INSTRUCTION FOR USE

QClamp® BRAF Mutation Detection Test

For Real-Time PCR Assays

CATALOG NUMBER

DC-10-1039R (10 samples)
DC-10-0197R (30 samples)
DC-10-0169R (60 samples)

MANUFACTURER

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INTENDED USE

For Research Use

*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® BRAF Mutation Detection Test is a research-use-only real-time PCR assay for qualitative detection of somatic mutations in and near codon 600 on exon 15, in the BRAF serine/threonine protein kinase gene, using purified DNA extracted from FFPE (formalin fixed paraffin embedded), plasma (cell-free DNA), 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 most frequent mutations found in the BRAF gene that can be detected by the kit. The QClamp® BRAF Cancer Mutation Detection assay is a sensitive tool intended to facilitate research in cancer, development, early detection, disease monitoring and therapeutic interventions. The kit is to be used within a laboratory environment for research only, not for clinical diagnostic purposes.

Table 1. List of Most Frequent BRAF Mutations and Cosmic Identities Detected by the QClamp® BRAF Assay

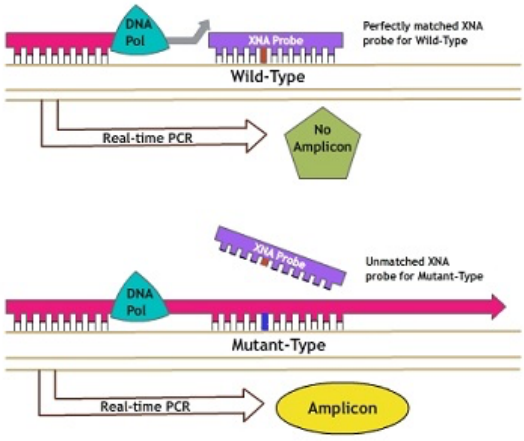
Exon	Amino Acid	Cosmic No.
15	p. V600E	COSM476, COSM475,
	p. V600K	COSM473, COSM26487
	p. V600R	COSM474, COSM21617
	p. V600D	COSM477, COSM36128

PART 2. BRAF MUTATIONS AND CANCER

The B-type Raf Kinase (BRAF) protein is a serine/threonine kinase that has important roles in regulating the MAP kinase/ERK signaling pathways, affecting cellular proliferation, differentiation, and programmed cell death. A BRAF mutation is commonly found in many human cancers including malignant melanoma, colorectal cancer, lung cancer, papillary thyroid carcinoma, non-small cell lung carcinoma, and adenocarcinoma of lung. The most common mutations in BRAF occur in codon 600, where an amino acid substitution in the activation segment of the kinase domain creates a constitutively active form of the protein. The V600E and V600K mutations are found in high frequencies in human cancer at 70-90% and 10-15%, respectively. BRAF mutations are generally found in tumors that are wild type for KRAS, NRAS, and EGFR. Therefore, BRAF c600 mutation may serve as a biomarker for diagnosis, prognosis, and treatment options for cancer patients.

PART 3. QCLAMP® TECHNOLOGY FOR MUTATION DETECTION

Figure 1. Principle of the QClamp® BRAF Mutation Test in Targeted Genes



The QClamp® BRAF mutation detection assay 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 novel synthetic 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 is a complete match to wild-type DNA, into 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.

PART 4. REAGENTS AND INSTRUMENTS

4.1. Package Contents

The QClamp® BRAF Mutation Detection Test comes in one box and is assembled as shown in Table 2.

