INSTRUCTIONS FOR USE

QClamp® PIK3CA Mutation Detection Test

For Detection of Mutations E542K, E545K and H1047R

CATALOG NUMBER

DC-10-1053 (10 Samples)
DC-10-1072 (30 Samples)

MANUFACTURER

DiaCarta, Inc.
4385 Hopyard Rd, Suite 100
Pleasanton, CA 94588, USA
P: +1 510-878-6662
F: + 1 510-735-8636
E: information@diacarta.com

INTENDED USE

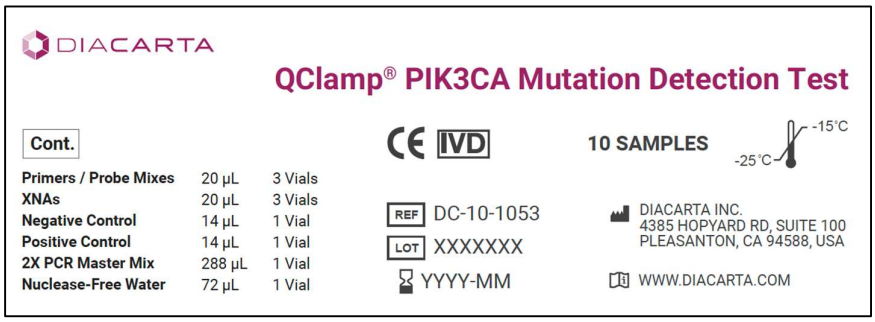
CE IVD EC REP

MDSS GmbH
Schiffgraben 41
30175 Hannover
Germany

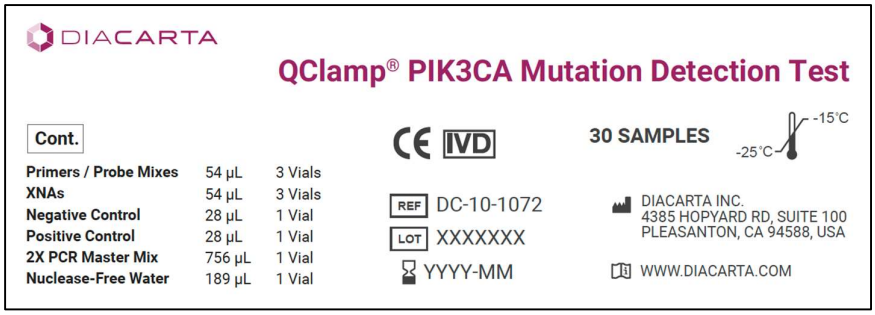
Document Number: MAN.0016 Instruction Version: Rev.4, Date of Revision: March 2024

PRODUCT LABEL

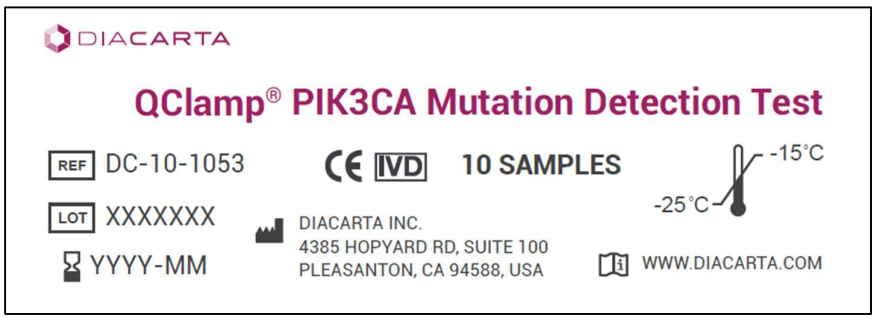
Outer Ziplock Bag Product Identification Label: DC-10-1053 (10 Samples)



Outer Ziplock Bag Product Identification Label: DC-10-1072 (30 Samples)



Pouch Label: DC-10-1053 (10 Samples)



Pouch Label: DC-10-1072 (30 Samples)

CONTENTS

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 purified DNA extracted from fresh or frozen tissues or formalin-fixed paraffin-embedded (FFPE) tissue specimens. The codon-specific assays are designed to detect three major PIK3CA mutations E542K, E545K, and H1047R. This kit is limited to testing only these mutations, but other common mutations in these three codons are also listed in Table 1. The QClamp® PIK3CA Mutation Detection Test is CE marked and is to be only used by trained laboratory professionals within a laboratory environment with CE compliance. Not for in vitro diagnostic use in the US.

