QClamp® NRAS Codon Specific Mutation Test In Codons 12, 13, 59, 61, 117, & 146

For Real-Time PCR Assays

Instruction Manual

Catalog numbers:
DC-10-2020R (10 samples)
DC-10-3020R (30 samples)
DC-10-4020R (60 samples)

Instruction Version: Rev. 0
Date of Revision: June 2016

This instruction manual also applies to:
*QClamp® NRAS Codon Specific Mutation Test in Codons 12, 13, & 61
DC-10-1043R (10 samples) # DC-10-0048R (30 samples) # DC-10-0051R (60 samples)
*QClamp® NRAS Codon Specific Mutation Test in Codons 12, 13, 61, & 146
DC-10-1034R (10 samples) # DC-10-0172R (30 samples) # DC-10-0173R (60 samples)
*Where appropriate, please disregard assay mixes that are not part of your kit

For Research use only

DiaCarta Inc.
2600 Hilltop Dr., Bldg B, 3rd Floor,
Richmond CA 94806
Tel: 510-878-6662
Email: info@diacarta.com
FAX: (510) 735-8636

RUO

Table of Contents

Intended Use

The QClamp® NRAS Codon Specific Mutation Test in Codons 12, 13, 59, 61, 117, & 146 is a real-time qualitative PCR assay for the detection of somatic mutations in and near NRAS Codon 12 and 13 in Exon 2, Codon 59 and 61 in Exon 3, and Codon 146 in Exon 4 in the human NRAS gene, using purified DNA extracted from FFPE (formalin fixed paraffin embedded), 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 NRAS gene that can be detected by the kit. The kit is to be used by trained laboratory professionals within a laboratory environment.

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

Exon	Amino Acid Change	Nucleotide change	Cosmic No.
2	G12>D	c.35G>A	564
	G12>C	c.34G>T	562
	G12>S	c.34G>A	563
	G13>R	c.37G>C	569
	G13>V	c.38G>T	574

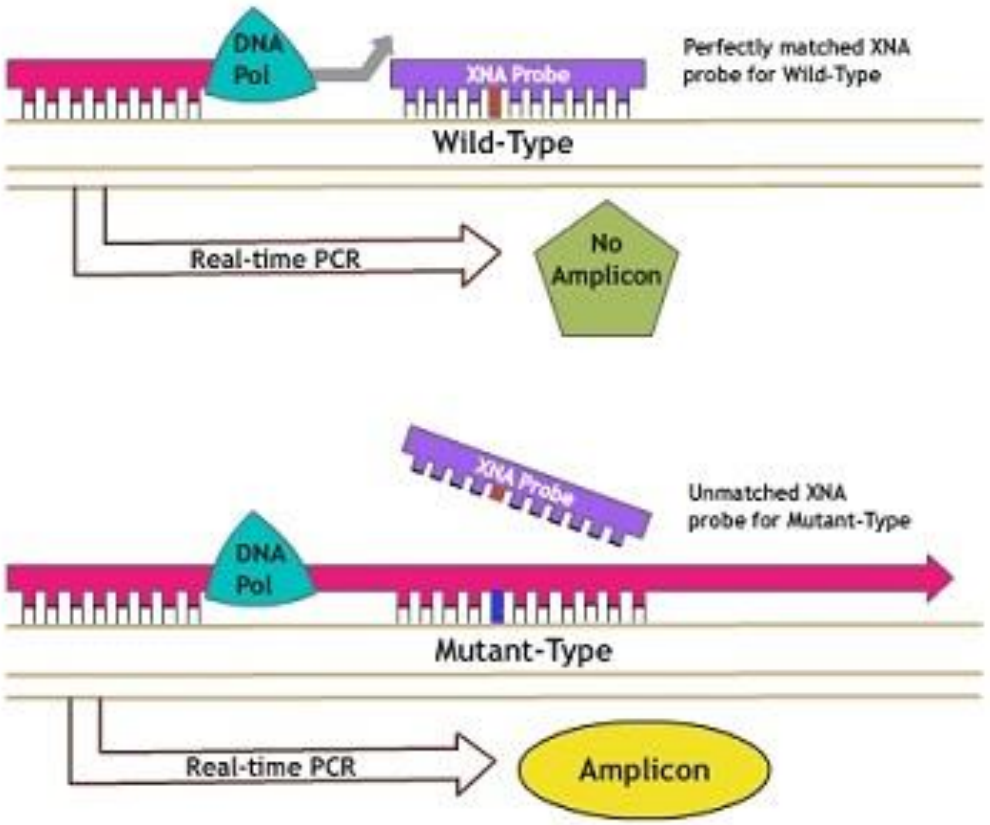
Exon	Amino Acid Change	Nucleotide change	Cosmic No.
3	A59>T	c.175G>A	578
	A59>D	c.176C>A	253327
	G60>R	c.178G>C	46507
	G60>E	c.179G>A	28673
	Q61>E	c.181C>G	581
	Q61>H	c.183A>C & c.183A>T	586/585
	Q61>L	c.182A>T, c.182_183AA>TG, & c.181_182CA>TT	583/30646/ 12725
	Q61>K	c.181C>A & c.181_183CAA>AAG	580/53223
	Q61>P	c.182A>C	582
	Q61>R	c.182A>G, c.181_182CA>AG, & c.182_183AA>GG	584/579/ 33696
	Q61>Q	c.183A>G	587

