INSTRUCTIONS FOR USE

QClamp® KRAS Mutation Detection Test

For Real-Time PCR Assays

CATALOG NUMBERS

DC-10-2010R (10 samples)
DC-10-3010R (30 samples)
DC-10-4010R (60 samples)

MANUFACTURER

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INTENDED USE

For Research Use Only

*Where appropriate, please disregard assay mixes that are not part of your kits.

Instruction Version: Rev. 3, Date of Revision: October 2022

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PART 1. INTENDED USE

The QClamp® KRAS Mutation Detection Test is a research-use-only real-time qualitative PCR assay for the detection of somatic mutations in and near Codons 12 and 13 in Exon 2, Codons 59 and 61 in Exon 3, and Codons 117 and 146 in Exon 4 in the human KRAS gene, using purified DNA extracted from FFPE (formalin fixed paraffin embedded), plasma, cells or tissue. The kit identifies the presence or absence of mutations in the targeted regions but does not specify the exact nature of the mutation. Table 1 shows a list of mutations commonly found in the KRAS gene that can be detected by the kit. The kit is to be used within a laboratory environment for research only, not for clinical diagnostic purposes.

Table 1. List of Mutations and Cosmic Identities Found in KRAS

Exon	Amino Acid	Nucleotide Change	Cosmic No.
2	G12>A	c.35G>C	522
	G12>R	c.34G>C	518
	G12>D	c.35G>A	521
	G12>C	c.34G>T	516
	G12>S	c.34G>A	517
	G12>V	c.35G>T	520
	G13>D	c.38G>A	532
	G13>C	c.37G>T	527
	G13>R	c.37G>C	529
3	A59>T	c.175G>A	546
	Q61>H	c.183A>C & c.183A>T	554/555
	Q61>K	c.181C>A	549
	Q61>L	c.182A>T	553
	Q61>R	c.182A>G	552
4	K117>E	c.349A>G	1360831
	K117>N	c.351A>C & c.351A>T	19940/28519
	K117>R	350A>G	
	A146>A	c.438A>G	1360826
	A146>G	c.437C>G	1360829
	A146>P	436G>C	19905
	A146>T	436G>A	19404
	A146>V	437C>T	19900

PART 2. KRAS MUTATIONS AND CANCER

KRAS mutations are found in several cancers including colorectal, lung, thyroid, and pancreatic cancers and cholangiocarcinoma. KRAS mutations are often located within codons 12 and 13 of exon 2, codons 59 and 61 in exon 3 and codons 117 and 146 in exon 4 which may lead to abnormal growth signaling by the p21- ras protein. These alterations in cell growth and division may trigger cancer development as signaling is excessive. A KRAS mutation often serves as a useful prognostic marker in drug response. For example, a KRAS mutation is considered to be a strong prognostic marker of response to tyrosine kinase inhibitors such as gefitinib (Iressa) or erlotinib (Tarceva). KRAS mutations have also been detected in many colorectal cancer patients and are associated with responses to cetuximab (Erbitux) or panitumumab (Vectibix), which are used in colon cancer therapy. The recent PEAK Phase 2 clinical study of Panitumumab and Bevacvizumab plus mFOLFOX6 for first line treatment of metastatic colorectal cancer exemplified the need for extended testing of KRAS and KRAS mutations at codons 61, 117 and 146 for selection of patients that would respond to anti-KRAS antibody combination therapy.

PART 3. QCLAMP® WORKFLOW

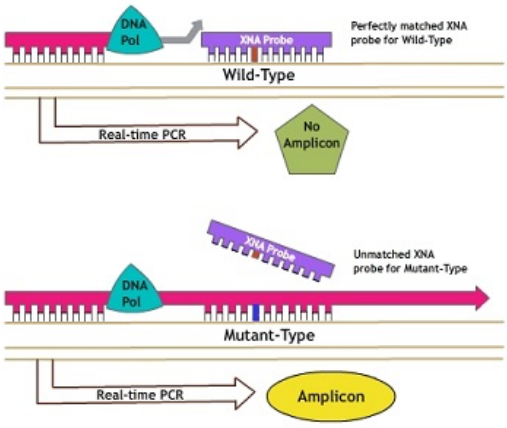
The assay workflow consists of four major steps:

1. **DNA isolation:** Extract DNA from FFPE or plasma tissue using commercial DNA extraction kit
2. **Set up qPCR:** Mix the assay reagents, load into PCR plate and add extracted DNA – 30-60 min
3. **Enter amplification parameters** on real-time PCR machine, load PCR plate and start the run – 2.5h
4. **Data analysis:** Determine the presence or absence of mutations according to the Cq value cutoffs – 15 min

PART 4. QCLAMP® TECHNOLOGY FOR MUTATION DETECTION

The QClamp® KRAS Mutation Detection Test is based on xenonucleic acid (XNA) mediated PCR clamping technology. XNA is a synthetic DNA analog in which the phosphodiester backbone has been replaced by a repeat formed by units of DiaCarta's proprietary novel uncharged backbone chemistry. XNAs hybridize tightly to complementary DNA target sequences only if the sequence is a complete match. Binding of XNA to its target sequence blocks strand elongation by DNA polymerase. When there is a mutation in the target site, and therefore a mismatch, the XNA:DNA duplex is unstable, allowing strand elongation by DNA polymerase. Addition of an XNA, whose sequence with a complete match to wild-type DNA, to a PCR reaction, blocks amplification of wild-type DNA allowing selective amplification of mutant DNA. XNA oligomers are not recognized by DNA polymerases and cannot be utilized as primers in subsequent real-time PCR reactions.