Table 2. Kit components

Table 2a. Pack-Size: 10 Samples

Name of Component	Part #	Description	Cap Color	Pack Size: 10 Samples	Label Volume for each vial	Storage Temp
Primer/Probe Mix	1001462	BRAF c600 Primers and probe	Amber	1 vial	38 µL	-25°C to -15°C
XNA	1001472	BRAF c600 XNA	Clear	1 vial	19 µL	-25°C to -15°C
2X qPCR Master Mix	1002102	PCR Reaction Premix	Blue	1 vial	95 µL	-25°C to -15°C
Negative Control	1002122	Wild-type DNA	Red	1 vial	12 µL	-25°C to -15°C
Positive Control	1002112	BRAF c600 mutant Template	Green	1 vial	12 µL	-25°C to -15°C
Non-Template Control	1002132	Nuclease-Free Water	Orange	1 vial	24 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
Primer/Probe Mix	1001463	BRAF c600 Primers and probe	Amber	1 vial	96 µL	-25°C to -15°C
XNA	1001473	BRAF c600 XNA	Clear	1 vial	48 µL	-25°C to -15°C
2X qPCR Master Mix	1002103	PCR Reaction Premix	Blue	1 vial	240 µL	-25°C to -15°C
Negative Control	1002123	Wild-type DNA	Red	1 vial	24 µL	-25°C to -15°C
Positive Control	1002113	BRAF c600 mutant Template	Green	1 vial	24 µL	-25°C to -15°C
Non-Template Control	1002133	Nuclease-Free Water	Orange	1 vial	36 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
Primer/Probe Mix	1001464	BRAF c600 Primers and probe	Amber	1 vial	156 µL	-25°C to -15°C
XNA	1001474	BRAF c600 XNA	Clear	1 vial	78 µL	-25°C to -15°C
2X qPCR Master Mix	1002104	PCR Reaction Premix	Blue	1 vial	390 µL	-25°C to -15°C
Negative Control	1002124	Wild-type DNA	Red	1 vial	24 µL	-25°C to -15°C
Positive Control	1002114	BRAF c600 mutant Template	Green	1 vial	24 µL	-25°C to -15°C
Non-Template Control	1002134	Nuclease-Free Water	Orange	1 vial	36 µL	-25°C to -15°C

4.2. Materials Required but Not Provided in the Kit

4.2.1. Reagents for DNA Isolation

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

4.2.2. Consumables

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

4.2.3. Equipment

- Permanent marker
- Real time PCR instrument
- 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

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

4.3. Instruments

The assays have been developed and validated on the instruments as listed in Table 3. 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
Roche	Light cycler 96
Roche	Light cycler 480
Bio-Rad	CFX384
ABI	Quant Studio 5

4.4. 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 1) in a pre-amplification area and the controls (Box 2) in a 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 on the box and labels of all components. Do not use expired or incorrectly stored components.

4.5. 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 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.

4.6. 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 2X PCR Master mix to room temperature for 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 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.

PART 5. QCLAMP® WORKFLOW

The assay workflow consists of five major steps:

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

PART 6. INSTRUCTIONS FOR USE

6.1. DNA Isolation

Human DNA must be extracted from plasma or FFPE tissue prior to use. Several methods exist for DNA isolation. For consistency, we recommend using a commercial kit listed below as per the manufacturer's protocol.

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

This QClamp® BRAF assay requires a total of 2.5 - 5 ng of DNA per sample. 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 (PC), WT Clamping Control (CC), Nuclease-Free Water, and 2X PCR master mix provided. Thaw all reaction mixes at room temperature for a minimum of 30 minutes. Vortex all components except the PCR Master Mix and Primer/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 shows the component volumes for each 10 µl reaction.

Table 4. QClamp® TaqMan Assay Components and Reaction Volume

Components	Volume/Reaction
2X PCR Master mix	5 µl
Primer and Probe Mix	2 µl
XNA	1 µl
DNA Sample or	2 µl
Total volume	**10 µl**

Notes: For accuracy, 2x PCR Master Mix, Primers and XNA should be pre-mixed into assay mixes as described in Table 5 below.

6.3. Preparation of Assay Mixes

Assay mixes should be prepared just prior to use. 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. Label a micro centrifuge tube (not provided) for the BRAF reaction mix, as shown in Table 5. For the mutation detection reaction, prepare sufficient working assay mixes for the DNA samples, one Positive Control (PC), one Nuclease-Free Water for No-Template Control (NTC), and one WT Clamping Control (CC), according to the volumes in Table 5. The assay mixes contain all the components needed for PCR except the sample or control DNA template. Include reagents for one extra sample to allow sufficient overage for the PCR set up.

Table 5. Preparation of Assay Mix

	Volume of 2X PCR Master	Volume of Primer and probe	Volume of XNA
BRAF c600	5 µl x(n+1)	2 µl x(n+1)	1µl x(n+1)

- n=number of samples to test (patient/unknown samples, PC, CC and NTC). We recommend preparing one extra sample to allow room for adequate pipetting. You may want to consider increasing volume of mix to (n+2) when processing larger number of samples.
- For accuracy, do not pipette less than 8 µl.

