TRUPCR™

TRUPCR®KRAS Kit

Detection of *KRAS* codons 12, 13, 59, 61, 117 & 146 mutation

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CE IVD

TRUPCR® KRAS Kit

Version 1.0

Detection of *KRAS* codons 12, 13, 59, 61, 117 & 146 mutation

CE IVD

REF Product No.: 3B1261/3B1262

24 tests/ 48 tests

Temperature limitation

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Manufactured By-

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INTENDED USE

The TRUPCR® KRAS Mutation Kit (K-RAS Kit) is an in vitro diagnostic test intended for the qualitative detection of *KRAS* somatic mutations in the genomic DNA extracted from fresh, frozen or formalin fixed paraffin-embedded (FFPE) tissue.

KRAS MUTATIONS AND CANCER

The KRAS gene encodes a small GTPase that plays a key role in transducing signals from the epidermal growth factor receptor (EGFR) to downstream effectors. KRAS mutations have been commonly found in several types of human malignancies, such as metastatic colorectal cancer (mCRC), lung adenocarcinoma and thyroid cancer (1-3). The most common mutations are found in codons 12, 13 and 61. Several studies have demonstrated that tumors carrying any of these mutant forms of the KRAS gene are less likely to respond to anti-EGFR antibody therapy (4-5). Such mutations in the KRAS oncogene are present in around 40% of cases (6). The American Society of Clinical Oncology (ASCO) recently released its first Provisional Clinical Opinion (PCO) suggesting that all patients to be administered anti-EGFR monoclonal antibody therapy (e.g. cetuximab, panitumumab and erlotnib) should be screened for KRAS mutations.

KRAS Detectable mutations:

Exon	Codon	Mutation	Nucleotide Change
2	12	G12C	c.34G>T
		G12S	c.34G>A
		G12R	c.34G>C
		G12V	c.35G>T
		G12D	c.35G>A
		G12A	c.35G>C
2	13	G13D	c.38G>A
3	59	A59T	c.175G>A
		A59E	c.176 C>A
		A59G	c. 176 C>G
3	61	Q61K	c.181C>A
		Q61L	c.182A>T
		Q61R	c.182A>G
		Q61H	c.183A>T
		Q61H	c.183A>C
4	117	K117E	c.349A>G
		K117R	c.350A>G
		K117N	c.351A>C
		K117N	c.351A>T
4	146	A146T	c.436G>A
		A146P	c.436G>C
		A146V	c.437C>T

PRINCIPLE

The TRUPCR KRAS Kit is based on allele specific amplification and is achieved by ARMS PCR.

Taq DNA polymerase is extremely effective at distinguishing between a match and a mismatch at the 3'-end of a PCR primer. Specific mutated sequences can be selectively amplified, even in samples where the majority of the sequences do not carry the mutation as:

- When the primer is fully matched, the amplification proceeds with full efficiency.
- When the 3'-base is mismatched, no efficient amplification occurs.

The kit is designed to selectively amplify mutant specific sequences in samples that contain a mixture of wild-type and mutated DNA. In real-time PCR, the fluorescent signal is generated from the presence of an oligonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5' end and a quencher molecule on its 3' end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Fluorescence resonance energy transfer (FRET).The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. As a result, the fluorescent dye and quencher dye are separated, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of DNA copies in a sample and its value allows qualitative comparisons of analyzed and control samples.

The detection is achieved using fluorescent probes labelled with FAM and HEX. The TRUPCR KRAS Kit is composed of 11 assays for the detection of the *KRAS* mutations and a reference control gene of *KRAS* region without any known polymorphism/mutation. The kit contains primers and probes for the detection of the all target (FAM) as well as an endogenous control gene (HEX). Endogenous control gene is included to verify the amplification procedure and also the possible presence of inhibitors, which may cause false negative results.