Figure 1. Principle of the QClamp® KRAS Mutation Detection Test



PART 5. REAGENTS AND INSTRUMENTS

5.1. Package Contents

Table 2. Kit Component

Table 2a. Pack size: 10 Samples

	Name of Component	Part #	Description	Cap Color	Pack Size: 10 Samples	Label Volume for each vial	Storage Temp
Grey Box 1	c12 Primer/Probe	1001652	G12 Primers and probes	Red	1 vial	22 µL	-25°C to -15°C
	c13 Primer/Probe	1001662	G13 Primers and probes	Violet	1 vial	22 µL	-25°C to -15°C
	c59 Primer/Probe	1001672	A59 Primers and probes	Blue	1 vial	22 µL	-25°C to -15°C
	c61 Primer/Probe	1001682	Q61 Primers and probes	Yellow	1 vial	22 µL	-25°C to -15°C
	c117 Primer/Probe	1001692	K117 Primers and probes	Green	1 vial	22 µL	-25°C to -15°C
	c146 Primer/Probe	1001702	A146 Primers and probes	Amber	1 vial	22 µL	-25°C to -15°C
	Negative Control	1001782	Wild-type gDNA	Orange	1 vial	36 µL	-25°C to -15°C
	Mixed Positive Control	1001802	G12D, G13D, A59T, Q61H, K117N, & A146T gDNA Template at 5% for each target mutation	Red	1 vial	36 µL	-25°C to -15°C
	Nuclease-Free Water	1001792	Nuclease-Free Water	Clear	1 vial	168 µL	-25°C to -15°C
Grey Box 2	c12 XNA Mix	1001712	G12 XNA	Red	1 vial	22 µL	-25°C to -15°C
	c13 XNA Mix	1001722	G13 XNA	Violet	1 vial	22 µL	-25°C to -15°C
	c59 XNA Mix	1001732	A59 XNA	Blue	1 vial	22 µL	-25°C to -15°C
	c61 XNA Mix	1001742	Q61 XNA	Yellow	1 vial	22 µL	-25°C to -15°C
	c117 XNA Mix	1001752	K117 XNA	Green	1 vial	22 µL	-25°C to -15°C
	c146 XNA Mix	1001762	A146 XNA	Amber	1 vial	22 µL	-25°C to -15°C
	2X PCR Master Mix	1001772	PCR Reaction Premix	Clear	1 vial	660 µL	-25°C to -15°C

Table 2b. Pack size: 30 Samples

	Name of Component	Part #	Description	Cap Color	Pack Size: 30 Samples	Label Volume for each vial	Storage Temp
Grey Box 1	c12 Primer/Probe	1001653	G12 Primers and probes	Red	1 vial	54 µL	-25°C to -15°C
	c13 Primer/Probe	1001663	G13 Primers and probes	Violet	1 vial	54 µL	-25°C to -15°C
	c59 Primer/Probe	1001673	A59 Primers and probes	Blue	1 vial	54 µL	-25°C to -15°C
	c61 Primer/Probe	1001683	Q61 Primers and probes	Yellow	1 vial	54 µL	-25°C to -15°C
	c117 Primer/Probe	1001703	K117 Primers and probes	Green	1 vial	54 µL	-25°C to -15°C
	c146 Primer/Probe	1001693	A146 Primers and probes	Amber	1 vial	54 µL	-25°C to -15°C
	Negative Control	1001783	Wild-type gDNA	Orange	1 vial	72 µL	-25°C to -15°C
	Mixed Positive Control	1001803	G12D, G13D, A59T, Q61H, K117N, & A146T gDNA Template at 5% for each target mutation	Red	1 vial	72 µL	-25°C to -15°C
	Nuclease-Free Water	1001793	Nuclease-Free Water	Clear	1 vial	396 µL	-25°C to -15°C
Grey Box 2	c12 XNA Mix	1001713	G12 XNA	Red	1 vial	54 µL	-25°C to -15°C
	c13 XNA Mix	1001723	G13 XNA	Violet	1 vial	54 µL	-25°C to -15°C
	c59 XNA Mix	1001733	A59 XNA	Blue	1 vial	54 µL	-25°C to -15°C
	c61 XNA Mix	1001743	Q61 XNA	Yellow	1 vial	54 µL	-25°C to -15°C
	c117 XNA Mix	1001753	K117 XNA	Green	1 vial	54 µL	-25°C to -15°C
	c146 XNA Mix	1001763	A146 XNA	Amber	1 vial	54 µL	-25°C to -15°C
	2X PCR Master Mix	1001774	PCR Reaction Premix	Clear	2 vials	810 µL	-25°C to -15°C

Table 2c. Pack size: 60 Samples

	Name of Component	Part #	Description	Cap Color	Pack Size: 60 Samples	Label Volume for each vial	Storage Temp
Grey Box 1	c12 Primer/Probe	1001654	G12 Primers and probes	Red	1 vial	84 µL	-25°C to -15°C
	c13 Primer/Probe	1001664	G13 Primers and probes	Violet	1 vial	84 µL	-25°C to -15°C
	c59 Primer/Probe	1001674	A59 Primers and probes	Blue	1 vial	84 µL	-25°C to -15°C
	c61 Primer/Probe	1001684	Q61 Primers and probes	Yellow	1 vial	84 µL	-25°C to -15°C
	c117 Primer/Probe	1001694	K117 Primers and probes	Green	1 vial	84 µL	-25°C to -15°C
	c146 Primer/Probe	1001704	A146 Primers and probes	Amber	1 vial	84 µL	-25°C to -15°C
	Negative Control	1001784	Wild-type gDNA	Orange	1 vial	72 µL	-25°C to -15°C
	Mixed Positive Control	1001804	G12D, G13D, A59T, Q61H, K117N, & A146T gDNA Template at 5% for each target mutation	Red	1 vial	72 µL	-25°C to -15°C
	Nuclease-Free Water	1001794	Nuclease-Free Water	Clear	1 vial	576 µL	-25°C to -15°C
Grey Box 2	c12 XNA Mix	1001714	G12 XNA	Red	1 vial	84 µL	-25°C to -15°C
	c13 XNA Mix	1001724	G13 XNA	Violet	1 vial	84 µL	-25°C to -15°C
	c59 XNA Mix	1001734	A59 XNA	Blue	1 vial	84 µL	-25°C to -15°C
	c61 XNA Mix	1001744	Q61 XNA	Yellow	1 vial	84 µL	-25°C to -15°C
	c117 XNA Mix	1001754	K117 XNA	Green	1 vial	84 µL	-25°C to -15°C
	c146 XNA Mix	1001764	A146 XNA	Amber	1 vial	84 µL	-25°C to -15°C
	2X PCR Master Mix	1001774	PCR Reaction Premix	Clear	2 vials	1251 µL	-25°C to -15°C

5.2. Materials Required but Not Provided in the Kit

A. Reagents for DNA Isolation

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, Cat. No. 60404) or equivalent
- QIAamp Circulating Nucleic Acid Kit (QIAGEN, Cat. No. 55114) or equivalent
- QIAamp MiniElute ccfDNA Kit (QIAGEN, Cat. No. 55204) or equivalent
- DNeasy Blood & Tissue kit (QIAGEN, Cat. No. 69504 or 69506) for tissue and blood specimens

B. Consumables

- 0.2 ml DNase-free PCR tubes or plates
- Nuclease-free, low-binding micro centrifuge tubes
- Nuclease-free pipet tips with aerosol barriers

C. Equipment

- Permanent marker
- Real time PCR instrument capable of TaqMan
- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR master mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA
- Micro centrifuge
- Bench top centrifuge* with rotor for 1.5 ml tubes
- Vortexer
- PCR rack
- Reagent reservoir

Note: Prior to use ensure that instruments have been maintained and calibrated according to the manufacturer's recommendations.