Exon	Amino Acid Change	Nucleotide change	Cosmic No.
4	A146>T	c.436G>A	19404
	K117>N	c.231A>G	N/A

QClamp® Technology for Mutation Detection

The QClamp® NRAS Codon Specific Mutation Test in Codons 12, 13, 59, 61, 117, & 146 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® NRAS Codon Specific Mutation Test in Codons 12, 13, 59, 61, 117, & 146



QClamp® Workflow

The assay workflow consists of five major steps:

- DNA Isolation: Extract DNA from FFPE tissue using commercial DNA extraction kit
- qPCR assay preparation: Mix the assay reagents and extracted DNA
- Set up qPCR: Enter amplification parameters on real-time PCR machine
- qPCR run: Load the reaction plate into a real-time PCR machine and start the run
- Data analysis: Determine the presence or absence of mutations according to the Cq value cutoffs

Reagents and Instruments

Package Contents

Table 2. Package Contents

No.	Name of Component	Description	Cap Color	Volume (10 tests)	Volume (30 tests)	Volume (60 tests)
1	External Control Primer Mix	Exon 5 Primers	Orange	1 X 88 uL	1 X 216 uL	1 X 336 uL
2	G12 Primer Mix	G12 Primers	Red	1 X 88 uL	1 X 216 uL	1 X 336 uL
3	G12 XNA	G12 XNA	Red	1 X 44 uL	1 X 108 uL	1 X 168 uL

4	G13 Primer Mix	G13 Primers	Purple	1 X 88 uL	1 X 216 uL	1 X 336 uL
5	G13 XNA	G13 XNA	Purple	1 X 44 uL	1 X 108 uL	1 X 168 uL
6	A59 Primer Mix	A59 Primers	Blue	1 X 88 uL	1 X 216 uL	1 X 336 uL
7	A59 XNA	A59 XNA	Blue	1 X 44 uL	1 X 108 uL	1 X 168 uL
8	Q61 Primer Mix	Q61 Primers	Yellow	1 X 88 uL	1 X 216 uL	1 X 336 uL
9	Q61 XNA	Q61 XNA	Yellow	1 X 44 uL	1 X 108 uL	1 X 168 uL
10	K117 Primer Mix	K117 Primers	Green	1 X 88 uL	1 X 216 uL	1 X 336 uL
11	K117 XNA	K117 XNA	Green	1 X 44 uL	1 X 108 uL	1 X 168 uL
12	A146 Primer Mix	A146 Primers	Clear	1 X 88 uL	1 X 216 uL	1 X 336 uL
13	A146 XNA	A146 XNA	Clear	1 X 44 uL	1 X 108 uL	1 X 168 uL
*14	2X PCR Master Mix	PCR Reaction Premix	Amber	3 X 450 uL	5 X 760 uL	5 X 1180 uL
*15	Clamping Control	Wild-type DNA	Clear	1 X 84 uL	1 X 168 uL	1 X 168 uL
*16	Mixed Positive Control	G12V, G13D, A59T, Q61H, K117N, & A146T Template	Clear	1 X 84 uL	1 X 168 uL	1 X 168 uL
*17	Nuclease-Free Water	Nuclease-Free Water	Clear	1 X 128 uL	1 X 276 uL	1 X 336 uL

* The volumes listed are for catalog products DC-10-2020, DC-10-3020, and DC-10-4020. For catalog products DC-10-1043, DC-10-0048, DC-10-0051, DC-10-1034, DC-10-0172, and DC-10-0173 fill volumes will vary. See product insert for appropriate reagent volume provided in kit.

