INSTRUCTION MANUAL

QClamp® EGFR Mutations Detection Test

For Real-Time PCR Assays

CATALOG NUMBER

DC-10-1038R (10 samples)
DC-10-0012R (30 samples)
DC-10-0011R (60 samples)

MANUFACTURER

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INTENDED USE

For Research Use Only

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

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CONTENTS

Part 1. Intended Use ... 3

Part 2. EGFR Mutations and Cancer ... 4

Part 3. QClamp® Workflow ... 4

Part 4. QClamp® Technology for Mutation Detection ... 5

Part 5. Reagents and Instruments ... 6

Part 6. Instructions for Use ... 11

Part 7. Assessment of Real-Time PCR Results ... 14

Part 8. Assay Performance Characteristics ... 16

Part 9. Symbols Used in Packaging ... 19

References ... 20

Part 1. Intended Use

The QClamp® EGFR Mutation Detection Test is a real-time PCR assay for the detection of somatic mutations in and near EGFR codon 719 in Exon 18, Exon 19 deletions, D769_v770 insertion ASV as well as mutations S768I, T790M in Exon 20, and L858R and L861Q in Exon 21 in the human EGFR (epidermal growth factor receptor) gene, using purified DNA extracted from FFPE (formalin fixed paraffin embedded), plasma or cells. The kit identifies the presence or absence of mutations in the targeted regions. Unless an assay targets a specific mutation the codon specific detection kits are designed to detect any mutation at or near the stated codon site without specifying the exact nucleotide change. Table 1 shows a list of mutations commonly found in the EGFR gene that can be detected by the kit. The kit is to be used by trained laboratory professionals within a laboratory environment.

Table 1. List of Mutations and Cosmic Identities Commonly Found in EGFR

Exon	Amino Acid	Nucleotide Change	Cosmic No.
18	G719>A	c.2156G>C	6239
	G719>S	c.2155G>A	6252
	G719>C	c.2155G>T	6253
19	E746_A750del	c.2235_2249 del 15	6223
	E746_T751>I	c.2235_2252 > AAT	13551
	E746_T751del	c.2236_2253 del 18	12728
	E746_T751>A	c.2237_2251 del 15	12678
	E746_S752>A	c.2237_2254 del 18	12367
	E746_S752>V	c.2237_2255>T	12384
	E746_A750del	c.2236_2250 del 15	6225
	E746_S752>D	c.2238_2255 del 18	6220
	L747_A750>P	c.2238_2248 >GC	12422
	L747_T751>Q	c.2238_2252 >GCA	12419
	L747_E749del	c.2239_2247 del 9	6218
	L747_T751del	c.2239_2253 del 15	6254
	L747_S752del	c.2239_2256 del 18	6255
	L747_ A750>P	c.2239_2248	12382
	L747_P753>Q	c.2239_2258 >CA	12387
	L747_T751>S	c.2240_2251 del 12	6210
	L747_P753>S	c.2240_2257 del 18	12370
	L747_T751del	c.2240_2254 del 15	12369
	L747_T751>P	c.2239_2251>C	12383
	K745_E749del	2233_2247del15	26038
	S752_I759del	2253_2276del24	13556
	E746_A750>IP	2235_2248>AATTC	13550
	E746_T751>V	2237_2252>T	12386
	E746_T751>IP	2235_2251>AATTC	13552
	E746_S752>I	2235_2255>AAT	12385
	E746_T751>VA	2237_2253>TTGCT	12416
	E746_P753>VS	2237_2257>TCT	18427
	L747_T751del	2238_2252del15	23571
	L747_S752>Q	2239_2256>CAA	12403
20	S768I	c.2303G>T	6241/1848
	V769_D770insAS	c.2307_2308insGCCAGC...	12376/263
	V769_D770insAS	c.2308_2309insCCAGCG...	12426
	T790>M	c.2369C>T	6240
21	L858R2303 G>T	2573_2574TG>GTS768I	12429/624
	L858>R	c.2573T>G	6224
	L861>Q	c.2582T>A	6213

Part 2. EGFR Mutations and Cancer

The epidermal growth factor receptor (EGFR) gene encodes a membrane protein that possesses an intracellular receptor tyrosine kinase and is expressed on the surface of epidermal cells. EGFR plays a central role in transmitting signals that promote cell growth and proliferation. Its tyrosine kinase (TK) domain activates several downstream effectors that lead to activation of the Ras-Raf-MAPK pathway. Overexpression and oncogenic mutations that constitutively activate the TK domain of EGFR have been found in various solid tumors.