5.3. Instruments

The assays have been developed and validated on the instruments shown in the table below. Instrument platforms not listed in the table should be validated by the individual labs. Guidance for validation can be obtained from DiaCarta upon request.

Table 3. List of Instruments Validated with this Kit

Compan	Model
Roche	LightCycler 96
Roche	LightCycler
Bio-Rad	CFX384
ABI	QuantStudio 5

5.4. Handling and Storage

This kit is shipped on dry ice. If any component of the kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packaging note or the reagents, please contact DiaCarta or the local distributors as soon as possible.

The kit should be stored at -20 °C immediately upon receipt, in a constant-temperature freezer and protected from light. When stored under the specified storage conditions, the kit is stable until the stated expiration date. It is recommended to store the PCR reagents (Box 1and 2) in a pre-amplification area and the controls (Box 3) in a postamplification (DNA template-handling) area. The kit can undergo up to 6 freeze-thaw cycles without affecting performance.

All reagents must be thawed at ambient temperature for a minimum of 30 minutes before use. Do not exceed 2 hours at ambient temperature. The primer and probe mixes contain fluorophore labeled probes and should be protected from light. Attention should be paid to expiration dates and storage conditions printed in the box and labels of all components. Do not use expired or incorrectly stored components.

5.5. General Considerations

Effective use of real-time PCR tests requires good laboratory practices, including maintenance of equipment that is dedicated to molecular biology. Use nuclease-free lab ware (pipettes, pipette tips, reaction vials) and wear gloves when performing the assay. Use aerosol-resistant pipette tips for all pipetting steps to avoid cross contamination of the samples and reagents.

Prepare the assay mixes in designated pre-amplification areas using only equipment dedicated to this application. Add template DNA in a separate area (preferably a separate room). Use extreme caution to prevent DNase contamination that could result in degradation of the template DNA, or PCR carryover contamination, which could result in a false positive signal.

Reagents supplied are formulated specifically for use with this kit. Make no substitutions to ensure optimal performance of the kit. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data.

5.6. Warnings and Precautions

- Use extreme caution to prevent contamination of PCR reactions with the positive and wild type DNA controls provided.
- Minimize exposure of the 2 X PCR Master mixes to room temperaturefor optimal amplification.
- Avoid overexposing the primer-probe mixes to light for optimal fluorescent signal.
- Use of non-recommended reagent volumes may result in a loss of performance and may also decrease the reliability of the test results.
- Use of non-recommended volumes and concentrations of the target DNA sample may result in a loss of performance and may also decrease the reliability of the test results.
- Use of non-recommended consumables with instruments may adversely affect test results.
- Do not re-use any remaining reagents after PCR amplification is completed.
- Additional validation testing by user may be necessary when using non-recommended instruments.
- Perform all experiments under proper sterile conditions using aseptic techniques.
- Perform all procedures using universal precautions.
- Wear personal protective apparel, including disposable gloves, throughout the assay procedure.
- Do not eat, drink, smoke, or apply cosmetics in areas where reagents or specimens are handled.
- Dispose of hazardous or biologically contaminated materials according to the practices of your institution.
- Discard all materials in a safe and acceptable manner, in compliance with all legal requirements.
- Dissolve reagents completely, then mix thoroughly by vortexing.
- If exposure to skin or mucous membranes occurs, immediately wash the area with large amounts of water. Seek medical advice immediately.
- Do not use components beyond the expiration date printed on the kit boxes.
- Do not mix reagents from different lots.
- Return all components to the appropriate storage condition after preparing the working reagents.
- Do not interchange vial or bottle caps, as cross-contamination may occur.
- Keep all the materials on ice when in use.
- Do not leave components out at room temperature for more than 2 hours.
- Reagents supplied are formulated specifically for use with this kit. Make no substitutions to ensure optimal performance of the kit. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data.

PART 6. INSTRUCTIONS FOR USE

6.1. DNA Isolation

Human genomic DNA must be extracted from plasma or fixed paraffin-embedded tissue prior to use. Several methods exist for DNA isolation. For consistency, we recommend using a commercial kit as follows:

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, Cat. No. 60404) or equivalent
- QIAamp Circulating Nucleic Acid Kit (QIAGEN, Cat. No. 55114) or equivalent
- QIAamp MiniElute ccfDNA Kit (QIAGEN, Cat. No. 55204) or equivalent
- DNeasy Blood & Tissue kit (QIAGEN, Cat. No. 69504 or 69506) for tissue and blood specimens

Follow the DNA isolation procedure according to manufacturer's protocol. This QClamp assay requires a total of 15- 60 ng of DNA per sample (2.5-10ng/reaction). After DNA isolation, measure the concentration using fluorometric analysis (i.e. Qubit) and dilute to 1.25-2.5 ng/µl. If using spectrophotometric analysis, make sure the A260/A230 value is greater than 2.0 and A260/A280 value is between 1.8 and 2.0.

6.2. Preparation of Reagents

Thaw all primer and probe mixes, XNAs, Positive Control, WT Negative Control, Nuclease-Free Water and 2X PCR mastermix provided. Thaw all reaction mixes at room temperature for a minimum of 30 minutes. Vortex all components except the PCR Master Mix and Primer and probe Mix for 5 seconds and perform a quick spin. The PCR Master Mix and Primer/probe mix should be mixed gently by inverting the tube a few times. Prior to use, ensure that the PCR Master Mix is re-suspended by pipetting up and down multiple times. Do not leave kit components at room temperature for more than 2 hours. The PCR reactions are set up in a total volume of 10 µl/reaction.

- A 10-sample test kit contains enough material for 3 runs (minimum 3 samples per run).
- A 30-sample test kit contains enough material for 6 runs (minimum 5 samples per run).
- A 60-sample test kit contains enough material for 6 runs (minimum 10 samples per run).

Table 4. QClamp® Assay Components and Reaction Volume

Components	Volume/Reaction
2X PCR Master mix	5 µl
Primer and probe	1 µl
XNA	1 µl
DNA sample or	2 µl
Nuclease Free	1 µl
Total volume	10 µl

Notes:

- For accuracy, 2x PCR Mastermix, primers and XNA should be pre-mixed into assay mixes as described in Table 5 below
- If needed, the sample volume can be increased to 3 µl by eliminating 1 µl water

6.3. Preparation of Assay Mixes

Table 5. Preparation of Assay Mixes

	Volume of 2X	Volume of Primer	Volume of XNA*
G12 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
G13 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
A59 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
Q61 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
K117 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
A146 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)

Notes:

- n = number of reactions (DNA samples plus 3 controls). Prepare enough for 1 extra sample (n +1) to allow for sufficient overage for the PCR set.
- For accuracy, do not pipette less than 8 µl.