Materials Required but Not Provided in the Kit

Reagents for DNA Isolation

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) or equivalent

Consumables

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

Equipment

- Permanent marker
- Real time PCR instrument capable of SYBR green detection
- 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
- Distilled water

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

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

Company	Model
ABI	ABI 7900
QIAGEN	Rotor-Gene Q

Handling and Storage

This kit is shipped on dry ice and/or cold packs, depending on recipient's country of import. 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.

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.

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 4 hours at ambient temperature. The 2X PCR master mix contains SYBR Green dye 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.

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 in order 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.

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 2X PCR Master mix to room temperature for optimal amplification.

- Avoid overexposing the 2X PCR Master mix 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.
- Additional purification may be required if DNA has been extracted from a paraffin block.
- 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 4 hours.

Instructions for Use

DNA Isolation

Human genomic DNA must be extracted from tissue or blood, or fixed paraffin-embedded tissue prior to use. Several methods exist for DNA isolation. For consistency, we recommend using a commercial kit, such as Qiagen DNA extraction kit (QIAamp DNA FFPE Tissue Kit, cat No. 56404, for paraffin embedded specimens; DNeasy Blood & Tissue kit, cat. No. 69504 or 69506, for tissue and blood specimens). Follow the genomic DNA isolation procedure according to manufacturer's protocol. Sufficient amounts of DNA can be isolated from FFPE blocks or fresh frozen sections (approx. 2-10 µg).

This QClamp assay requires a total of 25- 50 ng of DNA per sample (5-10ng/reaction).
After DNA isolation, measure the concentration using fluorometric or spectrophotometric analysis (i.e. Nanodrop or UV spectrophotometer) and dilute to 1.25-2.5 ng/µl. For spectrophotometric analysis, make sure the A260/A230 value is greater than 2.0 and A260/A280 value between 1.8 and 2.0.

Preparation of Reagents

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).

Thaw all primers, XNAs, Positive Control, WT Clamping 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 for 5 seconds and perform a quick spin. The PCR Master Mix should be mixed gently by inverting the tube a few times. Prior to use, ensure that any precipitate in the PCR Master Mix is resuspended by pipetting up and down multiple times. Do not leave kit components at room temperature for more than 4 hours. The PCR reactions are set up in a total volume of 20 µl/reaction. *Table 4* shows the component volumes for each 20ul reaction.

Table 4. QClamp Assay Components and Reaction Volume

Components	Volume/Reaction
2X PCR Master mix	10 µl
Primer Mix	4 µl
XNA	2 µl
DNA sample or Controls	4 µl
Total volume	20 µl

For accuracy, 2x PCR Mastermix, primers and XNA should be pre-mixed into assay mixes as described in *Table 5* below

Preparation of Assay Mixes

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.

Table 5. Preparation of Assay Mixes

	Volume of 2X PCR Master Mix	Volume of Primer Mix	Volume of XNA*
Ext Control Mix*	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)
G12 Mix	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)
G13 Mix	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)
A59 Mix	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)
Q61 Mix	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)
K117 Mix	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)
A146 Mix	10 µl x (n+1)	4 µl x (n+1)	2 µl x (n+1)

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 the External Control Mix, use 2µl of water provided in the kit in place of XNA.
For accuracy, do not pipette less than 8 µl.

Prepare sufficient working assay mixes for the DNA samples, one NRAS Mixed Positive Control, one Nuclease-Free Water for No-Template Control (NTC), and one WT Clamping Control, according to the volumes in *Table 5*. Include reagents for 1 extra sample to allow sufficient overage for the PCR set up. The assay mixes contain all of the components needed for PCR except the sample.

Each sample requires one reaction for each mutation site detected by the kit and an External Control assay. The External Control assay uses primers in Exon 5 of the NRAS gene to determine if an appropriate level of amplifiable DNA is present in the sample.

A set of controls: Clamping Control and Positive Control Mix must be run with each reaction mix, every time the assay is run. Clamping 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 External Control Mix with the Clamping 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.