The presence of mutations in the EGFR oncogene correlates with response to certain tyrosine kinase inhibitor (TKI) cancer therapies in patients with non-small cell lung cancer (NSCLC) (3–8). Such mutations in the EGFR oncogene are present in the general population of patients with NSCLC at a frequency of approximately 10% in patients from the USA, Europe, or Australia and up to 30% in patients from Japan and Taiwan (1, 2, 9). Excessive activation of EGFR has been shown to be associated with advanced stages of cancer and a poor prognosis.

Due to its association with malignancies, epidermal growth factor receptor (EGFR) has become the target of an expanding class of anti-cancer therapies, such as GILOTRIF, GEFITINIB (Iressa) and ERLOTINIB (Tarceva), which are tyrosine kinase inhibitors (TKIs).

The presence of the T790M 'gatekeeper' mutation correlates with resistance to tyrosine kinase inhibitor (TKI) therapy. Detection of somatic mutations in the EGFR gene provides a powerful strategy to predict the response of cancer patients to selected therapeutic regimens (e.g., tyrosine kinase inhibitors in lung cancer), in an effort to increase the survival rate of cancer patients receiving targeted therapies.

Part 3. QClamp® Workflow

The assay workflow consists of four major steps:

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

Part 4. QClamp® Technology for Mutation Detection

The QClamp® EGFR 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.

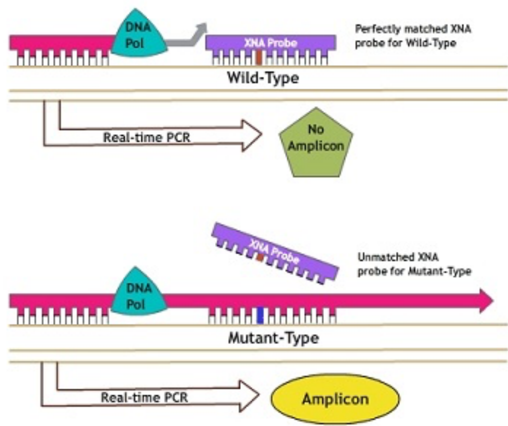

Figure 1. Principle of the QClamp® EGFR Mutation Detection Test

Part 5. Reagents and Instruments

5.1. Package Contents

Table 2. Package Contents

Table 2a. Pack size: 10 samples

	Name of Component	Part #	Description	Pack Size	Label Volume	Cap Color	Storage Temperature
Grey Box 1	c719 Primer/Probe Mix	1007192	c719 Primers and probes	1 vial	22 µL	Orange	-25°C to -15°C
	Ex19del Primer/Probe Mix	1007202	Ex19del Primers and probes	1 vial	22 µL	Red	-25°C to -15°C
	c768 Primer/Probe Mix	1007242	c768 Primers and probes	1 vial	22 µL	Purple	-25°C to -15°C
	c769_c770insASV Primer/Probe Mix	1007252	c769_c770insASV Primers and probes	1 vial	22 µL	Blue	-25°C to -15°C
	c790 Primer/Probe Mix	1007212	c790 Primers and probes	1 vial	22 µL	Yellow	-25°C to -15°C
	c858 Primer/Probe Mix	1007222	c858 Primers and probes	1 vial	22 µL	Green	-25°C to -15°C
	c861 Primer/Probe Mix	1007232	c861 Primers and probes	1 vial	22 µL	Amber	-25°C to -15°C
	Nuclease-Free Water	1007362	Nuclease-Free Water	1 vial	64 µL	Clear	-25°C to -15°C
	Negative Control	1007342	Wild-type gDNA	1 vial	42 µL	Clear	-25°C to -15°C
	Mixed Positive Control	1007332	G719S, Ex19 Del, Ex20insASV, I768I, T790M, L858R, & L861Q Mixed Template	1 vial	42 µL	Clear	-25°C to -15°C
Grey Box 2	c719 XNA	1007262	c719 XNA	1 vial	22 µL	Orange	-25°C to -15°C
	Ex19del XNA	1007272	Ex19del XNA	1 vial	22 µL	Red	-25°C to -15°C
	c768 XNA	1007312	c768 XNA	1 vial	22 µL	Purple	-25°C to -15°C
	c769_c770insASV XNA	1007322	c769_c770insASV XNA	1 vial	22 µL	Blue	-25°C to -15°C
	c790 XNA	1007282	c790 XNA	1 vial	22 µL	Yellow	-25°C to -15°C
	c858 XNA	1007292	c858 XNA	1 vial	22 µL	Green	-25°C to -15°C
	c861 XNA	1007302	c861 XNA	1 vial	22 µL	Amber	-25°C to -15°C
	2X PCR Master Mix	1007352	PCR Reaction Premix	1 vial	770 µL	Clear	-25°C to -15°C