Assay mixes should be prepared just prior to use. Label a micro centrifuge tube (not provided) for each reaction mix available, as shown in Table 5. Please disregard any assay mixes listed that are not part of your kit.

A reaction mix containing all reagents except for the DNA samples or controls should be prepared for the total number of samples and controls to be tested in one run. The KRAS Mixed Positive Control (PC), KRAS Clamping Control (Negative Control, CC), and a No Template Control (NTC) should be included in each run. Negative Controls use wild-type DNA as the template. Wild-type DNA should have no mutations; therefore, the XNA probes will bind strongly, blocking the polymerase from making amplicons. However, the Internal Control assay (HEX) with the Negative Control should make amplicons efficiently, providing another way to monitor performance of the primers, polymerase, and sample.

A set of positive controls must also be run with each reaction mix, every time the assay is run. The Positive Control contains one mutant template for each reaction mix. Positive controls contain mutations; therefore, XNA probes will not bind, allowing amplification of the mutant template. Positive controls must show the appropriate values for the reaction to be valid. A set of no template control (tube NTC) is run with each reaction mix every time the assay is run. Nuclease-Free Water is used in the place of template. The NTC serves as a negative control and assesses potential contamination during assay set-up.

6.4. Suggested Run Layout (96-well plate, 384-well plate, tube strips, or tubes)

Add 7 (if using 3µl template) or 8µl of the appropriate assay mix to the plate or tubes. Add 2 or 3µl of template.

Table 6. Suggested Plate Layout

	1	2	3	4	5	6
A	NTC	PC	CC	S1	S2	S3
	G12 Mix	G12 Mix	G12 Mix	G12 Mix	G12 Mix	G12 Mix
B	NTC	PC	CC	S1	S2	S3
	G13 Mix	G13 Mix	G13 Mix	G13 Mix	G13 Mix	G13 Mix
C	NTC	PC	CC	S1	S2	S3
	A59 Mix	A59 Mix	A59 Mix	A59 Mix	A59 Mix	A59 Mix
D	NTC	PC	CC	S1	S2	S3
	Q61 Mix	Q61 Mix	Q61 Mix	Q61 Mix	Q61 Mix	Q61 Mix
E	NTC	PC	CC	S1	S2	S3
	K117 Mix	K117 Mix	K117 Mix	K117 Mix	K117 Mix	K117 Mix
F	NTC	PC	CC	S1	S2	S3
	A146 Mix	A146 Mix	A146 Mix	A146 Mix	A146 Mix	A146 Mix

Notes:

- PC: Positive Control, NTC: No-Template Control (water), CC: Clamping Control (Wild-type DNA), S1-3: Samples 1-3.
- When all reagents have been added to the plate, tightly seal the plate to prevent evaporation. Spin at 2000rpm for 1 minute to collect all the reagents. Place in the real-time PCR instrument immediately. Table 6 is a suggested plate set-up for a single experiment analyzing 3 unknown samples.

6.5. Instrument Set-Up

These parameters apply to BioRad CFX384, Roche LC480, Roche 9C96, ABI QuantStudio 5 Instruments. For Applied Biosystems Platforms select NONE for the Passive Reference Dye.

Table 7. Cycling Parameters

Step	Temperature	Time	Ramp	Cycles	Data Collection
PreIncubation	95	300	4	1	OFF
Denaturation	95	20	1.55	X45	OFF
XNA	70	40	1.75		OFF
Primer	60	30	1.2		OFF
Extension	72	30	1.4		FAM and HEX

PART 7. ASSESSMENT OF REAL-TIME PCR RESULTS

The real-time PCR instrument generates a cycle threshold (Ct) value for each sample. Ct is the cycle number at which a signal is detected above the set threshold for fluorescence. The lower the cycle number at which signal rises above background, the stronger the PCR reaction it represents (**please see MIQE Guidelines under References for more information).

Negative Controls use wild-type DNA as the template. Wild-type DNA contains none of the targeted mutations, therefore the XNA probes will bind strongly, inhibiting the target amplification. In contrast, the Internal Control Assay assessed with HEX channel should make amplicons efficiently for the Negative Control, providing another way to monitor performance of the primers, polymerase, and sample DNA quality/quantity. The Positive Control contains one mutant template for each assay mix. Positive controls contain mutations; therefore XNA probes will not bind, allowing amplification of the mutant template. Positive controls must show the appropriate values in both HEX and FAM channels for the run to be valid.

A set of no-template control (NTC) is run with each assay mix every time the assay is run. Nuclease-Free Water is used in the place of template. The NTC serves as a negative control and assesses potential contamination during assay set-up.

7.1. Data Analysis

For the Bio Rad CFX, the entire run is on default and no manual adjustment should be performed. Choose all channels when prompted.

For the Light Cycler 480, open the LightCycler480 SW 1.5.1.61, choose Dual Color Hydrolysis Probe and select Abs Quant/2nd Derivative Max algorithm to analyze the run file data. Adjust ramp rates as shown in Table 7.

For ABI Quant Studio 5, under Properties select Taqman and under Plate, select Fam for Target 1 and Vic for Target 2. For the Passive reference select None.

The thresholds can be adjusted as needed for each run separately for each assay based on the levels of noise.

7.1.1. No-Template Controls

Verify that no amplification is observed in the no-template controls (NTC) for each of the reaction mixes. Cq should be undetermined. For each control or sample, calculate the difference in Cq value between the mutation assay and the Internal Control Assay as follows:

Cq difference (ΔCq) = Mutation Assay Cq - Internal Control Assay Cq

7.1.2. Analysis of Clamping and Positive Controls

For the assay to be valid, the Clamping Control and Positive Control must meet the criteria in Table 8a-c.