LIST OF DETECTABLE MUTATIONS BY TRUPCR® KRAS KIT

Mutation	Nucleotide Change Detection	Remarks
KRAS G12C	34G>T	
KRAS G12S	34G>A	
KRAS G12R	34G>C	
KRAS G12V	35G>T	
KRAS G12D	35G>A	
KRAS G12A	35G>C	
KRAS G13D	38G>A	
KRAS A59x	A59T (175G>A)	It detects 3 mutations but does not distinguish between them.
	A59E (176C>A)	
	A59G (176C>G)	
KRAS Q61x	Q61K (181C>A)	It detects 5 mutations but does not distinguish between them.
	Q61L (182A>T)	
	Q61R (182A>G)	
	Q61H (183A>C)	
	Q61H (183A>T)	
KRAS K117x	K117E (349A>G)	It detects 4 mutations but does not distinguish between them.
	K117R (350A>G)	
	K117N (351A>T)	
	K117N (351A>C)	
KRAS A146x	A146T (436G>A)	It detects 3 mutations but does not distinguish between them.
	A146P (436G>C)	
	A146V (437C>T)	
KRAS Reference	Detects *KRAS* region without any polymorphism/mutation.	

REAGENTS

The Kit contains amplification reagents for performance of 24/48 amplification reactions. Thaw and handle reagents on ice. Do not freeze/thaw Kit vials repeatedly.

Reagent	Description	Volume in µL 24 reactions	Volume in µL 48 reactions
Multiplex Master Mix	• Reaction Buffer • $MgCl_2$ and stabilizers • Hot-start DNA polymerase • dNTPs (dATP, dCTP, dGTP, dTTP)	720 µL x 4	720 µL X 8
KRAS G12C PPM (1)	• Primer and probe mix for G12C Mutation detection	60 µL	60 µL X 2
KRAS G12S PPM (2)	• Primer and probe mix for G12S Mutation detection	60 µL	60 µL X 2
KRAS G12R PPM (3)	• Primer and probe mix for G12R Mutation detection	60 µL	60 µL X 2
KRAS G12V PPM (4)	• Primer and probe mix for G12V Mutation	60 µL	60 µL X 2
KRAS G12D PPM (5)	• Primer and probe mix for G12D Mutation detection	60 µL	60 µL X 2
KRAS G12A PPM (6)	• Primer and probe mix for G12A Mutation detection	60 µL	60 µL X 2
KRAS G13D PPM (7)	• Primer and probe mix for G13D Mutation detection	60 µL	60 µL X 2
KRAS A59x PPM (8)	• Primer and probe mix for A59T, A59G and A59E Mutation detection	60 µL	60 µL X 2
KRAS Q61x PPM (9)	• Primer and probe mix for Q61K, Q61L, Q61R, Q61H, Q61H Mutation detection	60 µL	60 µL X 2
KRAS K117x PPM (10)	• Primer and probe mix for K117E, K117R, K117N, K117N Mutation detection	60 µL	60 µL X 2
KRAS A146x PPM (11)	• Primer and probe mix for A146T, A146P and A146V Mutation detection	60 µL	60 µL X 2
KRAS Reference PPM (12)	• Primer and probe mix for KRAS gene free from any known polymorphism/ mutation	60 µL	60 µL X 2
Internal Control PPM (13)	• Primer and probe mix for internal control	360 µL X 2	360 µL X 4
KRAS Positive Control	• Positive Control mix	300 µL	300 µL X 2
KRAS Negative Control	• Sterile Water	1000 µL	1000 µL X 2

DNA EXTRACTION

Specimen material must be human genomic DNA, extracted from fresh or formalin-fixed paraffin embedded tumour samples. DNA Extraction kits are available from various manufacturers. The procedure for extraction should be carried out according to the manufacturer's instruction. DNA quality should be assessed by Fluorometer or Spectrophotometer. The A260/A280 ratio should be 1.7–2.0. Smaller ratios usually indicate contamination by protein or organic chemicals. The qPCR reaction is optimized for minimum 20 ng (Fluorometer) or 40 ng (Spectrophotometer) of purified genomic DNA to detect the mutation in a background of wild type DNA.

Sample Material	Nucleic Acid Isolation Kit	Cat No.
Formalin fixed paraffin embedded tissue	QIAamp DNA FFPE Tissue Kit	56404

REAL TIME PCR PROTOCOL

REACTION PREPARATION: Prepare the reaction mix as follows:

Each sample must be amplified with 12 different mixes: **KRAS G12C (1)**, **KRAS G12S (2)**, **KRAS G12R (3)**, **KRAS G12V (4)**, **KRAS G12D (5)**, **KRAS G12A (6)**, **KRAS G13D (7)**, **KRAS A59x (8)**, **KRAS Q61x (9)**, **KRAS K117x (10)**, **KRAS A146x (11)**, **KRAS Ref (12)**.