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

Exon	Amino Acid Change	Nucleotide Change	COSM ID	COSV ID
9	p.E542K	c.1624G>A	COSM760	COSV55873227
	p.E542G	c.1625A>G	COSM761	COSV55876800
	p.E542V	c.1625A>T	COSM762	COSV55881194
	p.E545K	c.1633G>A	COSM763	COSV55873239
	p.E545G	c.1634A>G	COSM764	COSV55873220
	p.E545D	c.1635G>T	COSM765	COSV55874040
20	p.H1047Y	c.3139C>T	COSM774	COSV55876499
	p.H1047R	c.3140A>G	COSM775	COSV55873195

PART 2. PIK3CA AND CANCER

PIK3CA is the gene encoding the catalytic subunit alpha of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3 Kinase), a lipid kinase that is involved in cell growth, proliferation, differentiation, motility, and survival [1]. Mutant PIK3CA has been implicated in the pathogenesis of several cancers, including breast cancer, colon cancer, lung cancer and other cancers such as glioma, gastric cancer, and endometrial cancer [1-3]. The most PIK3CA missense mutations include H1047R, E545K and E542K [3].

PART 3. QCLAMP® WORKFLOW

The assay workflow consists of four major steps:

1. **DNA Isolation:** Extract DNA from FFPE tissue or plasma using a commercial DNA extraction kit.
2. **Set up qPCR:** Mix the assay reagents, load the controls and extracted DNA samples into a PCR plate (30-60 min).
3. **Run qPCR:** Enter amplification parameters on a qPCR machine, load PCR plate and start the run (2.5 h).
4. **Data analysis:** Determine the presence or absence of mutations according to the Cq value cutoffs (~20 min).

PART 4. QCLAMP® TECHNOLOGY FOR MUTATION DETECTION

The QClamp® PIK3CA Mutation Detection Test is based on xenonucleic acid (XNA) mediated PCR clamping technology [4-7]. 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 [5]. 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 wildtype DNA, to a PCR reaction, blocks amplification of wildtype DNA allowing selective amplification of mutant DNA. DNA polymerases do not recognize XNA oligomers and cannot be utilized as primers in subsequent PCR reactions. *The advantages of applying XNA in the PCR assay include improvement of sensitivity (blocking wildtype DNA to reduce false-negatives) and specificity (blocking wildtype DNA to reduce false-positives).*

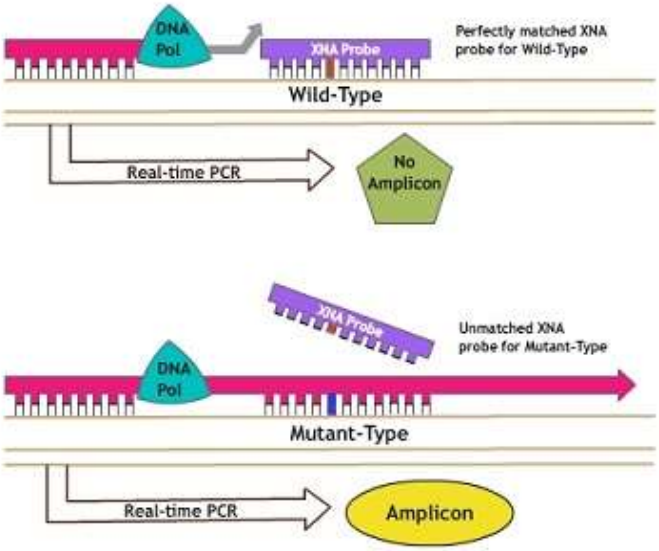


Figure 1. Principle of the QClamp® PIK3CA Mutation Detection Test

PART 5. REAGENTS AND INSTRUMENTS

5.1. Package Contents

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

Name of Component	Part #	Description	Pack Size: 10 Samples	Label Volume for Each Vial	Cap Color	Storage Temperature
E542K Primer/Probe Mix	1001612	PIK3CA E542K Primers and Probe	1 vial	20 µL	Red	-25°C to -15°C
E545K Primer/Probe Mix	1001622	PIK3CA E545K Primers and Probe	1 vial	20 µL	Violet	-25°C to -15°C
H1047R Primer/Probe Mix	1001632	PIK3CA H1047R Primers and Probe	1 vial	20 µL	Blue	-25°C to -15°C
E542K XNA	1001822	PIK3CA E542K XNA	1 vial	20 µL	Red	-25°C to -15°C
E545K XN	1001832	PIK3CA E545K XNA	1 vial	20 µL	Violet	-25°C to -15°C
H1047R XNA	1001842	PIK3CA H1047R XNA	1 vial	20 µL	Blue	-25°C to -15°C
Positive Control	1001852	PIK3CA E542K, E545K, H1047R mixed	1 vial	14 µL	Yellow	-25°C to -15°C
Negative Control	1001862	Wildtype DNA	1 vial	14 µL	Green	-25°C to -15°C
2X PCR Master Mix	1001882	PCR Reaction mix	1 vial	288 µL	Clear	-25°C to -15°C
Nuclease-Free Water	1000592	Nuclease-Free Water	1 vial	72 µL	Orange	-25°C to -15°C