Table 8a. Acceptable Values for Positive Controls and Clamping Controls (BioRad CFX384)

	Positive Control	Clamping Control
G12 Mix	ΔCq ≤ 7	ΔCq > 10.5
G13 Mix	ΔCq ≤ 7	ΔCq > 10.5
A59 Mix	ΔCq ≤ 8.4	ΔCq > 10.5
Q61 Mix	ΔCq ≤ 11	ΔCq > 13.5
K117	ΔCq ≤ 11.5	ΔCq > 12.5
A146	ΔCq ≤ 11	ΔCq > 13

Table 8b. Acceptable Values for Positive Controls and Clamping Controls (Roche LC480)

	Positive Control Acceptable Values	Clamping Control Acceptable Values
G12 Mix	ΔCq ≤ 6.7	ΔCq > 11
G13 Mix	ΔCq ≤ 6	ΔCq > 10.7
A59 Mix	ΔCq ≤ 8	ΔCq > 10.3
Q61 Mix	ΔCq ≤ 9.2	ΔCq > 12.4
K117	ΔCq ≤ 10.7	ΔCq > 12
A146	ΔCq ≤ 11.4	ΔCq > 13

Table 8c. Acceptable Values for Positive Controls and Clamping Controls (ABI QuantStudio 5)

	Positive Control Acceptable Values	Clamping Control Acceptable Values
G12 Mix	ΔCq ≤ 7.2	ΔCq > 12
G13 Mix	ΔCq ≤6.8	ΔCq > 11.8
A59 Mix	ΔCq ≤ 9.3	ΔCq > 11
Q61 Mix	ΔCq ≤ 11.	ΔCq > 15
K117	ΔCq ≤ 9.5	ΔCq > 11.7
A146	ΔCq ≤ 11.6	ΔCq > 14.2

7.1.3. Judging Validity of Sample Data Based on Internal Control Test Results (HEX)

The Cq value of the Internal Control Mix can serve as an indication of the purity and concentration of DNA. Thus, the validity of the test can be decided by the Cq value of the Int Control mix. Cq values of any sample with Internal Control Mix should be in the range of 18-24. If the Cq values fall outside this range, the test results should be considered invalid. The experiment should be repeated following the recommendations in Table 9.

Table 9. Acceptable Cq Ranges for Samples with Internal Control Mix

Validity	Cq Value of Int Control Mix	Descriptions and Recommendations
Optimal	22 < Cq < 29	The amplification and amount of DNA sample were optimal.
Invalid	Cq ≤22	Possibility of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ≥ 29	Not enough DNA or DNA not pure. The amplification is not optimal. Check DNA amount and purity. Repeat the experiment with more DNA or a new DNA prep may be required

7.1.4. Scoring Mutational Status

If a Cq value is Undetermined, assign a Cq of 45 and proceed to analysis. The table below should be used to determine mutational status.

Table 10. Scoring Mutational Status

Instrument	Call		KRAS Target Codon					
			G12*	G13	A59**	Q61**	K117	A146
BioRad CFX384	Positive:	ΔCq	≤10.5	≤10.	≤10.6	≤13.65	≤12.5	≤13
	Negative:		>10.5	>10.	>10.6	>13.65	>12.5	>13
Roche LC480	Positive:		≤11.1	≤10.	≤10.3	≤12.4	≤12	≤13
	Negative:		>11.1	>10.	>10.3	>12.4	>12	>13
ABI QuantStudio 5	Positive:		≤12.1	≤11.	≤11.1	≤15	≤11.6	≤14.2
	Negative:		>12.1	>11.	>11.1	>15	>11.6	>14.2

Notes:
- Refer to Table 11 for interpretation of G12/G13 Mutational Status
- Refer to Table 12 for interpretation of A59/Q61 Mutational Status

7.1.4.1. Differentiating KRAS c12/KRAS c13 Mutational Status

The KRAS c12 and c13 reaction mixes detect both KRAS c12 and KRAS c13 mutations, although each assay is more sensitive to its own target especially at low copy numbers. Therefore, in order to differentiate between KRAS c12 and KRAS c13 Mutations a combination of results from the 2 mixes should be used, as described in Table 11 below.

Table 11. Interpretation of G12/G13 Mutational Status

Reaction Mix	Result Based on Tables 10,	Mutational Status
KRAS c12 Reaction	Positive	G12 Mutation
KRAS c13 Reaction	Negative	
KRAS c12 Reaction	Positive	Either G12 or G13 Mutation
KRAS c13 Reaction	Positive	
KRAS c12 Reaction	Negative	G13 Mutation
KRAS c13 Reaction	Positive	

7.1.4.2. Differentiating A59/Q61 Mutational Status

The A 59 and Q61 reaction mixes detect both A59 and Q61 mutations, although each assay is more sensitive to its own target especially at low copy numbers. Therefore, in order to differentiate between A59 and Q61 mutations, a combination of results from the 2 mixes should be used, as described in Table 12 below.

Table 12. Interpretation of A59/Q61 Mutational Status

Reaction Mix	Result Based on Table 12	Mutational Status
A59 Reaction Mix	Positive	A59 Mutation
Q61 Reaction Mix	Negative	
A59 Reaction Mix	Positive	Either A59 or Q61 Mutation
Q61 Reaction Mix	Positive	
A59 Reaction Mix	Negative	Q61 Mutation
Q61 Reaction Mix	Positive	

PART 8. ASSAY PERFORMANCE CHARACTERISTICS

The performance characteristics of this product were established on the BioRadCFX384, ABI QuantStudio 5, Roche LightCycler 96, Roche LightCycler 480 real-time PCR instruments.

8.1. Analytical Performance

The specific performance characteristics of the QClamp® KRAS Mutation Detection Test were determined by studies involving KRAS-defined genomic DNA reference samples and FFPE samples of the cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England). These samples have been characterized genetically as containing heterozygous mutations in the coding sequence of the KRAS gene at exons 2, 3 and 4. These single nucleotide polymorphisms in the KRAS gene have been confirmed by droplet digital PCR (ddPCR) and genomic DNA sequencing. Additional samples consisted of formalin-fixed, paraffin-embedded (FFPE) reference and patient tissue samples, as well as wild-type DNA (no KRAS mutations).

8.2. Analytical Accuracy and Comparison to Reference Method

QClamp analytical accuracy is verified and validated through testing of well-characterized samples with known mutations verified by sequencing. Three studies were done to demonstrate concordance in mutation status of FFPE samples tested with the QClamp® KRAS Mutation Detection Test relative to sequencing. A set of samples were chosen for evaluation based on mutation status. Samples were chosen blindly to test with the QClamp® KRAS Mutation Detection Test for comparison to the mutation status returned from sequencing. The results demonstrated a 100% match between sequencing and the QClamp® KRAS Mutation Detection Test.

Both reference and patient samples were tested in the accuracy and reproducibility studies. Mutant reference materials were purchased from Horizon Discovery: 12 gDNA controls, 3 cfDNA reference mixed positive and negative controls, 7 FFPE controls were tested multiple times.

8.3. Cut-Offs

Along with studies for analytical accuracy, both reference gDNA and samples extracted from FFPE and plasma were tested to establish cut-offs for the assay. Cutoffs are presented in Table 10 of the manual.