Reagent	12C	12S	12R	12V	12D	12A	13D	A59x	Q61x	K117x	A146x	Ref
Multiplex Master Mix	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
12C PPM	2.5µl	-	-	-	-	-	-	-	-	-	-	-
12S PPM	-	2.5µl	-	-	-	-	-	-	-	-	-	-
12R PPM	-	-	2.5µl	-	-	-	-	-	-	-	-	-
12V PPM	-	-	-	2.5µl	-	-	-	-	-	-	-	-
12D PPM	-	-	-	-	2.5µl	-	-	-	-	-	-	-
12A PPM	-	-	-	-	-	2.5µl	-	-	-	-	-	-
13D PPM	-	-	-	-	-	-	2.5µl	-	-	-	-	-
A59x PPM	-	-	-	-	-	-	-	2.5µl	-	-	-	-
Q61x PPM	-	-	-	-	-	-	-	-	2.5µl	-	-	-
K 117x PPM	-	-	-	-	-	-	-	-	-	2.5µl	-	-
A146x PPM	-	-	-	-	-	-	-	-	-	-	2.5µl	-
Reference PPM	-	-	-	-	-	-	-	-	-	-	-	2.5µl
Internal Control PPM	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
Total	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**	**15µl**

a) Transfer 15 µl of the above prepared PCR Master Mix in 0.2 ml PCR tubes and close the tubes.

b) Add to the respective tubes/wells for each of the twelve assays

Negative Control: 5µl Sterile Water/well

Sample: 20-40 ng DNA/well (up to 5µl/well)

20 ng - Fluorometer

40 ng - Spectrophotometer

Positive Control: 5µl KRAS Positive Control/well

PROGRAM SET UP

a) Define the following setting for Temperature Profile and Dye Acquisition

Step	Program name	Temperature, °C	Time	Dye Acquisition	Cycles
1	Initial Denaturation	94	10 min	-	1
2	Denaturation	94	15 sec	-	40
	Annealing/Extension	60	60 sec	YES	

b) Define the following setting for channel selection

Detection	Detector Name	Reporter	Quencher	Gain Setup
KRAS	KRAS	FAM (Green)	None	Auto
Internal Control	IC	HEX (Yellow)	None	Auto

Reference Dye- Rox

REACTION CONTROL ANALYSIS

Reaction Control	KRAS Reference Mix	G12A, G12D, G12V, G12R, G12S, G12C, G13D, A59x, Q61x, K117x, A146x mix	Internal Control	
	Green	**Green**	**Yellow**	**Results**
Negative Control	Any Ct	Any Ct	Any Ct	Contamination: It is not possible to proceed with the analysis.
Positive Control	10 ≤ Ct ≤ 26	12≤ Ct ≤ 26	15 ≤ Ct ≤ 30	Proceed with the analysis of the sample.
	Ct<10or Ct >26	Ct<12 or Ct >26	Ct<15 or Ct >30	Error in the set up of the reaction or PCR, not possible to proceed with the analysis of the sample.

KRAS REFERENCE MIX ANALYSIS (FOR DNA ASSESSMENT)

KRAS Reference PPM	Green	Yellow	Results
DNA Samples	12≤ Ct ≤ 30	15≤ Ct ≤33	Proceed with the analysis of the sample
	Ct<12	Ct <15	Excess amount of DNA is present. Samples must be diluted with sterile water so that Ct falls in the range indicated above. Fall in the ranges indicated above.
	Ct>30	Ct>33	PCR inhibition or suboptimal amount of DNA: Not possible to proceed with analysis of the sample. 1. Proceed with a new DNA extraction to obtain higher concentration of DNA template or DNA of higher quality. 2. If inhibitors are present dilute the sample to decrease the inhibitors, it will affect the concentration of DNA.

MUTATION ANALYSIS

Samples that follows the passes the DNA assessment analysis are suitable and can be analysed to search for mutations

Compare ΔCt values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔCt values should be calculated with the following formula, taking care that the Ct value in FAM for the mutation and the equivalent for the reference assay belong to the same sample:

ΔCt = Ct Mutation – Ct Reference

Assay	Internal Control Amplification (Yellow Channel)	ΔCt ★	Results
G12C	OK (Almost similar Ct value for all mixes)	≤3.5	G12C Positive
G12S		≤7.0	G12S Positive
G12R		≤8.5	G12R Positive
G12V		≤6.5	G12V Positive
G12D		≤4.5	G12D Positive
G12A		≤7.5	G12A Positive
G13D		≤5.5	G13D Positive
A59x		≤4.0	A59x Positive
Q61x		≤4.0	Q61x Positive
K117x		≤5.5	K117x Positive
A146x		≤8.0	A146x Positive
G12C	OK (Almost similar Ct value for all mixes)	≥3.5	Wild Type or below LOD
G12S		≥7.0	
G12R		≥8.5	
G12V		≥6.5	
G12D		≥4.5	
G12A		≥7.5	
G13D		≥5.5	
A59x		≥4.0	
Q61x		≥4.0	
K117x		≥5.5	
A146x		≥8.0	
G12C	No Signal	-	PCR Reaction Failed: insufficient DNA/ Error in PCR set up or dispensing/PCR inhibition.
G12S		-	
G12R		-	
G12V		-	
G12D		-	
G12A		-	
G13D		-	
A59x		-	
Q61x		-	
K117x		-	
A146x		-	

- If multiple assays show a ΔCt equal to or below the cut-off value, the signal giving the higher ΔCt is probably due to cross-reactivity. Although double mutants have been observed, these are rare. In this case the sample should be considered positive only for the mutation with the lowest ΔCt.

General recommendations for analysis of the sample:

1. Analyse the negative control and the positive control. If they are in range of expected values, proceed with the analysis of the samples or the session should be considered invalid and the results of the samples should be rejected.
2. Analyse internal control of each sample in all assay:

- If the internal control assay gives a positive result continue with the analysis.
- If the internal control assay has failed but the FAM Channel reaction has amplified strongly, continue with the analysis as the FAM channel reaction has out-competed the internal control reaction.
- If both the FAM Channel and internal control reactions have failed, the data must be discarded, as there may be inhibitors present and the inhibitors could lead to false negative results.

NOTE

1. The users must be trained and familiar with real time PCR technology prior to the use of this kit.
2. Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings.
3. It is the user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by the TRUPCR performance studies.
4. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

PERFORMANCE CHARACTERISTIC

Clinical specificity

In order to evaluate the TRUPCR ® KRAS kit specificity DNA samples isolated from FFPE tumor tissue have been tested. Samples were suitable in terms of starting DNA amount and for the presence of mutations detected by TRUPCR ® KRAS kit and have been already genotyped through bidirectional sequencing technology with Allelic Frequency determination through droplet digital PCR. If no FFPE samples were available, Horizon Diagnostics standards, cell lines or plasmids have been tested

Mutation	No sample tested	No of sample correctly genotyped
G12D	22	22
G12C	17	17
G12S	24	24
G12R	32	32
G12A	29	29
G12V	20	20
G13D	36	36
A59X	8	8
Q61X	12	12
K117X	9	9
A146X	10	10
Wild Type	67	67
Total	286	286

Limit of detection (LOD)

The LOD of TRUPCR ® KRAS kit is defined as the lowest amount of mutant DNA in a background of wild -type DNA at which a mutant sample will provide mutation-positive results in 95% of tests. To determine the LOD, Horizon Diagnostics standards with 50% allelic frequency were diluted to give different percentage of mutation. Each percentage has been tested in triplicate in three different days.

The LOD of the TRUPCR ® KRAS kit is

Mutation	LOD
G12C	1%
G12S	0.5%
G12R	0.5%
G12V	0.5%
G12D	1%
G12A	0.5%
G13D	1%
A59X	5%
Q61X	2%
K117X	1%
A146X	0.5%

Reproducibility

System reproducibility (inter-assay variability) has been evaluated analyzing the data deriving from three independent runs with Horizon Diagnostics standards. Results were reproducible in terms of genotyping for all assays and samples analyzed.

Robustness

Different batches of kit have been tested with the same Horizon Diagnostics standards. Results from the different lots are comparable. Two different batches of primers and probes have been tested with the same DNA samples. Results from the different lots are comparable.

