rev	

Note: For each sample, forward reactions have light shading, and reverse reactions have darker shading.

2.4. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x g.

2.5. Insert the plate into a thermal cycler and run the following program:

Parameter	Polymerase activation	Post-PCR inactivation	Polymerase activation	Cycling (25 cycles)	
				Denaturation	Annealing
Temperature	37°C	80°C	96°C	96°C	50°C
Time	15 min	2 min	1 min	10 sec	5 sec

3. Sequencing cleanup

3.1. Spin the reaction plate at 1,000 x g for 1 minute, then remove the seal.

3.2. Prepare a mix with SAM Solution and XTerminator Solution in an appropriately sized tube. Cleanup will require 45 µL of SAM Solution and 10 µL of XTerminator bead solution per well.

3.2.1. Calculate the amount of SAM Solution and XTerminator bead solution needed for all samples.

3.2.2. Add the calculated volume of SAM Solution to a new tube using a conventional pipette tip.

Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to dissolve. Cool to room temperature before using.

3.2.3. Vortex the XTerminator bead solution bulk container at maximum speed for at least 10 seconds until the solution is homogeneous.

3.2.4. Using a wide-bore pipette tip, add the calculated volume of XTerminator Solution to the tube.

Important: Avoid pipetting from the top of the liquid.

3.2.5. Mix the tube of combined reagents until homogeneous.

3.3. Add 55 µL of the SAM Solution/XTerminator Solution mix to each well.

Important: Avoid pipetting from the top of the liquid. When pipetting into the plate, vortex the SAM Solution/XTerminator Solution mix every 8–10 wells to homogenize the bead mixture.

3.4. Seal the plate with MicroAmp Clear Adhesive Film. Make sure the plate is sealed well.

3.5. Vortex the reaction plate at 2,000 rpm for 20 minutes.

3.6. In a swinging-bucket centrifuge, spin the plate at 1,000 x g for 2 minutes.

4. Data collection

4.1. Make sure the instrument is calibrated with the correct sequencing standard (Z-dye set matrix and sequencing standard).

4.1.1. For details, see the user guide for your instrument.

4.2. Remove the MicroAmp Clear Adhesive Film and replace with a 96-well plate septum.

4.3. Load the plates into the genetic analyzer.

4.4. Select or create an appropriate run module according to your capillary length, number of capillaries, and polymer type on your instrument. The recommended default run modules are listed below:

4.4.1. For SeqStudio Flex Series instruments with 50 cm capillaries:

4.4.1.1. Instrument protocol: BDxFastSeq50_POP7

Note: If a 36 cm capillary is installed, the protocol name will be adjusted accordingly.

4.4.1.2. Dye set: Z_BigDye Direct.

4.4.1.3. Analysis settings: sequencing default.

4.4.2. For SeqStudio instruments:

4.4.2.1. Instrument protocol: MedSeqBDX.

4.4.3. For 3500xL instruments with 50 cm capillaries:

4.4.3.1. Instrument protocol: BDxFastSeq50_POP7xl_Z.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

4.4.3.2. Analysis module: BDTv3.1_PA_Protocol-POP7.

4.4.4. For 3730xl instruments with 50 cm capillaries:

4.4.4.1. Instrument protocol: BDX_FastSeq_POP7_Z.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

4.4.4.2. Analysis protocol: 3730BDTv3-KB-DeNovo_v5.2.

5. Analysis of results using a sequencing program

In our example the sequencing results were analyzed by SeqScape v4.1 software. SeqScape Software is a resequencing package designed for mutation detection and analysis, SNP discovery and validation, pathogen sub-typing, allele identification, and sequence confirmation. Click here to learn more about purchasing a SeqScape 4.1 license.

Alternatively, Sequence Scanner Software v2.0 can be used. This is a free software for viewing electropherograms. It provides an easy way to perform a high-level quality check of sequencing data or a general data review that includes summary tables and electropherograms as well as a general raw or analyzed view for .ab1 files.

5.1. To obtain the software, go to resource.thermofisher.com/pages/WE28396.

5.2. Using Sequence Scanner Software v2.0, generate a QC report. For each sequencing trace, determine the trace score, CRL, and the QV20+ score.