8.4. Multiple Freeze-Thaw Cycles

The effect of 1-8 freeze-thaw cycles were tested in QClamp® KRAS Mutation Detection Test reagents. There is no effect up to 6 freeze-thaw cycles on the QClamp® KRAS Mutation Detection Test to distinguish between mutation positive and mutation negative samples. Caution: Repeated freeze-thaw cycles may decrease the reliability of test results.

8.5. Shelf-Life

Approximately 12 months after production of kit-see product labels for actual expiration date.

8.6. Reproducibility

The precision of the QClamp® KRAS Mutation Detection Test was determined with defined analyte levels of mutated DNA and DNA extracted from FFPE and cfDNA samples of known mutational status. To establish lot-to-lot variation, a reproducibility study of was performed using two different kit lots tested on each of the validated instruments. Each lot was tested on one wild-type control and two reference samples (mixed positive controls) with different mutation frequency (5% and 1%) for each mutation on three separate dates. Inter-assay %CV was established with results from two different users at three different instruments performing the assay using the same lot of reagents, with tests run one to two times a day for at least three days. Intra-assay %CV was established through evaluation of performance of kit on reference samples run in replicates (18 to 6 per run) separately for three different qPCR instrument types. All testing was done using sequence-verified mutant reference samples from Horizon Discovery, WT gDNA and WT patient FFPE and plasma samples. Reproducibility is demonstrated based on %CV of Cq values and rate of % correct mutation calls for all assays across multiple lots and operators for BioRadCFX 384, Roche LC480 and ABI QuantStudio 5 instruments.

Table 13. Summary of Reproducibility Results

Variation	%CV Reference
Intra-assay	≤ 4%
Inter-assay	≤ 3%
Lot-to-Lot	≤ 3%
Operator	≤ 3%

Table 14. Summary of Intra-assay Reproducibility Results for Reference Samples Perforzmed on BioRad CFX384

	WT			5% Mutant			1% Mutant		
Assay Target	**Aver. Cq**	**SD**	**%CV**	**Aver. Cq**	**SD**	**%CV**	**Aver. Cq**	**SD**	**%CV**
c12	39.82	1.56	3.92	31.86	0.35	1.09	33.69	0.47	1.39
c13	37.68	1.17	3.1	31.56	0.54	1.14	33.58	0.53	1.56
c59	37.24	0.77	2.08	32.58	0.44	1.35	34.44	1.05	3.06
c61	42.17	2.13	5.05	33.68	0.49	1.45	54.4	0.58	1.59
c117	39.4	0.48	1.22	36.2	0.54	1.49	37.8	1.02	2.7
c146	41.68	1.62	3.87	35.76	0.3	0.83	37.36	1.23	3.35
Internal Control	25.9	0.56	2.56%	24.96	0.31	1.23%	24.92	0.31	1.24%

Table 15. Summary of Inter-assay Reproducibility Results for Reference Samples Performed on BioRad CFX384

	WT			5% mutant			1% mutant		
Assay Target	**Ave. cq**	**SD**	**%CV**	**Ave. cq**	**SD**	**%CV**	**Ave. cq**	**SD**	**%CV**
c12	39.34	0.46	1.16	31.55	0.23	0.73	33.65	0.09	0.27
c13	37.93	0.76	2	31.45	0.13	0.41	33.53	0.09	0.28
c59	37.54	0.22	0.58	33.09	0.37	1.12	34.6	0.12	0.34
c61	42.46	0.28	0.66	34.77	0.82	2.54	36.35	0.41	1.1
c117	39.83	0.31	0.78	36.12	0.05	0.13	37.74	0.13	0.34
c146	40.72	0.82	2.02	35.33	0.34	0.95	37.01	0.21	0.58
Internal	25.74	0.27	1.04%	24.9	0.35	1.42%	24.77	0.32	1.27%

Table 16. Summary of Lot-to Lot ReproducibilityResults on Roche LC480

WT					
KRAS Assay	**Lot 1**	**Lot 2**	**Aver. Cq**	**St**	**%CV**
c12	43.81	41.35	42.58	1.23	2.90
c13	40.84	38.61	39.72	1.12	2.81
c59	38.21	39.32	38.77	0.56	1.44
c61	41.80	44.41	43.11	1.31	3.03
c117	40.03	40.02	40.02	0.01	0.02
c146	41.67	42.93	42.30	0.63	1.49
Internal Control	**24.82**	**25.07**	13	24.94	5
	5% Mutation				
c12	32.90	32.63	32.77	0.13	0.41
c13	32.47	32.25	32.54	0.11	0.33
c59	34.09	33.62	33.85	0.23	0.69
c61	35.18	34.83	35.00	0.17	0.49
c117	35.33	35.73	35.53	0.30	0.83
c146	36.83.63	37.07	36.95	0.22	0.60
Internal Control	**25.69**	**25.85**	0.1%	**25.77**	0.3
	1% Mutation				
c12	35.46	35.02	35.24	0.22	0.63
c13	35.35	34.93	35.14	0.21	0.59
c59	35.50	35.44	35.47	0.03	0.09
c61	36.94	36.53	36.74	0.21	0.56
c117	37.87	37.51	37.69	0.18	0.48
c146	38.44	38.87	38.65	0.22	0.56
Internal Control	**24.91**	**25.16**	0.13%	**25.03**	0.5

Table 17. Summary of Lot-to Lot Reproducibility Results on Roche LC480

WT					
KRAS Assay	**Lot 1**	**Lot 2**	**Aver. Cq**	**St**	**%CV**
c12	39.34	37.44	38.39	0.95	2.48
c13	37.93	38.09	38.01	0.08	0.20
c59	37.55	36.74	37.15	0.41	1.10
c61	42.46	44.44	43.45	0.99	2.28
c117	39.83	38.41	39.12	0.71	1.82
c146	40.72	42.44	41.58	0.86	2.07
Internal Control	**26.74**	**26.58**	0.1%	26.66	0.3
	5% Mutation				
c12	31.55	31.18	31.37	0.19	0.59
c13	31.45	30.94	31.19	0.25	0.81
c59	33.09	33.00	33.04	0.04	0.13
c61	34.77	35.39	35.08	0.31	0.88
c117	36.47	34.74	35.61	0.87	2.43
c146	35.33	35.67	35.50	0.17	0.48
Internal Control	26.11	26.09	0.01	26.1	0.03%
	1% Mutation				
c12	33.66	33.50	33.58	0.08	0.24
c13	33.53	33.39	33.46	0.07	0.22
c59	34.60	33.54	33.98	0.62	1.81
c61	36.95	38.22	37.59	0.63	1.69
c117	37.74	37.14	37.94	0.80	2.17
c146	37.01	37.63	37.32	0.31	0.83
Internal Control	26.05	26.04	0.01%	26.05	0.03

