

	Product Manual QClamp® PIK3CA Mutation Detection Test Manual_RUO	Document #: MAN.0017 Rev. 3
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Revision	Summary of Change	Originator	Date Initiated
0	Change Control #: 768 replaces instruction manual rev16.2, updated doc. numbering and formatting	E. Peletskaya	20JUN16
1	Change Control #: 953 Updated to TaqMan version	Q. Sun	25OCT17
2	Change Control #: 1423 Updated address, product name, content and format	C. Li	14JUL22
3	Change Control #: 1536 Update threshold, added LOD data, and cutoffs for CFX96, CFX384, ABI 7500, and LC480II instruments	R. Brown	23DEC2022



INSTRUCTION FOR USE

QClamp[®] PIK3CA Mutation Detection Test

Codons E542, E545 and H1047

CATALOG NUMBER

DC-10-1053R (10 Samples)
DC-10-1072R (30 Samples)
DC-10-1075R (60 Samples)

MANUFACTURER

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INTENDED USE

Research Use Only

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

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PART 1. INTENDED USE

The QClamp® PIK3CA Mutation Detection Test is a real-time qualitative PCR assay for the detection of somatic mutations in the human PIK3CA gene, using genomic DNA extracted from cells or FFPE (formalin fixed paraffin embedded). The codon-specific assays are designed to detect mutations at or near PIK3CA Codons 542 and 545 in Exon 9 and Codon 1047 in Exon 20 without specifying the exact nucleotide change. However, since the probe used in each reaction is mutation specific, there is no guarantee that the PIK3CA kit can detect other mutations at the same amino acid. *Table 1* shows a list of mutations commonly found in the PIK3CA gene. The kit should be used only by trained laboratory professionals within a laboratory environment. The QClamp® PIK3CA Mutation Detection Test is for research use only. Not for use in diagnostic procedures.

Table 1. List of Mutations and Cosmic Identities Found in PIK3CA

Exon	Amino Acid Change	Nucleotide Change	Cosmic No.
9	E542>K	c.1624G>A	COSM760
	E542>G	c.1625A>G	COSM761
	E542>V	c.1625A>T	COSM762
	E545>K	c.1633G>A	COSM763
	E545>G	c.1634A>G	COSM764
	E545>D	c.1635G>T	COSM765
20	H1047>Y	c.3139C>T	COSM774
	H1047>R	c.3140A>G	COSM775

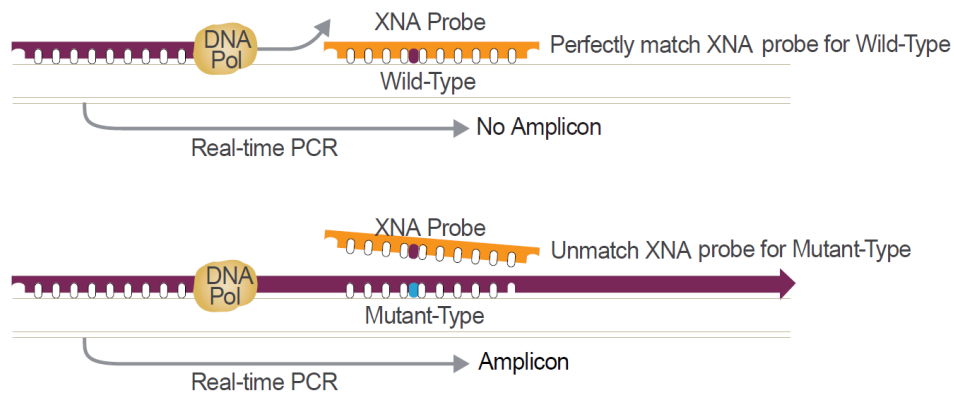
PART 2. QCLAMP® WORKFLOW

The assay workflow consists of four major steps:

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

PART 3. QCLAMP® TECHNOLOGY FOR MUTATION DETECTION

The QClamp® PIK3CA 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® PIK3CA Mutation Detection Test

PART 4. REAGENTS AND INSTRUMENTS

4.1. Package Contents

Table 2a. Package Contents – Pack Size: 10 samples (DC-10-1053R)