5.3. Suggested acceptance criteria:

5.3.1. A sequencing trace is acceptable if two of the three thresholds are met:

5.3.1.1. Trace score greater than 31

5.3.1.2. CRL greater than 50

5.3.1.3. QV20+ greater than 50

5.3.2. Sequencing traces that do not fit the above criteria are indeterminate and should be repeated.

5.3.3. Using BLAST software or other sequence alignment tool, align positive traces to the reference sequence of the target.

5.3.3.1. Ensure homology to the reference sequence meets your acceptance criteria.

5.4. Test validity, failures, and retests:

5.4.1. Test runs that fail for reasons not attributable to system performance, such as equipment malfunction, operator error, or other demonstrable cause will be designated as an invalid run. Invalid runs will be retested and documented in the study report(s).

Sample data

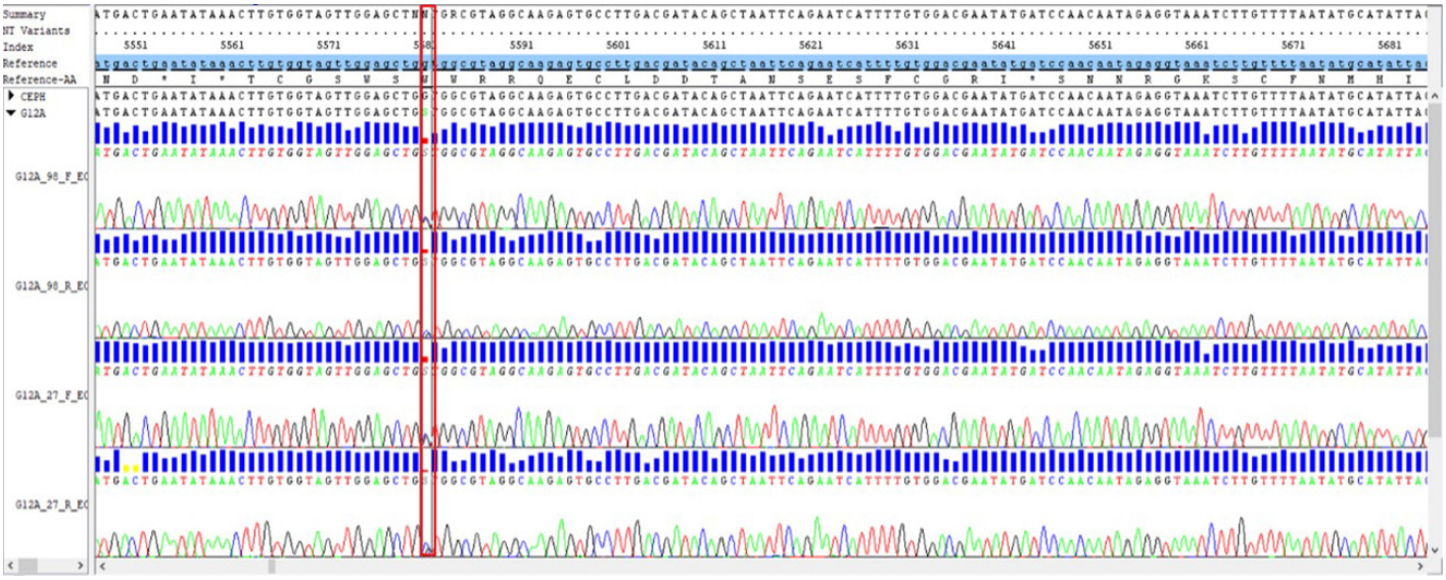

Fig. 5 Detection of KRAS G12A mutation using two different PCR primer assays and forward and reverse sequencing primers.