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

Name of Component	Part #	Description	Pack Size: 30 Samples	Label Volume for Each Vial	Cap Color	Storage Temp
E542K Primer/Probe Mix	1001613	PIK3CA E542K Primers and Probe	1 vial	54 µL	Red	-25°C to -15°C
E545K Primer/Probe Mix	1001623	PIK3CA E545K Primers and Probe	1 vial	54 µL	Violet	-25°C to -15°C
H1047R Primer/Probe Mix	1001633	PIK3CA H1047R Primers and Probe	1 vial	54 µL	Blue	-25°C to -15°C
E542K XNA	1001823	PIK3CA E542K XNA	1 vial	54 µL	Red	-25°C to -15°C
E545K XNA	1001833	PIK3CA E545K XNA	1 vial	54 µL	Violet	-25°C to -15°C
H1047R XNA	1001843	PIK3CA H1047R XNA	1 vial	54 µL	Blue	-25°C to -15°C
Positive Control	1001853	PIK3CA E542K, E545K, H1047R mixed	1 vial	28 µL	Yellow	-25°C to -15°C
Negative Control	1001863	Wildtype DNA	1 vial	28 µL	Green	-25°C to -15°C
2X PCR Master Mix	1001883	PCR Reaction mix	1 vial	756 µL	Clear	-25°C to -15°C
Nuclease-Free Water	1000593	Nuclease-Free Water	1 vial	189 µL	Orange	-25°C to -15°C

5.2. Materials Required but not Provided in the Kit

A. Reagents for DNA Isolation

- QIAamp® DNA FFPE Tissue Kit (QIAGEN, Cat. No. 56404) or equivalent.
- DNeasy Blood & Tissue kit (QIAGEN, Cat. No. 69504 or 69506) for tissue and blood specimens.

B. Consumables

- BIO-RAD 96-well (HSP9601) white shell clear wells for the CFX96™ instrument.
- BIO-RAD 384-well (HSP3805) clear shell white wells for the CFX384™ instrument.
- Globe Scientific Inc. 384-well PCR plate, A24/P24 two notch, white Roche style polypropylene non-sterile (Cat. PCR-384-ROCW) for the LightCycler® 480II instrument.
- Globe Scientific Inc. 384-well polypropylene non-sterile PCR plate, A24 single notch, white (Cat# PCR-384-ABIW; Lot#: 20322) for the QuantStudio™ 5 instrument.
- Applied Biosystems MicroAmp® 96-well Reaction Plate (0.1mL) (REF 4346907) for the ABI7500 Fast Dx instrument.
- Optical plate seal.
- Nuclease-free, low-binding microcentrifuge tubes.
- Nuclease-free pipette tips with aerosol barriers.

C. Equipment

- Permanent marker
- Real-time PCR instrument capable of detecting FAM (495 nm excitation/520 nm emission) and HEX (538 nm excitation/555 nm emission) or VIC (538 nm excitation/554 nm emission) instead of HEX.
- Dedicated pipettes* (adjustable) for sample preparation.
- Dedicated pipettes* (adjustable) for PCR Master Mix preparation.
- Dedicated pipettes* (adjustable) for dispensing of template DNA.
- Microcentrifuge
- Centrifuge* with rotor for 1.5 mL tubes.
- PCR plate centrifuge
- Vortexer
- PCR Rack
- Reagent reservoir.

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

5.3. Instruments

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

Table 3. List of Instruments Validated with this Kit

Company	Model
Bio-Rad	CFX384™, CFX96™
Roche	LightCycler® 480 II
Thermo Fisher	QuantStudio™ 5, ABI 7500

5.4. Handling and Storage

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

The kit should be stored at -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 post-amplification (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/Probe mixes contain fluorophore labeled probes and should be protected from light. Attention should be paid to expiration dates and storage conditions printed in the box and labels of all components. Do not use expired or incorrectly stored components.

5.5. General Considerations

Effective use of real-time PCR tests requires good laboratory practices (GLP), including maintenance of equipment that is dedicated to molecular biology. Use nuclease-free labware (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.

5.6. 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.
- 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 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 & PCR 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.
- 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 6. INSTRUCTIONS FOR USE

6.1. DNA Isolation

Human genomic DNA must be extracted from FFPE tissue prior to use. Several methods exist for DNA isolation. For consistency we recommend using a commercial kit, such as the 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 specimens). Follow the DNA isolation procedure according to the manufacturer's protocol.

This QClamp® assay requires a total of 30 ng of DNA per sample (10 ng/reaction). After DNA isolation, measure the concentration using a fluorometric analysis (i.e., Qubit® Cat. No. Q33216) with nuclease-free water to 5 ng/µL (do not use TE). If using spectrophotometric analysis, make sure the A260/A230 value is greater than 2.0 and A260/A280 value between 1.8 and 2.0. High quality DNA is required for the assay. Quantification of DNA by UV absorbance-based methods may result in an overestimation of the total DNA amount.