Name of Component	Part #	Pack Size: 10 samples	Label Volume for each vial	Cap Color	Storage Temp
E542K Primer/Probe Mix	1001612	1 vial	20 µL	red	-25°C to -15°C
E545K Primer/Probe Mix	1001622	1 vial	20 µL	violet	-25°C to -15°C
H1047R Primer/Probe Mix	1001632	1 vial	20 µL	blue	-25°C to -15°C
E542K XNA	1001822	1 vial	20 µL	red	-25°C to -15°C
E545K XNA	1001832	1 vial	20 µL	violet	-25°C to -15°C
H1047R XNA	1001842	1 vial	20 µL	blue	-25°C to -15°C
Positive Control	1001852	1 vial	14 µL	yellow	-25°C to -15°C
Negative Control	1001862	1 vial	14 µL	green	-25°C to -15°C
2X PCR Master Mix	1001882	1 vial	288 µL	clear	-25°C to -15°C
Nuclease-Free Water	1000592	1 vial	72 µL	orange	-25°C to -15°C

Table 2b. Package Contents – Pack Size: 30 samples (DC-10-1072R)

Name of Component	Part #	Pack Size: 30 samples	Label Volume for each vial	Cap Color	Storage Temp
E542K Primer/Probe Mix	1001613	1 vial	54 µL	red	-25°C to -15°C
E545K Primer/Probe Mix	1001623	1 vial	54 µL	violet	-25°C to -15°C
H1047R Primer/Probe Mix	1001633	1 vial	54 µL	blue	-25°C to -15°C
E542K XNA	1001823	1 vial	54 µL	red	-25°C to -15°C
E545K XNA	1001833	1 vial	54 µL	violet	-25°C to -15°C
H1047R XNA	1001843	1 vial	54 µL	blue	-25°C to -15°C
Positive Control	1001853	1 vial	28 µL	yellow	-25°C to -15°C
Negative Control	1001863	1 vial	28 µL	green	-25°C to -15°C
2X PCR Master Mix	1001883	1 vial	756 µL	clear	-25°C to -15°C
Nuclease-Free Water	1000593	1 vial	189 µL	orange	-25°C to -15°C

Table 2c. Package Contents – Pack Size: 60 samples (DC-10-1075R)

Name of Component	Part #	Pack Size: 60 samples	Label Volume for each vial	Cap Color	Storage Temp
E542K Primer/Probe Mix	1001614	1 vial	93 µL	red	-25°C to -15°C
E545K Primer/Probe Mix	1001624	1 vial	93 µL	violet	-25°C to -15°C
H1047R Primer/Probe Mix	1001634	1 vial	93 µL	blue	-25°C to -15°C
E542K XNA	1001824	1 vial	93 µL	red	-25°C to -15°C
E545K XNA	1001834	1 vial	93 µL	violet	-25°C to -15°C
H1047R XNA	1001844	1 vial	93 µL	blue	-25°C to -15°C
Positive Control	1001854	1 vial	28 µL	yellow	-25°C to -15°C
Negative Control	1001864	1 vial	28 µL	green	-25°C to -15°C
2X PCR Master Mix	1001884	1 vial	1296 µL	clear	-25°C to -15°C
Nuclease-Free Water	1000594	1 vial	324 µL	orange	-25°C to -15°C

4.2. Materials Required but not Provided in the Kit

A. DNA Samples

- Human genomic DNA extracted from samples using a kit appropriate for the sample type, such as the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, Cat. No. 60404)

B. Consumables

- Real-time PCR plate suitable for instrument of choice
- Optical plate seal
- Nuclease-free, low adhesion microcentrifuge tubes
- Nuclease-free pipet tips with aerosol barriers

C. Equipment

- Real-time PCR instrument capable of detecting FAM (495 nm excitation/520 nm emission) and HEX (538 nm excitation/555 nm emission)
- Dedicated pipettes for pre-PCR work covering a range of volumes between 1 µL and 200 µL
- Dedicated pipettes for dispensing of template nucleic acids with a volume of 2 µL
- Microcentrifuge for 1.5 mL and 2 mL tubes
- PCR plate centrifuge
- Vortexer

Note: Ensure that all equipment has been maintained and calibrated according to the manufacturer's recommendations prior to use.

4.3. Instruments

The analytical performance of the QClamp® PIK3CA Mutation Detection Test has been tested with genomic DNA templates and FFPE breast cancer biopsies using the instruments shown in the table below. The assay performance is dependent on the quantity and quality of the input DNA material.