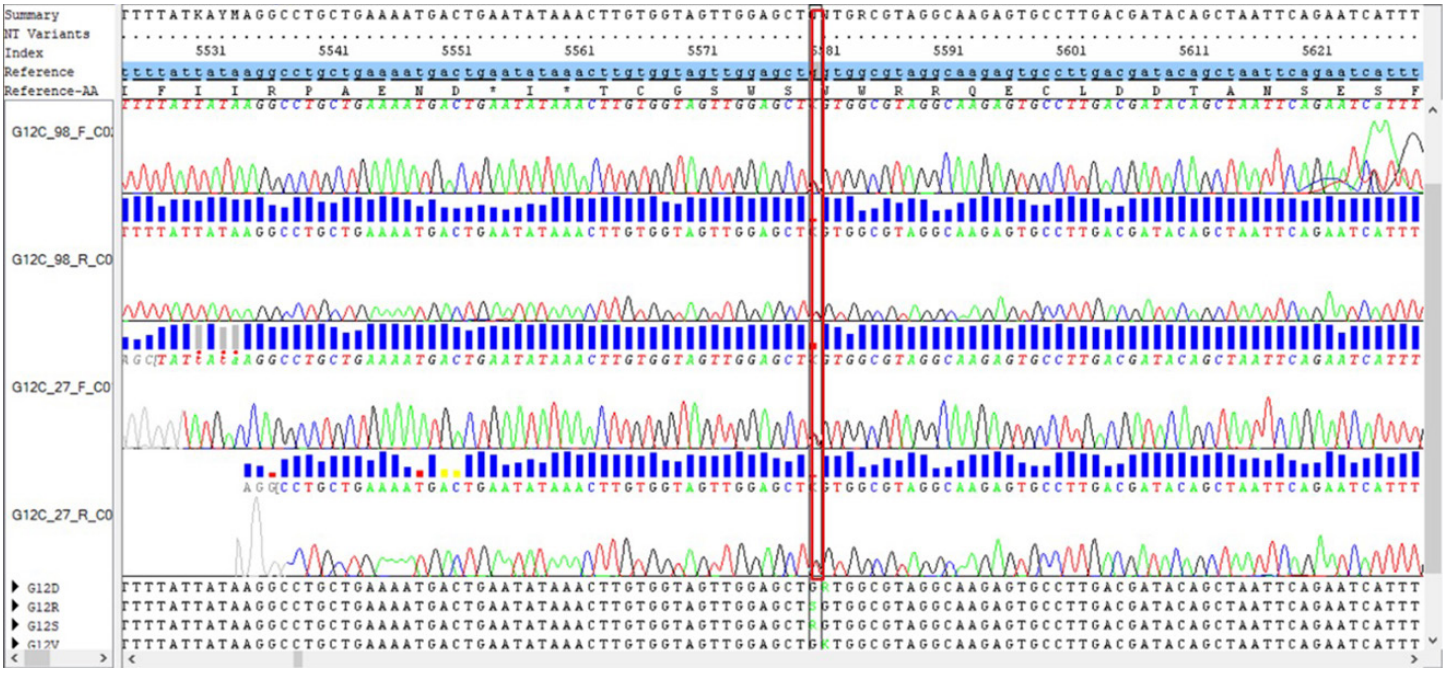

Fig. 6 Detection of KRAS G12C mutation using two different PCR primer assays and forward and reverse sequencing primers.