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

Add 16ul of the appropriate assay mix to the plate or tubes. Add 4ul of template.

Table 6 is a suggested plate set-up for a single experiment analyzing 3 unknown samples. Please disregard any assay mixes listed below that are not part of your kit.

NOTE: For setup on the Rotor-Gene Q Platforms, the layout must be changed such that the first tube contains Positive Control

Table 6. Suggested Plate Layout

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

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.

Instrument Set-Up

Applied Biosystems Platforms

1. Use SYBR Green as the Detector
2. Select NONE for the Passive Reference Dye
3. Setup the cycling parameters as shown in *Table 8* below

Table 7. Applied Biosystems Platforms Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Cycles	Data Collection
PreIncubation	95	30	1	OFF
Denaturation	95	20	X5	OFF
XNA Annealing	70	40		OFF
Primer Annealing	68	30		OFF

Extension	72	30		OFF
Denaturation	95	20	X5	OFF
XNA Annealing	70	40		OFF
Primer Annealing	66	30		OFF
Extension	72	30		OFF
Denaturation	95	20	X40	OFF
XNA Annealing	70	40		OFF
Primer Annealing	64	30		OFF
Extension	72	30		**ON**
Melting	Default	Default	Continuous	**ON**

QIAGEN Rotor-Gene Q

In the instrument software version 2.1 and above

4. Select File>New or click the "New" button from the menu bar.
5. Select the "Advanced" tab and choose "Three Step with Melt" and click New
6. Select the appropriate rotor, check the "Locking Ring Attached" box, click Next
7. Set Reaction volume to 20ul, click next
8. Click "Edit Profile…" and change the cycling parameters to match *Table 8.*
9. In Channel Setup, select the green channel
    a. Click Gain Optimization
    b. Click "Optimize Acquiring"
    c. In the pop-up box enter
        i. Target Sample Range 1FL up to 3FL
        ii. Acceptable Gain Range -10 to 10
    d. Click "Perform Optimization before 1st acquisition"
    e. Click "OK, then "Close", then "Next>>"
    f. Click "Save Template" to save if desired
    g. Click "Start Run"

Table 8. QIAGEN Rotor-Gene Q Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	# Cycles	Data Collection
PreIncubation	95	300	1	OFF
Denaturation	95	20	5	OFF
XNA Annealing	70	40		OFF
Primer Annealing	68	30		OFF
Extension	72	30		OFF
Denaturation	95	20	5	OFF
XNA Annealing	70	40		OFF
Primer Annealing	66	30		OFF
Extension	72	30		OFF
Denaturation	95	20	40	OFF
XNA Annealing	70	40		OFF
Primer Annealing	64	30		OFF

Extension	72	30		**ON**
Melting	Default	Default	1	**ON**

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).

Clamping Controls use wild-type DNA as the template. Wild-type DNA contains none of the targeted NRAS mutations, therefore the XNA probes will bind strongly, inhibiting the target amplification. In contrast, the External Control Assay should make amplicons efficiently for the Clamping Control, providing another way to monitor performance of the primers, polymerase, and sample.

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 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.

Data Analysis for Applied Biosystems Platforms

For the ABI 7900, manually change the analysis settings to use a threshold of 2 and Auto Baseline. Check that the threshold is within the exponential growth phase of the amplification plot (linear region of the curve when the Y-axis is set to Log); if not, the threshold value should be adjusted.

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. Occasionally, a Cq may be observed in the NTC. In such cases, check the melting curves obtained. If the dissociation peak is different than listed in *Table 9,* this indicates the formation of spurious products such as primer dimers rather than contamination and the performance is considered acceptable.

Table 9. Expected Dissociation Values

Assay	Tm (°C)
Ext Control	77.4 ± 0.5
G12	81.2± 0.5
G13	80.9± 0.5
A59	79.1± 0.5
Q61	81.5± 0.5
K117	83.2± 0.5
A146	83.2± 0.5

For each control or sample, calculate the difference in Cq value between the mutation assay and the External Control Assay as follows:

Cq difference (ΔCq) = Mutation Assay Cq – External Control Assay Cq

Analysis of Clamping and Positive Controls

For the assay to be valid, the Clamping Control and Positive Control must meet the criteria in Table 10.

