



QClamp® BRAF Codon Specific Mutation Test in Codon 600

Instruction Manual

Catalog #:

DC-10-1039R, 10 samples

DC-10-0197R, 30 samples

DC-10-0169R, 60 samples

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For Research use only



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Intended Use

The QClamp® BRAF Codon Specific Mutation Test in Codon 600 is a real-time PCR assay for the detection of somatic mutations in and near codon 600 in exon 15, in the BRAF serine/threonine protein kinase 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. The codon specific detection kits are designed to detect any mutation at or near the stated codon site without specifying the exact nucleotide change. *Table 1* shows a list of mutations commonly found in the BRAF 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 BRAF

Exon	Amino Acid Change	Nucleotide change
15	V600>E	c1799T>A
	V600>K	c1798_1799GT>AA
	V600>D	c1799_1800TG>AT
	V600>R	c1798_1799GT>AG
	V600>M	c1798G>A

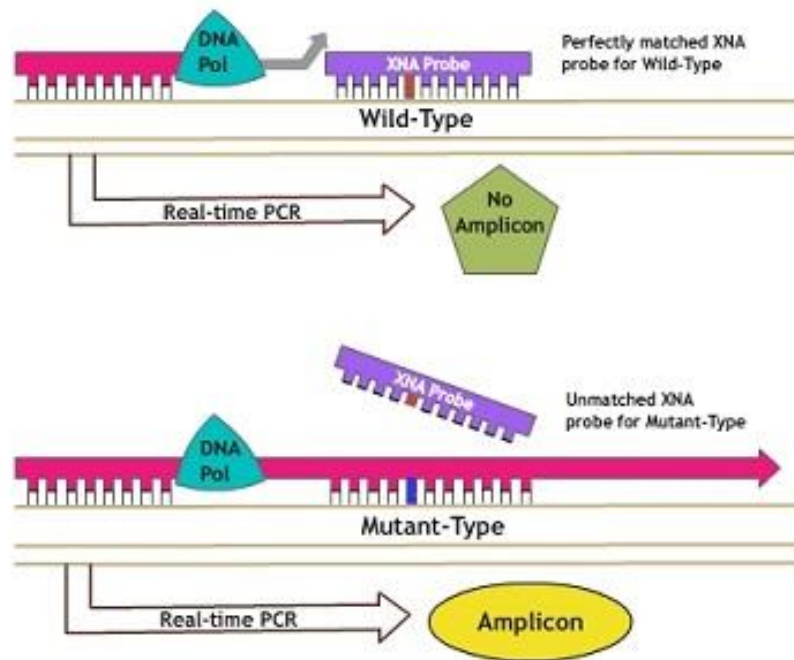
QClamp® Workflow

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
- Setup 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

QClamp® Technology for Mutation Detection

The QClamp® BRAF Codon Specific Mutation Test in Codon 600 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 XNA is a synthetic DNA analog in which the phosphodiester backbone has been replaced by a repeat formed by units of DiaCarta's proprietary novel uncharged backbone chemistry.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® BRAF Codon Specific Mutation Test in Codon 600

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 17 Primers	Orange	1 X 88 uL	1 X 216 uL	1 X 336 uL
2	V600 Primer Mix	V600 Primers	Red	1 X 88 uL	1 X 216 uL	1 X 336 uL
3	V600 XNA	V600 XNA	Red	1 X 44 uL	1 X 108 uL	1 x 168 uL
4	2X PCR Master Mix	PCR Reaction Premix	Amber	1 X 450 uL	1 X 760 uL	1 X 1180 uL
5	Clamping Control	Wild-type DNA	Clear	1 X 24 uL	1 X 48 uL	1 x 48 uL
6	Mixed Positive Control	V600E Template	Clear	1 X 24 uL	1 X 48 uL	1 x 48 uL
7	Nuclease-Free Water	Nuclease-Free Water	Clear	1 X 68 uL	1 X 156 uL	1 x 216 uL

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 DiaCarta Kits

Company	Model
ABI	ABI 7500, 7500FAST
ABI	ABI 7900
ABI	StepOnePlus
Qiagen	Rotor-Gene Q
Roche	LightCycler LC96
Roche	LightCycler 480 II

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 10– 20 ng of DNA per sample (5–10ng/reaction).

After DNA isolation, measure the concentration using spectrophotometric analysis (i.e. Nanodrop or UV spectrophotometer) and dilute to it to 1.25–2.5 ng/µl. Make sure 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 1 hour. 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. Do not leave kit components at room temperature for more than 4 hours. After thawing, keep materials on ice at all times. 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*.