6.2. Preparation of Reagents

Thaw all Primer/Probe Mixes, XNAs, Positive Control (PC), Negative Control (NC), Non-Template Control (NTC, Nuclease-Free Water) and 2X PCR Master Mix aliquots provided. Thaw all reaction mixes at room temperature for a minimum of 30 minutes. Vortex all components (except for 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 and performing a quick spin. Prior to use, ensure that the PCR Master Mix is resuspended by pipetting up and down multiple times. Do not leave kit components at room temperature for more than 2 hours. The final qPCR assay contains 10 µL of reagents (Table 4).

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

Table 4. QClamp® Assay Components and Reaction Volume

Components for E542K	Volume (per reaction)	Components for E545K	Volume (per reaction)	Components for H1047R	Volume (per reaction)
2X PCR Master Mix	5 µL	2X PCR Master Mix	5 µL	2X PCR Master Mix	5 µL
E542K Primer/Probe Mix	1 µL	E545K Primer/Probe Mix	1 µL	H1047R Primer/Probe Mix	1 µL
E542K XNA	1 µL	E545K XNA	1 µL	H1047R XNA	1 µL
DNA Sample (5 ng/µL) or Controls	2 µL	DNA Sample (5 ng/µL) or Controls	2 µL	DNA Sample (5 ng/µL) or Controls	2 µL
Nuclease Free Water	1 µL	Nuclease Free Water	1 µL	Nuclease Free Water	1 µL
Total Assay Volume	**10 µL**	**Total Assay Volume**	**10 µL**	**Total Assay Volume**	**10 µL**

6.3. Preparation of Assay Mixes

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)

Notes: n = number of reactions (DNA samples plus 3 controls). Prepare enough for 1 extra sample (n+1) to allow for sufficient volume for the PCR set.

Assay mixes should be prepared just prior to use. Label a microcentrifuge tube (not provided) for the reaction mix available, as shown in Table 5. **To maximize the XNA clamping efficiency, be sure that XNA is heated at 65°C for at least 15 min and add XNA Mix into assay mixture at the end.**

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 Positive Control (PC), Negative Control (NC), and Non-Template Control (NTC) should be included in each run. The controls should be spaced on the PCR plate away from each other and unknown samples to prevent cross-contamination.**

The Primer/Probe mixes contains the forward and reverse primers for PCR of the region of the *PIK3CA* gene to detect the target mutations (either E542K, E545K, or H1047R target). In each Primer/Probe mix, two probes are included: FAM-labeled *PIK3CA* probe for mutation detection and HEX-labeled *ACTB* probe as the internal control to monitor the validity of the assay.

The NC uses wildtype DNA as the template in each run. Wildtype DNA has no mutation in the *PIK3CA* gene, so the XNA probe will bind strongly, blocking the polymerase from making amplicons. Therefore, no PCR product from *PIK3CA* gene should be made from the negative control. However, the *ACTB* gene should be amplified as in the positive control.

A PC is also used with every assay run. The PC contains all three target mutation alleles (E542K, E545K, and H1047R) in the wildtype background; therefore, XNA probes will not bind to the mutant sequence, allowing amplification of the mutant template. The PC must show the appropriate Cq values of mutant amplification for the reaction to be valid.

A NTC is used with every assay run. Nuclease-Free Water is used in the place of template. The NTC serves as a general NC and assesses potential contamination during assay setup. Therefore, there should be no amplification products for NTC.

6.4. Suggested Plate Layout

Add 8 µL of the appropriate assay mix to each well of the plate and 2 µL of control or sample to ensure that each reaction has a final volume of 10 µL. **10 ng of DNA input is optimal for this assay.** We suggest the below layout (Table 6) to reduce possible contamination. **It is important to use the recommended PCR plates which were validated for this assay (see section *5.2 Materials Required but Not Provided in the Kit*).** Vortex DNA samples thoroughly and spin down before adding to each well. When all reagents have been added to the plate, pipette up and down 5 times to mix, and tightly seal the plate with the optical plate seal to prevent evaporation. Spin at 500 ×*g* for 1 minute to collect all the reagents. Place in the PCR instrument immediately. Table 6 is a suggested plate setup for a single experiment analyzing 3 unknown samples.

Table 6. Suggested Plate Layout (96-Well Plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC E542K Mix			NC E542K Mix			PC E542K Mix			S1 E542K Mix	S2 E542K Mix	S3 E542K Mix
B	NTC E545K Mix			NC E545K Mix			PC E545K Mix			S1 E545K Mix	S2 E545K Mix	S3 E545K Mix
C	NTC H1047R Mix			NC H1047R Mix			PC H1047R Mix			S1 H1047R Mix	S2 H1047R Mix	S3 H1047R Mix

Notes: NTC, Nuclease-free water; NC, wildtype DNA; PC, mutant DNA; S1 – S3, Samples 1 – 3. It is recommended that the controls be spaced on the PCR plate away from each other and unknown samples to prevent cross-contamination.