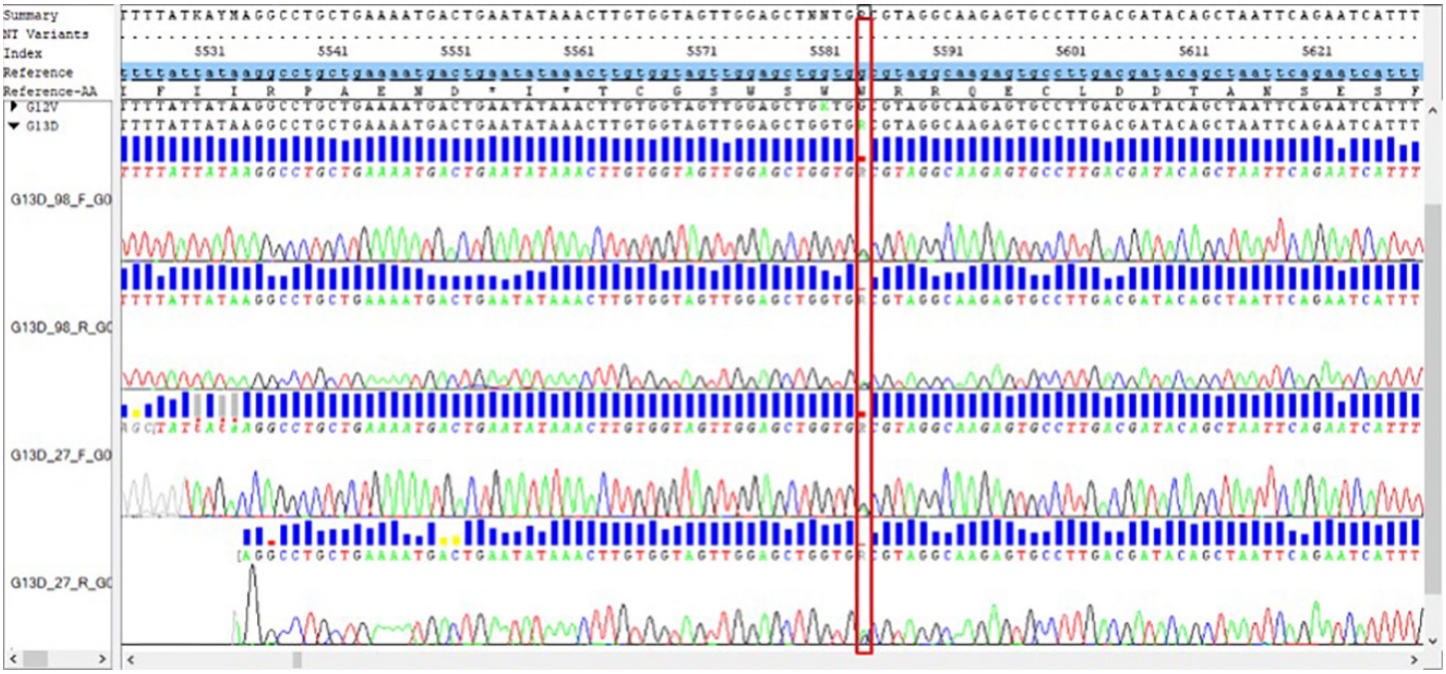

Fig. 7 Detection of KRAS G13D mutation using two different PCR primer assays and forward and reverse sequencing primers.