QClamp® TaqMan assay controls: Clamping Control, Positive Control and No template control must be run with each reaction mix, every time the assay is run.

1) Clamping Controls (CC) use wild-type DNA as the template. Wild-type DNA should have none of the targeted BRAF mutations, therefore the XNA probes will bind strongly, blocking the polymerase from making amplicons. However, the Internal Control (HEX channel) with the Clamping Control should make amplicons efficiently, providing another way to monitor performance of the polymerase and sample.
2) Positive controls (PC) must be run with each reaction mix, every time the assay is run. The Positive Control contains mutant template for each reaction; 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.
3) No Template Control (NTC) are 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.

The Internal Control assay uses ACTB housekeeping gene as a reference gene to assess the quality of amplifiable DNA and demonstrating if the reagents are working correctly. When assessed using the HEX channel, this control should make amplicons efficiently for all samples and controls except NTC, providing another way to monitor performance of the primers, probes, polymerase, and sample DNA quality/quantity.

6.4. Suggested Run Layout (96-well Plate, 384-well Plate, Tube strips or Tubes)

Table 6 is a suggested plate set-up for a single experiment analyzing 3 unknown samples. When all reagents have been added to the plate, tightly seal the plate to prevent evaporation. Spin at 1000rpm for 1 minute to collect all the reagents. Place in the real-time PCR instrument immediately.

For optimal fluorescence signal collection, white plates/PCR strip tubes/PCR tubes are recommended. In the pre-PCR area, add 8 µl of the BRAF assay mix to the plate or tubes. In the designated template area (post-PCR), add 2 µl of template.

Table 6. Suggested Plate Layout

	1	2	3	4	5	6
A	NTC BRAF c600 Mix	PC BRAF c600 Mix	CC BRAF c600 Mix	S1 BRAF c600 Mix	S2 BRAF c600 Mix	S3 BRAF c600 Mix

PC: Positive Control, NTC: No-Template Control (water), CC: Clamping Control, S1-3: Samples 1-3.

6.5. Instrument Set-Up

6.5.1. Roche Light cycler 96 and Roche Light cycler 480, Bio-Rad CFX 384 and ABI Quant Studio5

1) Selection of Detectors:
   i. Use 'FAM/HEX' as the Detector on Roche light cycler
   ii. Select 'All Channel' as detection format on BioRad384
   iii. For ABI Quant Studio, assign individual mutation target as 'FAM', and select all Targets and assign to VIC
2) Setup the cycling parameters as shown in Table 7a or Table 7b
3) Start the run

Table 7a. Roche Light Cycler and Bio-Rad CFX 384 Platforms Cycling Parameters

Step	Temperature	Time	Ramp Rate (°C/s)	Cycles	Data
Preincubation	95	300	4.4	1	OFF
Denaturation	95	20	4.4	X50	OFF
XNA Annealing	70	40	2.2		OFF
Primer	64	30	1		OFF
Extension	72	30	1		FAM and HEX

*On Bio-Rad CFX 384, use the default ramp rate

Table 7b. ABI Quant Studio 5 Cycling Parameters

Step	Temperature	Time	Ramp Rate (°C/s)	Cycles	Data
PreIncubation	95	300	1.6	1	OFF
Denaturation	95	20	1.6	X50	OFF
XNA Annealing	70	40	1.6		OFF
Primer	66	30	1		OFF
Extension	72	30	1		FAM and VIC

PART 7. ASSESSMENT OF REAL-TIME PCR RESULTS

The real-time PCR instrument generates a cycle threshold (Cq) value for each sample. Cq 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 higher concentration of the initial template and the stronger the PCR reaction it represents (**please see MIQE Guidelines under References for more information).