6.5. Instrument Setup

The parameters in Table 7 apply to Bio-Rad CFX384™, CFX96™, Roche LightCycler® 480II, ABI 7500, and QuantStudio™ 5 instruments. For Bio-Rad CFX384™ and CFX96™, the entire run is on default and no manual adjustment should be performed. Choose all channels when prompted. For ABI 7500 and QuantStudio™ 5, select NONE for the Passive Reference Dye. For Roche LightCycler® 480II the ramp rates should be adjusted according to Table 7.

Table 7. 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	×40	None
XNA Annealing	70	30	2.5		None
Primer Annealing	64	20	2.5		None
Extension	72	20*	4.8		**FAM and HEX**

Note: * For ABI 7500 Extension time for 30 s. This instrument does not allow a shorter extension time.

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 the signal rises above background, the stronger the PCR reaction it represents (**please see MIQE Guidelines under References [8] for more information*).

7.1. Data Analysis for Bio-Rad CFX384™

For the Bio-Rad CFX384™, use the Bio-Rad CFX Maestro software to analyze the data. Under the "Settings" tab, go to the "Baseline Setting" and select "Apply Fluorescence Drift Correction." Also, set the Cq Determination Mode to "Single Threshold" and set the "Baseline Setting" to "Baseline Subtracted Curve Fit" within the Quantification analysis tab. 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.

7.2. Data Analysis for Bio-Rad CFX96™

For the Bio-Rad CFX96™, use the Bio-Rad CFX Maestro software to analyze the data. Under the "Settings" tab, go to the "Baseline Setting" and select "Apply Fluorescence Drift Correction." Also, set the Cq Determination Mode to "Single Threshold" and "Baseline Setting" to "Baseline Subtracted Curve Fit" within the Quantification analysis tab. 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.

7.3. Data Analysis for Roche LightCycler® 480

For the Roche LightCycler® 480 II, open the LightCycler 480 SW 1.5.1.61 software (or later version) and select the Abs Quant/2nd Derivative Max analysis algorithm to analyze the run file data.

7.4. Data Analysis for ABI 7500

For the Thermo Fisher Scientific ABI 7500 use the *Design and Analysis* software version 2.6.0 (or newer version). 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 VIC. Verify that the thresholds for FAM and VIC are set slightly above the background fluorescence level so that negative controls or empty wells are not artificially called to be positive.

***Note:** The fluorescent dyes HEX and VIC have identical excitation wavelengths (538 nm) and near-identical emission wavelengths (555 and 554 nm, respectively). Choose the fluorescent dye for which your ABI instrument is calibrated.

7.5. Data Analysis for QuantStudio™ 5

For the Thermo Fisher Scientific ABI QuantStudio 5 use the *Design and Analysis* software version 2.6.0 (or newer version). Click "Analyze" button. Under the "Actions" tab, the Analysis Modules should be selected as "Presence Absence." Also, 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 ΔRn threshold to 25,000 for FAM and 10,000 for VIC for Internal Control. Verify that the thresholds for FAM and VIC are set slightly

above the background fluorescence level so that negative controls or empty wells are not artificially called to be positive. Also, set the baseline start at 3 and the baseline end at 28 for FAM, and set the baseline start at 3 and the baseline end at 22 for VIC.

7.6. Non-Template Controls

Verify that there is no amplification for both FAM and HEX (or VIC) signals in the non-template controls for each of the assay mixes. The Cq should be undetermined.

7.7. Analysis of Negative and Positive Controls

The PC, NC, and NTC should pass the criteria for the assay to be valid prior to analyzing data from your samples. The Cq values must be in the range of the following acceptable values for each instrument (Tables 9-14). *If the controls fail, the test is invalid and needs to be repeated. The sample data is analyzed and interpreted only after all the controls pass.*

Table 9. Acceptable Cq Values for Non-Template, Negative, and Positive Controls (Bio-Rad CFX384™)

Controls	Mutation Detection	FAM (target)	HEX (internal control)
Non-Template Control	E542, E545 and H1047	Undetected	Undetected
Negative Control	E542	Cq ≥ 37.0	Cq < 30.0
	E545	Cq ≥ 35.0	Cq < 30.0
	H1047	Cq ≥ 35.0	Cq < 30.0
Positive Control	E542	Cq < 35.0	Cq < 30.0
	E545	Cq < 32.0	Cq < 30.0
	H1047	Cq < 32.0	Cq < 30.0

Table 10. Acceptable Cq Values for Non-Template, Negative, and Positive Controls (Bio-Rad CFX96™)