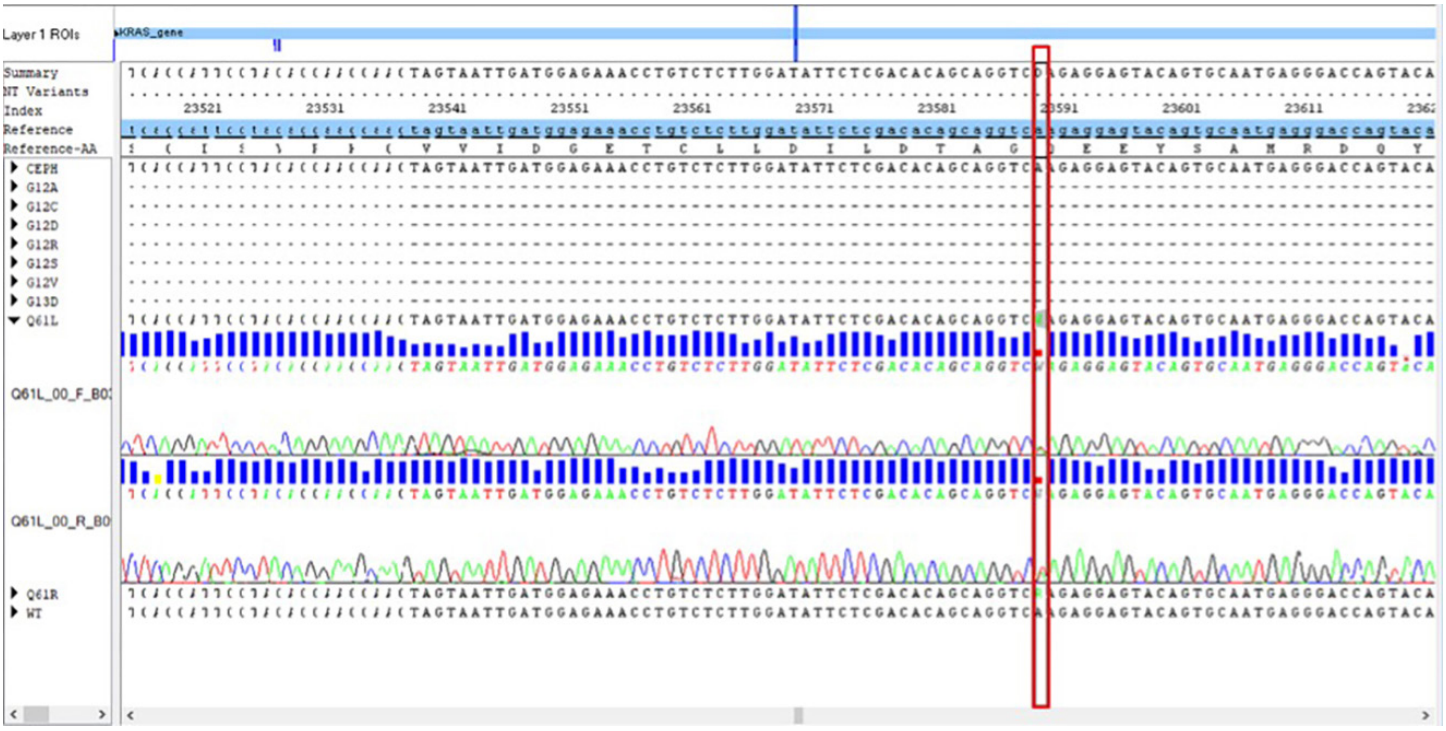

Fig. 8 Detection of KRAS Q61L mutation using two different PCR primer assays and forward and reverse sequencing primers.

Conclusion

Accurate detection of KRAS mutations is important for cancer treatment.

Using this protocol and kit you can:

- Detect new and known KRAS mutations within exons 2 and 3
- Save time and reduce steps by using Big Dye Terminator
- Be flexible in the instrument platform that you use

Choose the genetic analyzer that is right for you

	SeqStudio Genetic Analyzer	SeqStudio Flex Series genetic analyzers	3500 Series genetic analyzers	3730*xl* DNA Analyzer
	Easy-to-use, flexible system	**Easy-to-use, flexible, connected system**	**Meets the needs of verified and process-controlled environments**	**Maximum throughput, scalability, and flexibility**
				UPGRADED 
Number of capillaries	4	8 or 24	8 or 24	48 or 96
Capillary array length (cm)	28	36, 50	36, 50	36, 50
Sample capacity	1 plate; 96-well plate; 8-strip tube (compatible)	4 plates; 96- or 384-well plates; 8-strip tube (compatible)	2 plates; 96- or 384-well plates; 8-strip tube (compatible)	16 plates; 96- or 384-well plates
Continual plate loading	No	Yes	No	Yes
Sample reprioritization	No	Yes	Yes	No
Polymer type	POP-1, integrated into click-in cartridge	POP-6, POP-7, and POP-4	POP-6, POP-7, and POP-4	POP-6, POP-7, and conformational analysis polymer
Radio-frequency ID	Yes	Yes	Yes	No
Configuration	Integrated computer with touchscreen; optional desktop	Integrated computer with touchscreen; optional desktop	External desktop required	External desktop required
Amazon™ Alexa™ voice command	No	Yes	No	No
Remote monitoring and data sharing	Yes	Yes	No	No
Integrated remote troubleshooting tools	No	Yes	No	No
Connectivity	USB, Ethernet ports, and Wi-Fi dongle	USB, Ethernet ports, and Wi-Fi dongle	Ethernet port	Ethernet port
Connectivity with Thermo Fisher™ Connect Platform	Yes	Yes	No	Yes

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5. Chalela Rengifo RJ et al. (2021). EGFR and KRAS mutations in lung parenchyma of subjects with EGFR/KRAS wild-type lung adenocarcinoma. Pathol Oncol Res 27: 598292. doi:10.3389/pore.2021.598292.
6. Zhu G. (2021). Role of oncogenic KRAS in the prognosis, diagnosis and treatment of colorectal cancer. Molecular cancer 20(1):1-17. Doi:10.1186/s12943-021-01441-4.

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