Table 8. QClamp® TaqMan Assay Controls

Controls	Component	XNA binding	Target Amplification	Internal Control Amplification	Purpose
No Template Control	Water	No	No	No	Assess potential contamination
Clamping Control	WT genomic DNA	Strong	No	Yes	Monitor performance of the components in the kit
Positive Control	Mutant genomic DNA	No or weak	Yes	Yes	Monitor performance of the components in the kit

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. Data Analysis for Light Cycler 480

For the Light Cycler 480, open the LightCycler480 SW 1.5.1.61 and select Abs Quant/2nd Derivative Max algorithm to analyze the run file data.

7.2. Data Analysis for Bio-Rad CFX 384

For the Bio-Rad CFX 384, open the qPCR run file using Bio-Rad CFX manager. In the Log scale view, adjust the threshold to approximately 100 ± 20 for HEX and 300 ± 60 for FAM. Exact threshold setting may be different for individual instruments.

7.3. Data Analysis for ABI Quant Studio 5

For the ABI Quant Studio 5, adjust the threshold to approximately 5900 ± 600 for ACT-B (Internal control, VIC) and 20000 ± 2000 for BRAF (FAM). Exact threshold setting may be different for individual instruments.

Export the Cq data to excel. 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

7.3.1. Analysis of Clamping and Positive Control

For the assay to be valid, the Clamping Control and Positive Control must meet the criteria shown in the tables below.

Table 9a. Acceptable Values for Positive Controls and Clamping Controls (Roche Light Cycler 480)

Assay	Positive	Clamping
Internal Control	25 < Cq < 31	25< Cq <31
BRAF c600	ΔCq ≤ 4	ΔCq>7.5

Table 9b. Acceptable Values for Positive Controls and Clamping Controls (Bio-Rad CFX 384)

Assay	Positive	Clamping
Internal Control	25 < Cq < 31	25< Cq <31
BRAF c600	ΔCq ≤3.6	ΔCq>8.5

Table 9c. Acceptable Values for Positive Controls and Clamping Controls (ABI Quant Studio5)

Assay	Positive	Clamping
Internal Control	25 < Cq < 31	25< Cq <31
BRAF c600	ΔCq ≤ 3.7	ΔCq>7.6

7.4. Evaluating Validity of Sample Data Based on Internal Control Results

The Cq value of the Internal Control serves as an indication of the purity and concentration of DNA in each well. Thus, the validity of the test can be evaluated by the Cq value of the Internal Control. Internal Control Cq values of any sample should be in the range of 25<Cq<31. 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 10.

Table 10. Acceptable Internal Control Cq Ranges for Samples

Validity	Cq Value of Internal Control	Descriptions and Recommendations
Optimal	25 < Cq < 31	The amplification and amount of DNA sample were
Invalid	Cq <25	Possibility of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ⩾ 31	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 preparation may be required.

7.5. Scoring Mutational Status

Cut-off values for DNA extracted from FFPE and plasma (cfDNA) samples tested on different qPCR instruments are shown in the tables below. If a Cq value is “Undetermined”, assign a Cq of 50 and proceed to analysis. Please use Tables 11a-c below to determine mutational status.

Table 11a. Scoring Mutational Status for Roche Light Cycler 480 system

Mutation /sample	BRAF c600 / FFPE	BRAF c600/ cfDNA
Positive	< 7.5	<7.3
Negative	≥ 7.5	≥ 7.3

Table 11b. Scoring Mutational Status for Bio-Rad CFX384 system

Mutation/sample	BRAF c600 / FFPE	BRAF c600/cfDNA
Positive	< 8.5	<9.7
Negative	≥ 8.5	≥9.7

Table 11c. Scoring Mutational Status for ABI Quant Studio 5 system

Mutation/sample	BRAF c600 / FFPE	BRAF c600/cfDNA
Positive	< 7.6	<10.7
Negative	≥ 7.6	≥10.7

PART 8. ASSAY PERFORMANCE CHARACTERISTICS

The performance characteristics of this product were established on the Roche Light Cycler 96, Roche Light Cycler 480, Bio-Rad CFX 384, and ABI Quant Studio 5 real-time PCR instruments.