Controls	Mutation Detection	FAM (target)	HEX (internal control)
Non-Template Control	E542, E545 and H1047	Undetected	Undetected
Negative Control	E542	Cq ≥ 39.0	Cq < 30.0
	E545	Cq ≥ 36.0	Cq < 30.0
	H1047	Cq ≥ 36.0	Cq < 30.0
Positive Control	E542	Cq < 36.0	Cq < 30.0
	E545	Cq < 33.0	Cq < 30.0
	H1047	Cq < 33.0	Cq < 30.0

Table 11. Acceptable Cq Values for Non-Template, Negative, and Positive Controls (Roche LightCycler® 480II)

Controls	Mutation Detection	FAM (target)	HEX (internal control)
Non-Template Control	E542, E545 and H1047	Undetected	Undetected
Negative Control	E542	Cq ≥ 37.0	Cq < 30.0
	E545	Cq ≥ 35.0	Cq < 30.0
	H1047	Cq ≥ 35.0	Cq < 30.0
Positive Control	E542	Cq < 34.0	Cq < 30.0
	E545	Cq < 32.0	Cq < 30.0
	H1047	Cq < 32.0	Cq < 30.0

Table 12. Acceptable Cq Values for Non-Template, Negative, and Positive Controls (ABI 7500)

Controls	Mutation Detection	FAM (target)	VIC (internal control)
Non-Template Control	E542, E545 and H1047	Undetected	Undetected
Negative Control	E542	Cq ≥ 36.0	Cq < 30.0
	E545	Cq ≥ 33.0	Cq < 30.0
	H1047	Cq ≥ 34.0	Cq < 30.0
Positive Control	E542	Cq < 33.0	Cq < 30.0
	E545	Cq < 30.0	Cq < 30.0
	H1047	Cq < 31.0	Cq < 30.0

Table 13. Acceptable Cq Values for Non-Template, Negative, and Positive Controls (QuantStudio™ 5)

Controls	Mutation Detection	FAM (target)	VIC (internal control)
Non-Template Control	E542, E545 and H1047	Undetected	Undetected
Negative Control	E542	Cq ≥ 36.0	Cq < 30.0
	E545	Cq ≥ 34.0	Cq < 30.0
	H1047	Cq ≥ 35.0	Cq < 30.0
Positive Control	E542	Cq < 33.0	Cq < 30.0
	E545	Cq < 29.0	Cq < 30.0
	H1047	Cq < 30.0	Cq < 30.0

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

The Cq value of the Internal Control (*ACTB* gene) 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 Internal Control. Cq values of any sample with Internal Control should be in the range of Cq 24.0 to Cq 30.0. 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 14.

Table 14. Acceptable Cq values for Internal Controls

Validity	Cq Value of Internal Control Mix	Descriptions and Recommendations
Valid	24.0 ≤ Cq < 30.0	The amplification and amount of DNA sample were optimal.
Invalid	Cq < 24.0	The chance of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ≥ 30.0	The amplification is not optimal. Either not enough amount DNA or poor quality of DNA. Re-check DNA amount and quality. If DNA is degraded, a new DNA prep may be required.

7.9. Scoring Mutational Status

PIK3CA mutational status is determined by using the QClamp® PIK3CA Mutation Detection Test in 10 ng DNA input with a Cq cutoff value of 5% Variant Allele Frequency (VAF) for positivity. When all quality checks are passed, use the following Cq cutoff values to determine mutational status (Tables 15−19).

Note: The QClamp® PIK3CA Mutation Detection Test has been evaluated using PIK3CA E542K, E545K, and H1047R mutations.

Table 15. Scoring Mutational Status for Bio-Rad CFX384™

Mutation	Positive Cq cutoff	Negative Cq cutoff
E542K	Cq < 35.0	Cq ≥ 37.0
E545K	Cq < 32.0	Cq ≥ 35.0
H1047R	Cq < 32.0	Cq ≥ 35.0

Table 16. Scoring Mutational Status for Bio-Rad CFX96™

Mutation	Positive Cq cutoff	Negative Cq cutoff
E542K	Cq < 36.0	Cq ≥ 39.0
E545K	Cq < 33.0	Cq ≥ 36.0
H1047R	Cq < 33.0	Cq ≥ 36.0

Table 17. Scoring Mutational Status for Roche LightCycler® 480II

Mutation	Positive Cq cutoff	Negative Cq cutoff
E542K	Cq < 34.0	Cq ≥ 37.0
E545K	Cq < 32.0	Cq ≥ 35.0
H1047R	Cq < 32.0	Cq ≥ 35.0

Table 18. Scoring Mutational Status for ABI 7500

Mutation	Positive Cq cutoff	Negative Cq cutoff
E542K	Cq < 33.0	Cq ≥ 36.0
E545K	Cq < 30.0	Cq ≥ 33.0
H1047R	Cq < 31.0	Cq ≥ 34.0