TROUBLESHOOTING REAL TIME PCR

No.	Observation	Probable causes	Comments
1	Amplification signal in negative control	Cross contamination during handling	Check for contamination of kit's component
2	No amplification signal with positive controls	Incorrect PCR mixture	Check whether all components are added.
		Missing control sample during DNA mixing	Be careful when pipetting
		Changing DNA during DNA mixing	Write down sample number on the 1.5 ml micro centrifuge tube and the PCR tube
		Leaving reagents at room temperature for a long time or incorrect storage condition	Please check the storage condition and the expiration date(see the kit label) of the reagents and use a new kit, if necessary
		The PCR conditions do not comply with the protocol	Repeat the PCR with corrected settings
3	Weak or no signal of the Internal Control in Hex channel	Reagent has been thawed and frozen too often or exposed to inappropriate storage conditions	Please mind the storage conditions given in manual
		The PCR was inhibited	DNA of Poor quality may interfere with the PCR reaction, use a recommended isolation method

STORAGE AND HANDLING

All the components of kit should be stored at -20˚C and stable until the date of expiry stated. The reagents can be aliquoted and stored at -20˚C in-order to maintain the stability and sensitivity.

MATERIAL AND DEVICES REQUIRED BUT NOT PROVIDED

- Adjustable pipettes with sterile filter or positive displacement tips
- Disposable powder-free gloves
- Sterile bidistilled water
- Sterile 1.5 ml and 2 ml microcentrifuge tubes
- 50 ml conical tubes
- Vortex mixer
- 3B Termi-DNA-Tor or equivalent, in order to remove DNA from working surfaces
- Real time PCR

- Laminar airflow cabinet
- PCR vials (0.2 ml, thin-walled)
- Personal protection equipment (lab coat, gloves, goggles)
- DNA isolation kit

KIT IS COMPATIBLE TO USE WITH FOLLOWING REAL TIME PCR INSTRUMENTS

- Applied Biosystems™ 7500
- StepOne and StepOnePlus
- QuantStudio® 3, 5 and 12
- Rotor-Gene Q
- Bio-Rad CFX96, CFX384
- AriaMx Real-Time PCR
- Roche - LightCycler® 480 -II
- Line gene K real time PCR

GENERAL PRECAUTIONS

The user should always pay attention to the following:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Dispose of all samples and unused reagents in compliance with local authorities' requirements.

- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices. Infected material and disposable plasticware that was in contact with infected material must be treated with chlorine-containing solutions.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5 % sodium hypochlorite or other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact, immediately flush with water, seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be uni-directional; it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

NOTICE

- The user should always pay attention to the following:
- **Store DNA samples at -20°C until ready for use and keep on ice during use.**
- Avoid microbial contamination of reagents when removing aliquots from reagent tubes. The use of sterile disposable pipette tips is recommended.
- Specimens should be handled as if infectious using safe laboratory procedures. Thoroughly clean and disinfect all work surfaces with 0.5% Sodium Hypochlorite in de-ionized or distilled water.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

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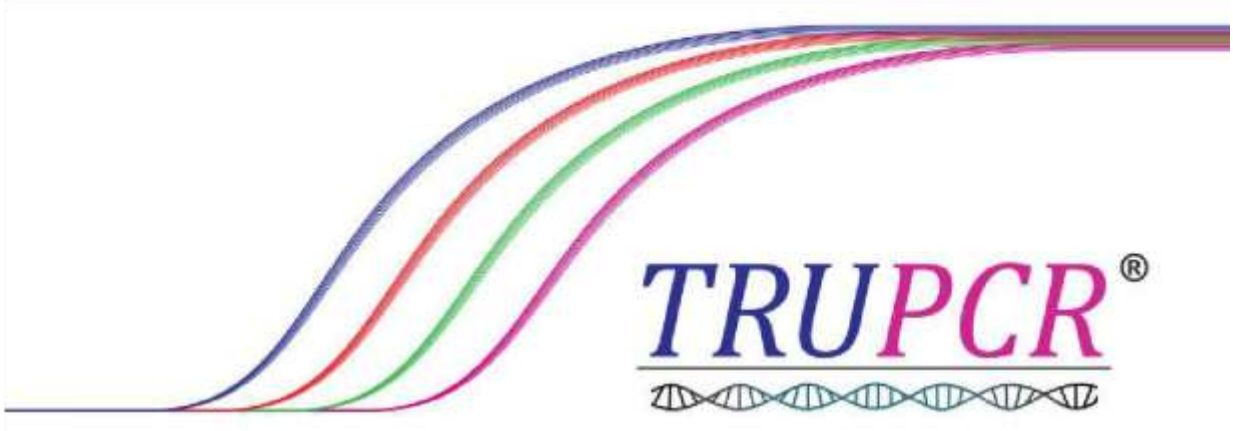

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