8.1. Analytical Performance

The specific performance characteristics of the QClamp® BRAF kit were determined by studies involving genetically defined BRAF reference standards (genomic DNA and FFPE) from cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England) and cfDNA reference standards from Seracare (Massachusetts, US). These samples have been characterized genetically as containing heterozygous or homozygous mutations in the coding sequence of the respective target regions. These single nucleotide polymorphisms in the target regions of the BRAF gene assay have been confirmed by genomic DNA sequencing and/or ddPCR. Additional samples consisted of cancer patient tissue, plasma samples and normal healthy donor DNA from tissue and plasma (no BRAF 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 NGS, Sanger sequencing or ddPCR. Studies were conducted to demonstrate concordance in mutation status of FFPE samples tested with the QClamp® BRAF Mutation Test kit. The results demonstrated a 100% match between reference methods and the QClamp® BRAF Mutation Test kit.

8.3. Precision and Reproducibility

Precision of the QClamp® BRAF kit was determined with defined analytical levels of genomic DNA with known mutational status and allelic frequencies. To establish lot-to-lot variation, a reproducibility study was performed using two different lots of the BRAF codon 600 kit. Each lot was tested on one wild-type control and two reference samples containing each mutation at 5% and 1% allelic frequency in nine replicates. Tests were performed on three separate dates. Inter-assay %CV was established for same lot of reagents tested on the same instrument by the same user. Operator variability was evaluated from different users and instrument variability was established for different instruments performing the assay using the same lot of reagents, with tests run one to two times a day for three days. Intra-assay %CV was established through performance of kit on reference samples run in replicates of nine. This was done separately for two different qPCR instrument types.

Reproducibility is demonstrated based on %CV of Cq values and rate of % correct mutation calls for all assays on two lots and operators for Roche and Bio-Rad instruments.

Table 12. Summary of Reproducibility Results

Variation	%CV
Intra-assay	⩽ 3%
Inter-assay	⩽ 4%
Lot-to-Lot Variation	⩽ 4%
Operator Variability	⩽ 3%

Table 13. Summary of Intra-Assay Reproducibility Results on LC480

Target	WT			0.5% mutant			1% mutant		
	Average Cq	SD	%CV	Average Cq	SD	%CV	Average Cq	SD	%CV
BRAF c600	37.8	0.9	2.2%	33.4	0.2	0.7%	32.4	0.3	1.0%
Internal Control	28.4	0.2	0.6%	28.9	0.8	2.1%	28.4	0.2	0.7%

Table 14. Summary of Inter-Assay Reproducibility Results on LC480

Assay	Run 1	Run 2	Run 3	Average	SD	CV%
	WT					
BRAF c600	39.2	39.0	41.1	39.7	0.9	2.3%
Internal Control	29.5	29.2	29.4	29.4	0.1	0.4%
	5% mutation					
BRAF c600	32.3	32.0	32.4	32.2	0.2	0.7%
Internal Control	29.3	29.7	28.4	29.1	0.5	1.8%
	1% mutation					
BRAF c600	34.2	35.3	34.7	34.7	0.6	1.6%
Internal Control	28.8	29.0	28.8	28.9	0.1	0.3%

Table 15. Summary of Lot-to Lot Reproducibility Results on Roche LC480

WT					
Assay	**Lot 1**	**Lot 2**	**Average**	**SD**	**%CV**
BRAF c600	50.0	50.0	50.0	0.0	0.0%
Internal Control	29.0	28.9	28.9	0.1	0.3%
	5% Mutation				
BRAF c600	30.6	30.2	30.4	0.3	1.0%
Internal Control	28.8	29.5	29.1	0.5	1.6%
	1% Mutation				
BRAF c600	33.0	32.8	32.9	0.1	0.4%
Internal Control	28.8	28.9	28.8	0.1	0.2%

Table 16. Summary of Lot-to Lot Reproducibility Results on Bio-Rad CFX384

Assay	Lot 1	Lot 2	Average	SD	%CV
WT					
BRAF c600	37.6	38.0	37.8	0.2	0.4%
Internal Control	29.0	29.0	29.0	0.1	0.2%
5% Mutation					
BRAF c600	31.0	31.2	31.1	0.2	0.5%
Internal Control	28.8	26.8	27.8	0.3	1.3%
1% Mutation					
BRAF c600	32.10	33.30	32.70	0.84	2.58%
Internal Control	27.27	27.27	27.27	0.49	1.83%

8.4. Analytical Sensitivity and Limit of Detection

To determine the limit of detection (LOD) and analytical sensitivity of the kit, the QClamp® BRAF assay was performed using serial dilutions of mutant DNA (reference FFPE and cfDNA) in wild-type background. The wild-type DNA used for dilution was obtained from mutant-free FFPE and normal human plasma respectively. BRAF mutant allelic frequencies tested were 1%, 0.5% and 0.1% at 2.5, 5 and 10ng/reaction DNA input levels. The mutant copy numbers present in genomic DNA with 1%, 0.5% and 0.1% allelic frequency at different DNA input levels are shown in Table 18.