Table 19. Scoring Mutational Status for ABI QuantStudio™ 5

Mutation	Positive Cq cutoff	Negative Cq cutoff
E542K	Cq < 33.0	Cq ≥ 36.0
E545K	Cq < 29.0	Cq ≥ 34.0
H1047R	Cq < 30.0	Cq ≥ 35.0

Above the cutoff value (including “undetermined” or “N/A”), the sample may either contain lower percentages of mutation which are close to but smaller than 5%, or the sample is mutation negative. When the cutoff is chosen, any testing results between the ‘Positive Cq cutoff’ and the ‘Negative Cq cutoff’ could be in grey zone or inconclusive, the test shall be repeated before making a conclusion (e.g. for the E542K target, the repeat range is 35.0 ≤ Cq <37.0 for the CFX384™).

Note: Since different commercial kits have different sensitivity and different amounts of DNA input, it is not always possible to compare sample results. We recommend that each clinical laboratory performs their own validation if comparison is desired.

PART 8. ASSAY PERFORMANCE CHARACTERISTICS

The performance characteristics of this product were established on the Bio-Rad CFX384™, Bio-Rad CFX96™, Roche LightCycler® 480 II, ABI 7500™, and ABI QuantStudio™ 5 real-time PCR instruments.

8.1. Limit of Blank (LOB)

To determine the limit of blank (LOB) for the kit, the QClamp® PIK3CA assay for each target was run using fifty replicates of 10 ng DNA input of wild-type genomic DNA. The Cq values for the fifty replicates were ranked in numerical order and the 95% confidence interval Cq value (lower 95% C.I.) was used as the LOB Cq value. The LoB Cq value for each assay varies on different instruments, as shown in Table 20.

Table 20. LOB Cq value Summary

Instrument	Assay	LOB (Cq Value)
CFX384™	E542K	undetectable
	E545K	≥ 39.7
	H1047R	≥ 45.0
CFX96™	E542K	undetectable
	E545K	≥ 41.4
	H1047R	≥ 43.4
LightCycler® 480II	E542K	undetectable
	E545K	≥ 40.7
	H1047R	≥ 42.2
ABI 7500	E542K	≥ 44.3
	E545K	≥ 45.4
	H1047R	≥ 42.3
ABI QuantStudio™ 5	E542K	≥ 47.7
	E545K	≥ 42.7
	H1047R	≥ 40.8

8.2. Limit of Detection (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® PIK3CA Mutation Detection assays were run using a serial dilution of mutant genomic DNA in wildtype background at different total DNA inputs and several Variant Allele Frequency (VAF) for each target. Mutant reference samples were verified by droplet digital PCR 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%, 0.13% and 0.06% VAF. Results shown in Table 21 demonstrate effective clamping of wild type sequences, providing a reproducible detection of mutations at concentrations as low as 0.1% VAF for some assays run in multiple replicates.

Table 21. LOD Summary

Instrument	Assay	LOD (% VAF)
CFX384™	E542K	0.125
	E545K	0.063
	H1047R	0.125
CFX96™	E542K	0.125
	E545K	0.125
	H1047R	0.063
LightCycler® 480II	E542K	0.125
	E545K	0.125
	H1047R	0.125
ABI 7500	E542K	0.063
	E545K	0.125
	H1047R	0.125
ABI QuantStudio™ 5	E542K	0.125
	E545K	0.250
	H1047R	0.250

8.3. Cutoff Values

PIK3CA wild-type, E542K, E545K, H1047R reference DNA diluted to 5% VAF were tested to establish the cutoff values for the assay. Fifty replicates each of 5% VAF samples were tested and the Cq values were ranked in numerical order. The 95% confidence interval Cq value (higher 95% CI) for 5% VAF data was determined and used to set the positive cutoff for each assay for each instrument (see Tables 9-13 and Tables 15-19). The negative samples (wildtypes) used to determine the LOB are used to set the negative cutoff.

8.4. 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.

8.5. 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 Cq values and rate of % correct mutation calls for all assays on two lots and operators for Roche LightCycler® 480II and Bio-Rad CFX384™ instruments.