Table 18. Summary of Operator Variability Results on BioRad CFX384

WT					
KRAS Assay	**Lot 1**	**Lot 2**	**Aver.**	**St**	**%CV**
c12	39.49	38.73	39.11	0.38	0.98
c13	37.16	38.96	38.06	0.90	2.37
c59	37.77	37.60	37.68	0.08	0.22
c61	42.37	42.84	42.60	0.23	0.55
c117	40.11	39.99	40.05	0.06	0.14
c146	39.67	40.80	40.23	0.56	1.40
Internal Control	**24.75**	**25.44**	0.3%	25.1.9	0.8
	5% Mutation				
c12	31.49	31.31	31.40	0.09	0.28
c13	31.27	31.51	31.39	0.12	0.40
c59	33.22	33.46	33.34	0.12	0.54
c61	34.96	35.67	35.31	0.35	1.00
c117	36.41	36.50	36.45	0.05	0.13
c146	34.94	35.28	35.11	0.17	0.48
Internal Control	25.47	26.04	0.28%	25.93	0.6
	1% Mutation				
c12	33.74	33.53	33.63	0.11	0.32
c13	33.40	33.61	33.50	0.10	0.31
c59	34.65	34.71	34.68	0.03	0.08
c61	37.09	37.37	37.23	0.14	0.37
c117	37.56	37.87	37.71	0.15	0.40
c146	36.88	37.31	37.09	0.21	0.57
Internal Control	24.75	24.70	0.1%	24.725	0.2

8.7. Analytical Sensitivity (LOD)

To determine the limit of detection (LOD) for the kit, the QClamp assay was run using a serial dilution of mutant DNA in wild-type background at different total DNA inputs and several mutation frequencies for each target. Mutant reference samples were sequence verified by Horizon Discovery. DNA was added to each well of the assay at 7.5, 5 and 2.5ng/well. Mutant allele concentrations tested were 5, 1, and 0.1%. Results demonstrate effective clamping of wild type, providing reproducible detection of mutations at concentrations as low as 0.1% for some assays run in multiple replicates.

DNA isolated from reference FFPE DNA with known mutational status, patient FFPE and plasma samples were used to determine the limit of detection (analytical sensitivity) of this assay. Serial dilutions of DNA extracted from FFPE reference materials with known mutations controls were made using wild-type DNA from samples to yield various percentages of mutant allele over wild-type background. The same procedure was applied for the plasma derived DNA. The normal plasma from healthy volunteers was used to extract WT cfDNA which was used as a background for the serial dilutions of the mutant cfDNA.

Table 19a. Gene Copy Number Correspondence for % in ng of Input DNA

Mutant DNA	Copy number of mutant DNA		
	2.5ng input DNA	5ng input DNA	10ng input
1%	7	14	29
0.5%	4	7	14
0.1%	0.7	1.5	3

Table 19b. LOD Summary of Data for Three Instruments Tested on Horizon Discovery Reference Standard gDNA

Assay	Amount of DNA per reaction needed for 0.5%		
	CFX384	LC480	QS5
KRAS c12	2.5	2.5	2.5
KRAS c13	2.5	5	2.5
KRAS c59	2.5	2.5 (1%)*	2.5
KRAS c61	2.5	2.5	2.5
KRAS c117	2.5 (1%)	2.5 (0.1%)	5
KRAS c146	5	5	2.5

Based on the data from the tables below, recommended DNA input is 5ng/well to achieve 0.5% sensitivity. Assays for c59 on LC480 and c117 on CFX384 have sensitivity of 1% at 5ng/well. For most assays 0.5% mutation is detected at 2.5ng/well. Recommended input of FFPE should not be higher than 10 ng/well due to possible PCR inhibition in FFPE samples. Optimal FFPE sample input is between 20and 29 Cq of the Internal Control reaction. Optimal cfDNA input is 5 - 2.5 ng/well.

Table 19c. Correct Calls of Different Percentage Reference Mutant Inputs in the Varying Amount of the WT gDNA

Note: If the sensitivity is different from 0.5%, it is shown in brackets.

BioRad CFX384		DNA Input, ng/well		
		7.5	5	2.5
		% Correct Call	% Correct Call	% Correct Call
G12D	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	0%	0%	0%
G13D	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	0%	0%	0%
A59T	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	0%	0%	67%
Q61H	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	33%	67%	67%
K117N	1% mutation	100%	100%	100%
	0.5% mutation	0%	67%	67%
	0.10% mutation	0%	0%	0%
A146T	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	67%
	0.10% mutation	0%	33%	0%

Roche LC480		DNA Input, ng/well		
		7.5	5	2.5
		% Correct Call	% Correct Call	% Correct Call
G12D	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	33%	0%	67%
G13D	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	67%
	0.10% mutation	0%	0%	0%
A59T	1% mutation	100%	100%	100%
	0.5% mutation	0%	33%	67%
	0.10% mutation	0%	33%	0%
Q61H	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	0%	33%	0%
K117N	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	100%	100%	100%
A146T	1% mutation	100%	100%	100%
	0.5% mutation	0%	100%	0%
	0.10% mutation	0%	0%	0%

QS5 ABI		DNA Input, ng/well		
		7.5	5	2.5
		% Correct Call	% Correct Call	% Correct Call
G12D	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	67%	67%	33%
G13D	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	67%	33%	0%
A59T	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	100%	67%	33%
Q61H	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	100%	33%	33%
K117N	1% mutation	100%	100%	100%
	0.5% mutation	0%	100%	33%
	0.10% mutation	0%	33%	33%
A146T	1% mutation	100%	100%	100%
	0.5% mutation	100%	100%	100%
	0.10% mutation	0%	100%	33%

8.8. Analytical Specificity

Analytical specificity of the KRAS test was determined as both the correct calling of the samples with no mutation at different concentrations of WT template and as cross-reactivity of the assays within the kit when one or more mutations are present. The test was performed on gDNA, FFPE DNA and cfDNA. Table 19 shows that all the assays in the kit are accurate in detecting the target mutations. Only in case of KRAS 13 mutations present both KRAS 12 and KRAS 13 signals were detected, but the pattern of lower Cq in KRAS 12 assay and higher Cq in KRAS 13 assay allows for discerning the two targets. Same is true for c59 in the c59/c61 pair.

For known WT templates specificity of the assay at 5ng per well is over 98%. There were no false positive calls for up to 320ng of gDNA per well and up to 20ng FFPE DNA except for c117 assay where upper LOD was 40ng/well. No false positive calls were observed for up to 20ng of FFPE DNA and 10ng of cfDNA. Higher amounts of DNA input were not tested for FFPE and cfDNA.