Table 10. Acceptable Values for Positive Controls and Clamping Controls

	Positive Control Acceptable Values	Clamping Control Acceptable Values
Ext Control	15 ≤ Cq ≤ 22	15 ≤ Cq ≤ 22
G12 Mix	ΔCq ≤ 7	ΔCq > 7
G13 Mix	ΔCq ≤ 6	ΔCq > 6
A59 Mix	ΔCq ≤ 6	ΔCq > 6
Q61 Mix	ΔCq ≤ 8	ΔCq > 8
K117 Mix	ΔCq ≤ 5	ΔCq > 5
A146 Mix	ΔCq ≤ 9	ΔCq > 9

Judging Validity of Sample Data Based on External Control Mix Results

The Cq value of the Ext 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 Ext Control mix. Cq values of any sample with Ext Control Mix should be in the range of 15–22. 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 11*.

Table 11. Acceptable Cq Ranges for Samples with External Control Mix

Validity	Cq Value of Ext Control Mix	Descriptions and Recommendations
Optimal	15 < Cq < 22	The amplification and amount of DNA sample were optimal.
Invalid	Cq ≤15	Possibility of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ≥ 22	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

Scoring Mutational Status

If a Cq value is Undetermined, assign a Cq of 40 and proceed to analysis.

For each sample that produces a positive Cq for any of the mutation assays, the dissociation peaks should be scored. Samples for which the dissociation peaks are not within the ranges specified in *Table 9*, a Cq of 40 (representing undetected amplification) should be assigned. (NOTE: failure to perform this step could result in false positive results due to primer-dimer formation or spurious amplification).

The table below should be used to determine mutational status.

Table 12. Scoring Mutational Status

Mutation		G12	G13	A59	Q61	K117	A146
Positive:	ΔCq	≤7	≤6	≤6	≤8	≤5	≤9
Negative	ΔCq	>7	>6	>6	>8	>5	>9

*Refer to *Table 17* for interpretation of G12/G13 Mutational Status

Data Analysis for QIAGEN Rotor-Gene Q Platforms

In the Analysis tool, select the Quantitation tab to analyze the cycling data. Manually set the threshold to 0.09 in the "CT Calculation" menu and set "Eliminate Cycles before" value to 12. In the "Quantitative Analysis" window, make sure that "Dynamic Tube" is selected. From the sample list, unselect any empty tubes.

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. Occasionally, a Cq may be observed in the NTC. In such cases, check the melting curves obtained. If the dissociation peak is different than listed in *Table 13,* this indicates the formation of spurious products such as primer dimers rather than contamination and the performance is considered acceptable.

Table 13. Expected Tm values

Assay	Tm (°C)
Ext Control	78.8 ± 0.5
G12	82.3± 0.5
G13	82.3± 0.5
A59	80.4± 0.5
Q61	82.7± 0.5
K117	84.2± 0.5
A146	84.2± 0.5

Analysis of Clamping and Positive Controls

For the assay to be valid, the Clamping Control and Positive Control must meet the criteria in *Table 14*.

Table 14. Acceptable Values for Positive Controls and Clamping Controls

	Positive Control Acceptable Values	Clamping Control Acceptable Values
Ext Control	18 ≤ Cq ≤ 23	18 ≤ Cq ≤ 23
G12 Mix	ΔCq ≤ 4	ΔCq > 4
G13 Mix	ΔCq ≤ 7	ΔCq > 7
A59 Mix	ΔCq ≤ 10	ΔCq > 10
Q61 Mix	ΔCq ≤ 6	ΔCq > 6
K117 Mix	ΔCq ≤ 2.5	ΔCq > 2.5
A146 Mix	ΔCq ≤ 9	ΔCq > 9

Judging Validity of Sample Data Based on External Control Mix Results

The Cq value of the Ext 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 Ext Control mix. Cq values of any sample with Ext

Control Mix should be in the range of 18-23. 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 15*.

Table 15. Acceptable Cq Ranges for Samples with External Control Mix

Validity	Cq Value of Ext Control Mix	Descriptions and Recommendations
Optimal	18 < Cq < 23	The amplification and amount of DNA sample were optimal.
Invalid	Cq ≤18	Possibility of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ≥ 23	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

Scoring Mutational Status

For each control or sample, calculate the difference in Ct value between the mutation assay and the External Control Assay as follows:

Ct difference (ΔCt) = Mutation Assay Ct – External Control Assay Ct

If a Ct value is Undetermined, assign a Ct of 40 and proceed to analysis.