PART 9. ASSAY TROUBLESHOOTING

Table 22. Frequently Occurring Problems, Their Causes and Solutions

Problem	Cause	Solution
Clear amplification seen in Non-Template Control (NTC)	The positive signal may be caused by contamination during setting-up of the PCR; or the signal is not true target amplification, but background curves generated by the software of the PCR instrument.	Repeat the PCR with new reagents. Follow the general rules of GLP in a PCR lab. It is recommended to set up the PCR reactions in a separate area, where no DNA is handled and with equipment designated for pre-PCR activities. Make sure the workspace and instruments are decontaminated regularly. Ignore the Cq value of NTC if the amplification curve looks not real but background noise.
The Positive Control (PC) did not meet the criteria set for acceptable values of the kit. The assay is invalid.	Kit was not stored at the recommended conditions; or Kit shelf-life expired.	Check the kit label for storage conditions and expiration date and use a new kit if necessary. Also, be sure that the Primer/Probe Mixes should be protected from light.
The Negative Control (NC) did not meet the criteria set for acceptable values of the kit. The assay is invalid.	Kit was not stored at the recommended conditions; or Kit shelf-life expired. Alternatively, XNA may not bind to it target sequences very efficiently.	Check the kit label for storage conditions and expiration date and use a new kit if necessary. In addition, be sure that XNA Mix is heated at 65°C for at least 15 min and add XNA Mix into assay mixture at the end.
The sample Cq value of HEX dye (Internal control) is not in the range of 25.0 < Cq < 30.0.	The amplification and amount of DNA input are not optimal.	Repeat the experiments following the recommendations described in the Kit Instruction Manual. The optimal input DNA amount should be 10 ng. Also, be sure that DNA quality and integrity are sufficient.
A sample that is positive for the mutation, but the Cq of HEX dye (Internal control) is not in the range of 25.0 < Cq < 30.0.	It is most likely due to the DNA input or quality is not optimal.	If the sample Cq of HEX is ≥ 30.0, not sufficient samples are used or samples are degraded. If the sample Cq of HEX is ≤ 25, the sample is overloaded. Either situation causes false-negative or false-positive results. Repeat the experiments following the recommendations described in the Kit Instruction Manual.
Intra-assay (well-to-well) variations even for the same sample repeat or replicates	Multiple reasons for the causes: (1) accuracy of pipetting for reagents, (2) mixing of samples/reagents before aliquoting, (3) use of the correct PCR plates, (4) XNA addition practice.	(1) Since only a few microliters of reagents are added, pipetting accuracy is critical. Make sure no extra liquid sticks on pipette tips or loose tips. (2) DNA should be diluted with H_2O (instead of TE) to 5 ng/µL. Make sure that DNA samples are mixed thoroughly by vortexing and spinning down before adding to each well. Also, all the reagents should be fully thawed and mixed well before use. (3) Use the correct PCR plates as different plates have different readings due to the material composition of the plates. (4) Preheat XNA before adding to the assay mix as described in the manual.

PART 10. SYMBOLS USED IN PACKAGING

Table 23. 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)		

PART 11. REFERENCES

1. Cathomas G. PIK3CA in colorectal cancer. Front. Oncol. 4 (35) https://doi.org/10.3389/fonc.2014.00035, 2014.
2. Reinhardt K., Stückrath K., Hartung C., Kaufhold S., Uleer C., Hanf V. Lantzsch T., Peschel S., John J., Pöhler M., Bauer M., Bürrig F.K., Weigert E., Buchmann J., Kantelhardt E.J., Thomssen C., and Vetter M. *PIK3CA*-mutations in breast cancer. Breast Cancer Res Treat. 196(3):483-493, 2022.
3. Martínez-Sáez O., Chic N., Pascual T., Adamo B., Vidal M., González-Farré B., Sanfeliu E., Schettini F., Conte B., Brasó-Maristany F., Rodríguez A., Martínez D., Galván P., Rodríguez A.B., Martinez A., Muñoz M., and Prat A. Frequency and spectrum of *PIK3CA* somatic mutations in breast cancer. Breast Cancer Res. 22(1):45. DOI: 10.1186/s13058-020-01284-9
4. Ørum, Henrik. PCR Clamping. Curr. Issues Mol. Biol. 2(1), 27-30, 2000.
5. Powell et. al., Detection of the hereditary hemochromatosis gene mutation by real-time fluorescence polymerase chain reaction and peptide nucleic acid clamping. Analytical Biochemistry 260: 142–8, 1998.
6. Sun Q., Paster L., Du J., Powell M., Zhang A., Bodmer W and Sha M. A Novel Xenonucleic Acid Mediated Molecular Clamping Technology for Early Colorectal Cancer Screening. PLOS ONE 2021 https://doi.org/10.1371/journal. pone.0244332
7. Tanaka H., Shen S., Scimia M., Pastor L., Li J., Fu AY., Jamba M., Sun Q., Zhang A., and Sha MY. The ColoScapeTM: A Novel QClamp® XNA Technology-based Assay to Detect Colorectal Cancer Associated Mutations and Methylations in Plasma Cell-free DNA. JOURNAL OF MOLECULAR DIAGNOSTICS 24 (10), S133-S133, 2022
8. **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
9. Mycancergenome.org. Genetically informed Cancer Medicine – My Cancer Genome. [online] Available at: https://www.mycancergenome.org/.