Table 5. Preparation of Assay Mixes

	Volume of 2X PCR Master Mix	Volume of Primer Mix	Volume of XNA (†use water for ext control)
Ext Control Mix	10 µl x (*n+1)	4 µl x (*n+1)	2 µl x (*n+1)
V600 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.

† Use 2ul of water provided in the kit as the Ext Control Mix does not require XNA.

For accuracy, do not pipette less than 10ul of the XNA.

Prepare sufficient working assay mixes for the DNA samples, one BRAF 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 17 of the BRAF 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 PCR plate. Add 4ul of template.

In the case of 96-well plates, the exact plate layout can be set to the user’s preference.

Table 6 is a suggested plate set-up for a single experiment analyzing 3 unknown samples.

Table 6. Suggested Plate Layout

	1	2	3	4	5	6
A	NTC <i>Ext Ctrl Mix</i>	PC <i>Ext Ctrl Mix</i>	CC <i>Ext Ctrl Mix</i>	S1 <i>Ext Ctrl Mix</i>	S2 <i>Ext Ctrl Mix</i>	S3 <i>Ext Ctrl Mix</i>
B	NTC <i>V600 Mix</i>	PC <i>V600 Mix</i>	CC <i>V600 Mix</i>	S1 <i>V600 Mix</i>	S2 <i>V600 Mix</i>	S3 <i>V600 Mix</i>

PC: Positive control, NTC: No template control (water), CC: Clamping control (Wild-type DNA), S1-3: Samples 1-3.

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

When all reagents have been loaded, tightly close the PCR tubes or 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

Roche LightCycler 96 or RocheLightCycler 480

1. Select New empty experiment > create
2. In the Run Editor>Measurement, choose SYBR Green 1 (470/514) channel on (LC96), SYBR Green 1 /HRM Dye on (LC480)
3. Set up run profile using parameters in Table 7. Ramp rates for the LC 96 and LC480 should match settings below
4. During the analysis:
 - a. Set Threshold to Auto
 - b. Under Cycle Range, set First Cycle to 15, Last Cycle is at 40

Table 7. Roche Light Cycler, LC96 and LC480 Parameters

Step	Temperature (°C)	Time (Seconds)	Cycles	Ramp Rate	Mode	Acquisition Mode
Preincubation	95	300	1	4.4		None
Denaturation	95	20	X40	2.2	Standard	None
XNA Annealing	70	40		2.2		None
Primer Annealing	64	30		2.2		None
Extension	72	30		1.0		Single
Melting	95	10		1		4.4
	65	60	2.2		None	
	97	1	0.20		Continuous (5 readings/°C)	
Cooling	37	30	1	2.2		None

* An HRM curve or melt analysis should be run at the end of the PCR reaction. This helps to verify the PCR amplification results and with troubleshooting.

Applied Biosystems Platforms

1. Select File>New Experiment
2. Enter an experiment name and select 7500 (96 wells) or as appropriate
3. Select Quantitation – Standard Curve

4. Select SYBR Green Reagents
5. Select Standard Ramp Rate if available
6. Click on Plate Setup in the left navigation panel
7. Select the Assign Targets and Samples tab and assign samples to the wells
8. Select NONE for the Passive Reference Dye
9. Click on Run Method on the left panel, set reaction volume to 20ul
10. Setup the cycling parameters as shown in the table below
11. Add Melt Curve at the end of the Cycling Stage. Use continuous and leave default setting for data collection
12. **During the analysis set threshold to 0.5 (ABI 7900) and 5000 (ABI 7500).**

Table 8. Applied Biosystems Platforms Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Cycles	Data Collection
Preincubation	95	300	1	OFF
Denaturation	95	20	X40	OFF
XNA Annealing	70	40		OFF
Primer Annealing	66	30		OFF
Extension	72	30		ON
Melt Curve	Default			Continuous

Rotor–Gene Q Platforms

In the instrument software version 2.1 and above

1. Select File>New, Select Three Step with Melt and click New
2. Select appropriate rotor, check the Locking Ring Attached box, click Next
3. Set Reaction volume to 20ul, click next
4. Set Temperature profile as shown in *Table 9*.
5. Channel Setup: Select Green Source 470nm, Detector 510nm, Gain 7
 - a. Click Gain Optimization
 - b. Set Temperature to 70C
 - c. Perform Optimization before 1st acquisition
 - d. Click optimize acquiring
 - e. In the pop-up box enter
 - i. Target Sample Range 5FL up to 10FL
 - ii. Acceptable Gain Range -10 to 10
 - f. Click OK, Click Close, Click Next
6. Start–run
7. **During the analysis set threshold to 0.01–0.02**