Table 3. List of Instruments Validated with this Kit

Company	Model
Roche	LightCycler® 480 II
Bio-Rad	CFX384
Bio-Rad	CFX96
Thermo Fisher	ABI 7500

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 -15°C to -25°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 postamplification (DNA template-handling) area. The kit can undergo up to 5 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.

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 Negative DNA Controls provided.
- Minimize exposure of the 2X PCR Master Mixes to room temperature for optimal amplification.
- Avoid overexposing the Primer Probe Mixes to light for optimal fluorescent signal.
- Using non-recommended reagent volumes may result in a loss of performance and may also decrease the reliability of the test results.
- Using 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.
- Using 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 users 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 equipment (PPE), 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, except for Primer Probe Mixes & Master Mixes.
- 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. INSTRUCTIONS FOR USE

5.1. DNA Isolation

Prior to performing this assay, genomic DNA should be extracted from FFPE samples using a kit such as the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN Cat. No. 60404) or equivalent. Since ethanol carryover into the elute may interfere with some downstream applications, such as QClamp® PIK3CA assay, we highly recommend the customs follow the procedure exactly that specified by the manufacturer's protocol. After extraction, DNA should be quantified using a fluorometric method such as the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or Q32854). Quantification of DNA by UV absorbance-based methods may result in an overestimation of the total DNA amount.

This QClamp® assay requires a minimum of 30 ng of DNA per sample (10 ng/reaction in 3 reactions).

5.2. Preparation of Reagents

All reagents must be thawed at ambient temperature for a minimum of 15 minutes before use or until completely thawed. Do not leave kit components at room temperature for more than 2 hours. Ensure that all reagents are completely thawed before use. After thawing, mix all reagents by flicking the vials with a finger several times. Centrifuge all components briefly to bring contents to the bottom of the tube. This is extremely important to prevent reagent loss. Extra reagents are provided in each kit to test three controls in each run.

The PCR reactions are set up in a total volume of 10 µl/reaction. *Table 4* shows the component volumes for each reaction.

- A 10-sample test kit contains enough material for 2 runs.
- A 30-sample test kit contains enough material for 4 runs.
- A 60-sample test kit contains enough material for 4 runs.

Table 4. Assay Setup

Components	Volume/Reaction
2X PCR Master Mix	5 µl
Primer and Probe Mix	1 µl
XNA Mix	1 µl
DNA Sample or Controls	2 µl
Nuclease Free Water	1 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 4 below
- If needed, the sample volume can be increased to 3 µl by eliminating 1 µl water

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 PIK3CA Mixed Positive Control (POS), Negative Control (NEG), and a Non-Template Control (NTC) should be included in each run. The Mixed Positive Control contains a mixture of genomic DNA harboring mutations in codons 542, 545, and 1047 of the PIK3CA gene. The Negative Control contains wild-type genomic DNA. For NTC, 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.

Each sample requires one reaction for each mutation site detected by the kit, for a total of three reactions per sample. The Internal Control Assay, part of each Primer Probe Mix, uses a reference gene to determine if an appropriate level of amplifiable DNA is present in each reaction, and ensures that the supplied primers and polymerase are working properly on the sample.

The DNA samples should be at a concentration of 2.5 ng/μL or diluted to this concentration if needed. Overloading of sample should be avoided to reduce the probability of a false positive signal or a false negative signal due to inhibition from the sample. A total input of less than 5 ng may result in a false negative signal. It is recommended to test each sample in duplicate if possible.

5.3. Preparation of Assay Mixes

IMPORTANT:

Assay mixes should be prepared just prior to use. Do not store assay mixes.

Calculate the volume of each component required for the reaction mix as shown in Table 5. For the total reaction number (n), including the number of test samples plus 3 controls. The calculations include an extra volume of 10% to account for reagent loss during pipetting.