For each sample that produces a positive Ct for any of the mutation assays, the dissociation peaks should be scored. Samples for which the dissociation peaks are not within 0.5°C of the positive control peaks, a Ct of 40 (representing undetected amplification) should be assigned. (NOTE: failure to perform this step could result in false positive results due to primer-dimer formation or spurious amplification).

The table below should be used to determine mutational status.

Table 16. Scoring Mutational Status

Mutation		G12	G13	A59	Q61	K117	A146
Positive:	ΔCq	≤4	≤7	≤10	≤6	≤2.5	≤9
Negative	ΔCq	>4	>7	>10	>6	>2.5	>9

*Refer to *Table 17* for interpretation of G12/G13 Mutational Status

Differentiating G12/G13 Mutational Status

The G12 reaction mix detects both G12 and G13 mutations, whereas the G13 reaction mix detects only G13 mutations. Therefore, in order to differentiate between G12 and G13 Mutations a combination of results from the 2 mixes should be used, as described in *Table 13* below.

Table 17. Interpretation of G12/G13 Mutational Status

Reaction Mix	Result Based on Table 12	Mutational Status
G12 Reaction Mix G13 Reaction Mix	Positive Negative	G12 Mutation
G12 Reaction Mix G13 Reaction Mix	Positive Positive	G13 Mutation
G12 Reaction Mix G13 Reaction Mix	Negative Positive	G13 Mutation

HRM Curves as a Tool to Confirm Analyses

In High Resolution Melting Analysis (HRM), the region of interest amplified by PCR is gradually melted. SYBR green is a dsDNA binding dye that is released as the dsDNA amplicon is melted. Emitted fluorescence is

measured to generate a characteristic curve. The Tm (Melting Temperature) is characteristic of the GC content, length and sequence of a DNA product and is a useful tool in product identification. The resulting melt profile reflects the mix of amplicons present.

Wild-type DNA (clamping control) is provided. Some amplification may occur in these reactions. Melt profiles of unknown samples should be compared to wild-type and positive controls. Enrichment of one or more peaks, resulting in a melt profile distinct from wild-type DNA profile, can serve as an indication of specific amplification of a mutation target. If the melt profile of an unknown sample is similar to wild-type DNA, and has been scored as a mutation due to Cq, the analysis should be repeated. The resulting PCR product can be sent for Sanger sequencing for further clarification.

A mixed positive control is provided which contains a mutant template for each codon investigated by the assay. HRM curves obtained from unknown samples can be compared to HRM curves obtained from positive controls. Amplicons of similar length and sequence will exhibit the same melt profile. Templates are not provided for every possible mutant target, so melt profiles obtained from an unknown sample, may not be represented in the positive control.

Small differences in melt curves can arise from sources other than sequence, such as genomic DNA quality, carryover of extraction impurities, amplicon length, primer design and PCR reagent choice. For best results, all DNA samples in an analysis should be prepared using the same method. DNA input amount into the PCR reaction should also be similar.

Assay Performance Characteristics

The performance characteristics of this product were established on the ABI 7500, ABI 7900, Roche LightCycler 96, Roche LightCycler 480 real-time PCR instruments.

Cut-Offs

Along with studies for analytical accuracy, reference DNA and FFPE samples were tested to establish cut-offs for the assay. Cutoffs are presented in *Tables 9-16* of the manual.

Multiple Freeze-Thaw Cycles

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

Shelf-Life

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

Symbols Used in Packaging

Table 22. Symbols Used in Packaging

Symbol	Definition
	Catalog Number
	Manufactured By
	Temperature Limitation
	Batch Code
	Expiration Date
RUO	For Research use only
2012-11-25	Date Format (year-month-day)
2012-11	Date Format (year-month)

References

1. Ørum, Henrik., PCR Clamping.. Curr. Issues Mol. Biol. 2000; 2(1), 27-30.
2. 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.
3. **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

DiaCarta Inc.

2600 Hilltop Drive
Richmond, CA 94806
Email: information@diacarta.com
Tel: +1 (510) 878-6662
Fax: +1 (510) 735-8636
www.diacarta.com