Table 17a. LOD Summary Determined Using Genomic DNA Reference Standards

Target mutation	DNA Input, ng/well			
		10	5	2.5
BRAF c600	1% mutation	100%	100%	90%
	0.5% mutation	100%	75%	70%
	0.10% mutation	65%	15%	20%

Table 17b. LOD Summary Determined Using cfDNA Reference Standards

Target mutation	DNA Input, ng/well		
		5	2.5
BRAF c600	1% mutation	100%	100%
	0.5% mutation	84.6%	78.6%
	0.10% mutation	58.3%	35.7%

Note: Considering the low amount of cfDNA that can be extracted from plasma, assay sensitivity at 10 ng DNA input was not tested.

Table 18. Mutant DNA Copy Numbers at Different Allelic Frequencies

Allelic frequency	Mutant DNA copy numbers at different DNA inputs		
	10ng DNA	5ng DNA	2.5ng DNA
1%	28 copies	14 copies	7 copies
0.50%	14 copies	7 copies	3.5 copies
0.10%	2.8 copies	1.4 copies	0.7 copies

Conclusion:

- FFPE DNA samples: the QClamp® BRAF assay can detect 1% allelic frequency at 5 ng DNA input.
- Plasma cfDNA samples: the QClamp® BRAF assay can detect 1% allelic frequency at 2.5 ng DNA input.
- **The recommended DNA input is a minimum of 5ng/well for FFPE DNA and 2.5ng/well for plasma cfDNA.**
- To increase sensitivity to 0.5% BRAF c600 mutation, increase DNA input to 10ng.

Recommended input of FFPE should not be higher than 20 ng/well due to possible PCR inhibition. Optimal FFPE sample input is between 25 and 31 Cq of the Internal Control reaction.

8.5. Analytical Specificity

Analytical specificity of the QClamp® BRAF test was determined as the correct calling of the samples with no mutation at different concentrations of WT template. There were no false positive calls for up to 320ng of gDNA per well and up to 20ng FFPE DNA. Higher amounts of DNA input were not tested.

8.6. Cut-Offs

DNA from FFPE and plasma samples were used to establish cut-offs for the assay. Please refer to Tables 9-11.

8.7. Limit of Blank

Two lots of reagents were run without template (NTC) in multiple runs (50 data points for each target) to assess the level of background noise when no template is present. No internal control or mutant signals below Cq 48 were detected in any of the runs.

8.8. Interfering Substances

A study was performed to evaluate the impact of potentially interfering substances on the performance of the QClamp® BRAF assay. Potentially interfering substances tested were paraffin (same as used in FFPE preparation) and ethanol. The impact of each 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 cut-offs. None of the potentially interfering substances evaluated at concentrations encountered in normal use impacted the ability of the QClamp® BRAF to distinguish between mutation-positive and mutation-negative control samples.

8.9. Multiple Freeze-Thaw Cycles

The effect of 1-8 freeze-thaw cycles were tested in QClamp® BRAF Mutation Test kit reagents. There is no effect up to 6 freeze-thaw cycles on the QClamp® BRAF Mutation detection kit to distinguish between mutation positive and mutation negative samples.

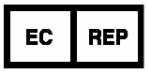
Caution: Repeated freeze-thaw cycles may decrease the reliability of test results.

8.10. Shelf-Life

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

PART 9. SYMBOLS USED IN PACKAGING

Table 19. Symbols Used in Packaging

Symbol	Definition
	Catalog Number
	Manufactured By
	Storage Temperature Range
	Batch Code
	Expiration Date
	Authorized Representative in the European Community
1011-11-17	Date Format (year-month-day)
1011-11	Date Format (year-month)

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