Table 5. Preparation of Assay Mixes

	Volume of 2X PCR Master Mix	Volume of Primer and Probe Mix	Volume of XNA	Volume of Water
PIK3CA E542 Mix	5 μl x (n x 1.1)	1 μl x (n x 1.1)	1 μl x (n x 1.1)	1 μl x (n x 1.1)
PIK3CA E545 Mix	5 μl x (n x 1.1)	1 μl x (n x 1.1)	1 μl x (n x 1.1)	1 μl x (n x 1.1)
PIK3CA H1047 Mix	5 μl x (n x 1.1)	1 μl x (n x 1.1)	1 μl x (n x 1.1)	1 μl x (n x 1.1)

Label three 1.5 mL microcentrifuge tubes (not provided), one for each assay mix. **Into each tube, add the calculated volume of each component.** Cap the tube and mix well by flicking the tube with a finger. Briefly centrifuge the tube to bring the contents to the bottom of the tube. Dispense 8 μL of the reaction mix into the designated wells of a PCR plate.

The exact plate layout can be set to the user's preference, although it is recommended to leave one well empty between each sample if possible. *Table 6* is an example plate set-up for a single experiment analyzing 3 unknown samples (S1-S3) in duplicate.

Table 6. Suggested Plate Layout (384-Well Plate)

	1	2	3	4	5	6
A	NTC PIK3CA E542 Mix	POS PIK3CA E542 Mix	NEG PIK3CA E542 Mix	S1 PIK3CA E542 Mix	S2 PIK3CA E542 Mix	S3 PIK3CA E542 Mix
B	NTC PIK3CA E545 Mix	POS PIK3CA E545 Mix	NEG PIK3CA E545 Mix	S1 PIK3CA E545 Mix	S2 PIK3CA E545 Mix	S3 PIK3CA E545 Mix
C	NTC PIK3CA H1047 Mix	POS PIK3CA H1047 Mix	NEG PIK3CA H1047 Mix	S1 PIK3CA H1047 Mix	S2 PIK3CA H1047 Mix	S3 PIK3CA H1047 Mix

Notes:

- POS: Positive Control, NTC: Non-Template Control (Nuclease-Free Water), NEG: Negative Control (Wild-Type DNA), S1-3: Samples 1-3.
- When all reagents have been loaded into the plate, add 2 μL of nuclease-free water to the wells designated as Non-Template Control.
- Transfer the plate to a workspace designated for handling nucleic acid templates (if available) and add 2 μL of the PIK3CA Mixed Positive Control, Negative Control, and other DNA samples to the designated wells.
- Tightly seal the wells of the plate with film to prevent evaporation and cross-contamination. Mix the plate by tapping it on a lab bench. Centrifuge the plate for at least 15 seconds. Place the plate into the real-time PCR instrument immediately.

5.5. Instrument Set-Up

These parameters apply to Bio-Rad CFX384, Bio-Rad CFX96, Roche LD480II, and ABI 7500 instruments. For Bio-Rad CFX384, the entire run is on default and no manual adjustment should be performed. Choose all channels when prompted. For ABI 7500 select NONE for the Passive Reference Dye. For Roche LC480II the ramp rates should be adjusted according to the Table 7.

Table 7. Thermal cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s)	Cycles	Acquisition Mode
Preincubation	95	300	4.8	1	None
Denaturation	95	10	4.4	X50	None
XNA Annealing	70	30	2.5		None
Primer Annealing	64	20	2.5		None
Extension	72	20*	4.8		FAM and HEX

* For ABI 7500 Extension time for 30 s.

PART 6. ASSESSMENT OF REAL-TIME PCR RESULTS

Real-time PCR instruments generate Cq values, which is the cycle number at which the fluorescence signal is detected above a background threshold. The higher the starting template amount in the reaction, the earlier the cycle number at which the signal rises above background.

Table 8. QClamp® TaqMan Assay Controls

Controls	Component	XNA Binding	Target Amplification	Internal Control Amplification	Purpose
Non-Template Control	Nuclease-Free Water	No	No	No	Assess potential contamination
Negative Control	Wild-Type 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 Non-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:

$$\text{Cq difference } (\Delta\text{Cq}) = \text{Mutation Assay Cq} - \text{Internal Control Assay Cq}$$

6.1. Data Analysis for Roche LightCycler® 480

For the Roche LightCycler® 480 II, the Abs Quant/Fit Points analysis method is recommended. For the FAM detector, in the Cycle Range tab, set the First Cycle to 15, and in the Noise Band tab, set the Noise Band value to 0.3. For the HEX detector, set the Noise Band level to just above any background signal from empty wells and negative controls.