Table 20. Cross-reactivity Tested on Reference gDNA

Assay	Expected Mutations in Tested 50% Templates, Reference gDNA											
	c12		C13		C59		C61		C117		C146	
	Average Cq, FAM	ΔCq	Average Cq, FAM	ΔCq	Average Cq, FAM	ΔCq	Average Cq, FAM	ΔCq	Average Cq, FAM	ΔCq	Average Cq, FAM	ΔCq
c12	29.83	**4.86**	29.89	**4.52**	37.93	11.6	39.26	14.0	40.54	15.3	40.14	15.0
c13	32.15	**7.01***	30.39	**4.98**	37.68	12.5	40.84	15.6	40.22	14.9	40.32	15.1
c59	40.58	15.52	37.61	12.37	30.1	**4.78**	32.51	**7.42**	40.05	14.9	41.68	16.6
c61	40.18	15.03	37.13	11.59	37.89	**8.61**	31.43	**6.32**	40.44	15.3	40.32	15.1
c117	40.22	15.17	37.96	11.73	37.06	11.6	39.87	14.5	34.61	**9.45**	41.07	15.7
c146	37.41	12.35	37.53	12.21	37.29	12.0	38.29	13.1	37.51	13.1	31.61	**6.62**
WT	39.13	14.3	37.83	12.83	37.48	12.4	37.61	12.6	40.51	15.5	40.59	15.7

Note: Shows Ct values corresponding to positive calls.
*2Cq higher FAM values than KRAS12
**4Cq higher FAM values than KRAS61

8.9. Interfering Substances

Matrix interference was tested by demonstrating compatibility of the intended human patient samples – gDNA from cells, FFPE DNA extracts and cfDNA from plasma with the assay. All these samples can be tested by KRAS with high sensitivity and specificity as shown above.

A study was performed to evaluate the impact of potentially interfering substances on the performance of the QClamp® KRAS Mutation Detection Test. Potentially interfering substance tested was ethanol. The impact of each concentration of substance on resultant ΔCq and mutation status of test samples was determined via spiking experiments conducted at three different concentrations, 0.1%, 1% and 5%. DNA extracted from FFPE samples was tested and shown to be compatible with the assay within established cutoffs. The potentially interfering substance evaluated at concentrations encountered in normal use did not impact the ability of the QClamp® KRAS Mutation Detection Test to distinguish between mutation-positive and mutation-negative samples.

Table 21. Interfering Substance: No Significant Interference from EtOH up to 10%

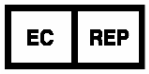
Target	0%		2%		5%		10%	
	Aver. Cq	ΔCt	Aver. Cq	ΔCt	Aver. Cq	ΔCt	Aver. Cq	ΔCt
KRAS c12-NC	42.22	17.11	42.02	16.81	39.56	14.34	41.65	16.58
KRAS c12-PC	31.69	5.77	31.55	5.63	31.5	5.79	31.75	6.18
KRAS c13-NC	38.86	13.63	38.85	13.52	39.79	14.63	40.27	15.22
KRAS c13-PC	31.58	5.71	31.47	5.47	31.54	5.82	31.84	5.97
KRAS c59-NC	37.83	12.66	37.52	12.49	38.39	13.38	40.62	15.77
KRAS c59-PC	32.82	6.85	32.58	6.69	31.94	6.4	31.61	6
KRAS c61-NC	41.26	16.04	43.53	18.13	43.17	17.91	44.39	19.33
KRAS c61-PC	34.59	8.71	34.18	8.17	34.53	8.92	35.95	10.38
KRAS c117-	37.34	11.1	37.27	12.16	38.24	13.21	39.93	15.03
KRAS c117-PC	33.64	7.85	33.27	7.72	33.12	7.37	33.38	7.9
KRAS c146-	41.63	16.56	43.67	18.48	43.55	18.42	44.66	19.82
KRAS c146-PC	35.23	9.52	35.14	9.54	34.43	8.78	34.48	8.89

8.10. Limit of Blank

Several lots of reagents were run without template (NTC) to assess the level of background noise when no template is present. No internal control or mutant signals were detected in any of the 50 replicates.

PART 9. SYMBOLS USED IN PACKAGING

Table 22. Symbols Used in Packaging

Symbol	Definition
	Catalog Number
	Manufactured By
	Temperature Limitation
	Batch Code
	Expiration Date
	Authorized Representative in the European Community
	CE Mark
2012-11-25	Date Format (year-month-day)
2012-11	Date Format (year-month)

REFERENCES

1. Beau-Faller et al., Detection of K-Ras mutations in tumour samples of patients with non-small cell lung cancer using PNA-mediated PCR clamping. Br J Cancer. 2009 Mar 24;100(6):985-92.
2. Chang et al., Fast simultaneous detection of K-RAS mutations in colorectal cancer. BMC Cancer. 2009 Jun 11;9:179.
3. Jeong et al., Rapid and Sensitive Detection of KRAS Mutation by Peptide Nucleic Acid based Real-time PCR Clamping: A Comparison with Direct Sequencing between Fresh Tissue and Formalin-fixed and Paraffin Embedded Tissue of Colorectal Cancer. The Korean Journal of Pathology 2011; 45: 151-169
4. Kobunai et al., The frequency of KRAS mutation detection in human colon carcinoma is influenced by the sensitivity of assay methodology : A comparison between direct sequencing and real-time PCR. Biochem Biophys Res Commun. 2010 Apr 23;395(1):158-62.
5. Kwon et al., Frequency of KRAS, KRAS, and KRAS mutations in advanced colorectal cancers: Comparison of peptide nucleic acid-mediated PCR and direct sequencing in formalin-fixed, paraffin-embedded tissue. Pathol Res Pract. 2011 Dec 15;207(12):762-8.
6. Ørum, Henrik., PCR Clamping.. Curr. Issues Mol. Biol. 2000; 2(1), 27-30.
7. Powell et. al., Detection of the hereditary hemochromatosis gene mutation by real-time fluorescence polymerase chain reaction and peptide nucleic acid clamping. Analytical Biochemistry 1998; 260: 142–8.
8. Schwartzberg et. al., Analysis of KRAS/KRAS mutations in PEAK: A randomized phase 2 study of FOLFOX6 plus panitumumab (pmab) or bevacizumab as first-line treatment for wild-type KRAS (exon 2) metastatic colorectal cancer (mCRC). ASCO Meeting 2013,
   J Clin Oncol 31, 2013 (suppl; abstr 5431).
9. ****MIQE Reference:** "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments". Stephen A. Bustin et. al., Clin Chem. 55 (4): 611–22 (2009).
   http://www.clinchem.org/content/55/4/611