Table 9. Rotor–Gene Q Platforms Cycling Parameters

Hold		95°C	5 minutes	X1	Not Acquiring
Cycling	Timed Step	95°C	20 seconds	X40	Not Acquiring
	Timed Step	70°C	40 seconds		Not Acquiring
	Timed Step	64 °C	30 seconds		Not Acquiring
	Timed Step	72°C	40 seconds		Acquiring to Cycling A on Green
Melt		Ramp from 65 to 95, rising by 1degree each step Wait for 90 sec of pre-melt conditioning on first step Wait for 5 seconds for each step afterwards Gain Optimization Check optimize gain before melt on all tubes The gain giving the highest fluorecence less than 95 will be selected.			Acquire to melt A on green

Assessment of Real–Time PCR Results

For the analysis use Absolute Quantitation and Automatic Baseline. The threshold to be used with each instrument is listed above. Check threshold to ensure that the Threshold is within the exponential growth phase of the amplification plot. If not, the threshold may be adjusted depending on the run.

The real–time PCR instrument generates a Cq value. Cq is the cycle threshold, the cycle number at which a signal is detected above background 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).

No Template Controls

Verify that there is no amplification in no–template controls for each of the reaction mixes. Cq should be undetermined. For some mixes a Cq of 36 or higher may be observed in the NTC. In such cases, check the melting curves obtained. If the melting curve indicates the presence of primer dimers, the reaction may be acceptable. SYBR green binds to primer dimmers, resulting in a peak with a lower melting temperature, than the desired amplicon. In many cases formation of primer dimers can be avoided by setting up the PCR reactions on ice, until ready to load into the PCR instrument.

Analysis of Clamping and Positive Controls

The Cq values of the positive control (mixed mutant templates) should amplify in the presence of XNAs and yield Cq values given in *Table 10*.

Table 10. Acceptable Cq Ranges for Positive Controls

	Positive Control Acceptable Cq Range
Ext Control	20 ≤ Cq ≤ 26
V600 Mix	≤ 31

*The Cq value of the clamping control (WT DNA) with the Ext Control Mix should be within 20 and 26.

*In addition, the Cq of the Clamping Control with each of the mutation reaction mixes should be at least 3 Cq greater than the Cq of the Positive Control with the same reaction mix. If these criteria are not met, the reaction has failed and the results are not valid.

PASS: Cq of Clamping Control with mutation reaction mix – Cq of Positive Control with same mutation reaction mix ≥ 3

FAIL: Cq of Clamping Control with mutation reaction mix – Cq of Positive Control with same mutation reaction mix ≤ 3

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 the 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 20–27. If the Cq values fall outside the range given in *Table 11*, the test results should be considered invalid. The experiment should be repeated.

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

Validity	Cq Value of Ext Control Mix	Descriptions and Recommendations
Optimal	20 < Cq < 27	The amplification and amount of DNA sample were optimal.
Invalid	Cq ≤ 20	Possibility of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ≥ 27	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.

The table below should be used to determine mutational status

Table 12. Scoring Mutational Status

Mutation		V600
Strong Positive ¹ :	Cq	≤ 31
	Cq	31 < Cq ≤ 35*
Weak Positive ² :	ΔCq	≤ 10
	Cq	> 35

*If reaction has been set-up with 5ng of DNA, it is recommended that the experiment be repeated with 10ng of template DNA to confirm the results.

¹ Mutation Content: >5%

² Mutation Content: 1–5%

If the Cq value suggests mutation content between 1%–5%, a further calculation of ΔCq should be performed to determine mutational status.

$$\Delta Cq = [\text{Cq value of sample with mutant reaction mix}] - [\text{Cq value of sample with Ext Control Mix}]$$

For ex: $\Delta Cq = [\text{Cq of sample with V600 mutant reaction mix}] - [\text{Cq of sample with Ext Control Mix}]$

Refer to the table above to confirm mutational status of weak positives.

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, and Qiagen Rotor-Gene Q real-time PCR instruments.

Cut-Offs

Along with studies for analytical accuracy, both reference DNA and FFPE samples were tested to establish cut-offs for the assay by using different concentrations of input DNA for assays. Cutoffs are presented in *Tables 10-12* 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 15. Symbols Used in Packaging

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

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>

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