6.2. Data Analysis for Bio-Rad CFX384

For the Bio-Rad CFX384, under the "Settings" tab, set the Cq Determination Mode to "Single Threshold" and "Baseline Setting" to "Baseline Subtracted Curve Fit". Set the threshold for FAM and HEX channels individually. Check the FAM

channel, under the “Settings” tab, select “Baseline Threshold” and “User Defined” and set FAM to 500 RFU. Check the HEX channel, under the “Settings tab”, select “Baseline Threshold” and “User Defined” and set HEX to 250 RFU. Verify that the thresholds for FAM and HEX are set slightly above the background fluorescence level so that negative controls or empty wells are not artificially called to be positive.

6.3. Data Analysis for Bio-Rad CFX96

For the Bio-Rad CFX396, under the “Settings” tab, set the Cq Determination Mode to “Single Threshold” and “Baseline Setting” to “Baseline Subtracted Curve Fit”. Set the threshold for FAM and HEX channels individually. Check the FAM channel, under the “Settings tab”, select “Baseline Threshold” and “User Defined” and set FAM to 100 RFU. Check the HEX channel, under the “Settings” tab, select “Baseline Threshold” and “User Defined” and set HEX to 50 RFU. Verify that the thresholds for FAM and HEX are set slightly above the background fluorescence level so that negative controls or empty wells are not artificially called to be positive.

6.4 Data Analysis for ABI 7500

For the Thermo Fisher Scientific ABI 7500 use the *Design and Analysis* software. Click “Analyze” button. Under the “Actions” tab open “Primary Analysis Setting” and uncheck “Use Default”, uncheck “Auto Threshold”, and uncheck “Auto Baseline”, for FAM and VIC (HEX) channels. Set the “Threshold” to 20,000 for FAM and 10,000 for HEX. Verify that the thresholds for FAM and HEX are set slightly above the background fluorescence level so that negative controls or empty wells are not artificially called to be positive.

6.5. Non-Template Controls

Verify that there is no amplification for both FAM and HEX signals in the no template controls for each of the assay mixes. The Cq should be undetermined. If a Cq value is observed, check the amplification curves look abnormal, such as if the signal arises at a very early cycle in the reaction or if the curve is not S-shaped (sigmoidal). This indicates that the signal is not a real amplification. If the amplification curve shows a good S shape, it is highly likely due to contamination. Please carefully follow our instructions in the “Reagents and Instrument” section to avoid contamination.

6.6. Analysis of Negative and Positive Controls

The expected Cq values for the PIK3CA Negative and Positive Controls are shown in Table 9.

Table 9. Acceptable Values for Positive Controls and Negatives Controls (Bio-Rad CFX384)

	Assay	FAM	HEX
Non-Template Control	542, 545 and 1047	Undetected	Undetected
Negative Control	542, 545 and 1047	Cq ≥40	26 < Cq <32
Positive Control	542	Cq < 36	26 < Cq <32
	545	Cq < 36	26 < Cq <32
	1047	Cq < 35	26 < Cq <32

Table 10. Acceptable Values for Positive Controls and Negatives Controls (Bio-Rad CFX96)

	Assay	FAM	HEX
Non-Template Control	542, 545 and 1047	Undetected	Undetected
Negative Control	542, 545 and 1047	Cq ≥40	26 < Cq <32
Positive Control	542	Cq < 40	26 < Cq <32
	545	Cq < 37	26 < Cq <32
	1047	Cq < 37	26 < Cq <32

Table 11. Acceptable Values for Positive Controls and Negatives Controls (ABI 7500)

	Assay	FAM	HEX
Non-Template Control	542, 545 and 1047	Undetected	Undetected
Negative Control	542, 545 and 1047	Cq \geq 40	26 < Cq <32
Positive Control	542	Cq < 38	26 < Cq <32
	545	Cq < 35	26 < Cq <32
	1047	Cq < 35	26 < Cq <32

Table 12. Acceptable Values for Positive Controls and Negatives Controls (Roche LC480II)

	Assay	FAM	HEX
Non-Template Control	542, 545 and 1047	Undetected	Undetected
Negative Control	542, 545 and 1047	Cq \geq 40	26 < Cq <32
Positive Control	542	Cq < 38	26 < Cq <32
	545	Cq < 36	26 < Cq <32
	1047	Cq < 37	26 < Cq <32

If a Cq value is reported for the Negative Control in the FAM channel, follow the recommendations for No Template Controls.

If the HEX Cq values for the Positive and Negative Controls do not fall within the ranges given in Table 9-12, it is recommended to repeat the experiment.

6.7. Judging Validity of Sample Data Based on Internal Control Assay Results

The Cq value of the Internal Control assay (HEX channel) can serve as an indication of the purity and concentration of the input DNA. Thus, the validity of the test can be determined by the Cq value of the Internal Control assay. The HEX Cq values should be within the ranges specified in Table 13.

Table 13. Acceptable Ranges for Internal Control

Validity	Cq Value of Internal Control Mix	Descriptions and Recommendations
Valid	26 < Cq < 32	The amplification and amount of DNA sample were optimal.
Invalid	Cq \leq 26	Sample DNA is overloaded, which increases the possibility of a false positive. Repeat the test with less DNA.
Invalid	Cq \geq 32	Not enough DNA, DNA degraded, or inhibitors present in sample. If DNA is degraded, higher input amounts may be needed. If inhibitors are present, dilution of the DNA may help obtain an acceptable level of amplification. For samples with low DNA concentration, it may be necessary to combine multiple FFPE sections for extraction.

6.8. Scoring Mutational Status

If the DNA samples and PCR amplification condition were optimal (based on the Cq of HEX), the Cq result for the PIK3CA mutation assay (FAM channel) can be used to assess the presence or absence of a mutation. If a FAM Cq value is undetermined, assign a Cq of 50 and proceed to analysis using the criteria in Table 14 to assess the PIK3CA mutation result.

Note: The QClamp® PIK3CA Mutation Detection Test has been evaluated using PIK3CA E542K, E545K, and H1047R mutations. Other mutations should be evaluated by the end user.

Table 14. Result Determination

Cq Value of FAM Channel for Any of Assay Mixes	PIK3CA Mutation Result
Cq < 40	Positive
Cq ≥ 40	Negative

PART 7. ASSAY PERFORMANCE CHARACTERISTICS

The performance characteristics of this product were established on the Roche LightCycler® 480 II, Bio-Rad CFX384, Bio-Rad CFX96, and ABI 7500 real-time PCR instruments.

7.1. Analytical Sensitivity (LOD)

The specific performance characteristics of the QClamp® PIK3CA Mutations Detection Test were determined by studies involving the PIK3CA-defined genomic DNA reference standards of the cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England). These samples have been characterized genetically as containing heterogenous mutations in the coding sequence of the PIK3CA gene at exons 9 and 20.

To determine the limit of detection (LOD) for the kit, the QClamp® assay was run using a serial dilution of mutant genomic DNA in wild-type background at 10 ng DNA input and four Variant Allele Frequency (VAF) for each target. Mutant reference samples were verified by droplet digital PCR (ddPCR) by Horizon Discovery. DNA was added to each well of the assay at 10 ng/well. Mutant allele concentrations tested were 0.5%, 0.25%, 0.125%, and 0.0625% VAF. 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.

Table 15. LOD Summary: Reference gDNA Samples

Instrument	Assay	LOD (% VAF)
CFX384	E542K	0.125
	E545K	0.063
	H1047R	0.125
CFX96	E542K	0.125
	E545K	0.125
	H1047R	0.063
ABI 7500	E542K	0.063
	E545K	0.063
	H1047R	0.125
LC480II	E542K	0.125
	E545K	0.125
	H1047R	0.125

7.2. Cut-Offs

PIK3CA wild-type, E542K, E545K, H1047R reference DNA, reference FFPE samples, and FFPE breast cancer samples were tested to establish the cut-offs for the assay. Cutoffs are presented in Tables 9-14 of the manual. Other PIK3CA mutations were not tested.

7.3. Multiple Freeze/Thaw Cycles









The effect of multiple freeze-thaw cycles was tested for the QClamp® PIK3CA Mutation Detection Test. No effect was observed with up to 5 freeze-thaw cycles.

7.4. Shelf-Life

Approximately 12 months after manufacture of the kit when stored at -25°C to -15°C. Please see the product label for the expiration date of a specific lot. Reproducibility is demonstrated based on %CV of C_q values and rate of % correct mutation calls for all assays on two lots and operators for Roche LC480II and Bio-Rad CFX384 instruments.

PART 8. SYMBOLS USED IN PACKAGING

Table 12. Symbols Used in Packaging

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

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