Table 2b. Pack size: 30 samples

	Name of Component	Part #	Description	Pack Size	Label Volume	Cap Color	Storage Temperature
Grey Box 1	c719 Primer/Probe Mix	1007193	c719 Primers and probes	1 vial	54 µL	Orange	-25°C to -15°C
	Ex19del Primer/Probe Mix	1007203	Ex19del Primers and probes	1 vial	54 µL	Red	-25°C to -15°C
	c768 Primer/Probe Mix	1007243	c768 Primers and probes	1 vial	54 µL	Purple	-25°C to -15°C
	c769_c770insASV Primer/Probe Mix	1007253	c769_c770insASV Primers and probes	1 vial	54 µL	Blue	-25°C to -15°C
	c790 Primer/Probe Mix	1007213	c790 Primers and probes	1 vial	54 µL	Yellow	-25°C to -15°C
	c858 Primer/Probe Mix	1007223	c858 Primers and probes	1 vial	54 µL	Green	-25°C to -15°C
	c861 Primer/Probe Mix	1007233	c861 Primers and probes	1 vial	54 µL	Amber	-25°C to -15°C
	Nuclease-Free Water	1007363	Nuclease-Free Water	1 vial	138 µL	Clear	-25°C to -15°C
	Negative Control	1007343	Wild-type gDNA	1 vial	84 µL	Clear	-25°C to -15°C
	Mixed Positive Control	1007333	G719S, Ex19 Del, Ex20insASV, I768I, T790M, L858R, & L861Q Mixed Template	1 vial	84 µL	Clear	-25°C to -15°C
Grey Box 2	c719 XNA	1007263	c719 XNA	1 vial	54 µL	Orange	-25°C to -15°C
	Ex19del XNA	1007273	Ex19del XNA	1 vial	54 µL	Red	-25°C to -15°C
	c768 XNA	1007313	c768 XNA	1 vial	54 µL	Purple	-25°C to -15°C
	c769_c770insASV XNA	1007323	c769_c770insASV XNA	1 vial	54 µL	Blue	-25°C to -15°C
	c790 XNA	1007283	c790 XNA	1 vial	54 µL	Yellow	-25°C to -15°C
	c858 XNA	1007293	c858 XNA	1 vial	54 µL	Green	-25°C to -15°C
	c861 XNA	1007303	c861 XNA	1 vial	54 µL	Amber	-25°C to -15°C
	2X PCR Master Mix	1007353	PCR Reaction Premix	2 vials	945 µL	Clear	-25°C to -15°C

Table 2c. Pack size: 60 samples

	Name of Component	Part #	Description	Pack Size	Label Volume	Cap Color	Storage Temperature
Grey Box 1	c719 Primer/Probe Mix	1007194	c719 Primers and probes	1 vial	84 µL	Orange	-25°C to -15°C
	Ex19del Primer/Probe Mix	1007204	Ex19del Primers and probes	1 vial	84 µL	Red	-25°C to -15°C
	c768 Primer/Probe Mix	1007244	c768 Primers and probes	1 vial	84 µL	Purple	-25°C to -15°C
	c769_c770insASV Primer/Probe Mix	1007254	c769_c770insASV Primers and probes	1 vial	84 µL	Blue	-25°C to -15°C
	c790 Primer/Probe Mix	1007214	c790 Primers and probes	1 vial	84 µL	Yellow	-25°C to -15°C
	c858 Primer/Probe Mix	1007224	c858 Primers and probes	1 vial	84 µL	Green	-25°C to -15°C
	c861 Primer/Probe Mix	1007234	c861 Primers and probes	1 vial	84 µL	Amber	-25°C to -15°C
	Nuclease-Free Water	1007364	Nuclease-Free Water	1 vial	276 µL	Clear	-25°C to -15°C
	Negative Control	1007344	Wild-type gDNA	1 vial	84 µL	Clear	-25°C to -15°C
	Mixed Positive Control	1007334	G719S, Ex19 Del, Ex20insASV, I768I, T790M, L858R, & L861Q Mixed Template	1 vial	84 µL	Clear	-25°C to -15°C
Grey Box 2	c719 XNA	1007264	c719 XNA	1 vial	84 µL	Orange	-25°C to -15°C
	Ex19del XNA	1007274	Ex19del XNA	1 vial	84 µL	Red	-25°C to -15°C
	c768 XNA	1007314	c768 XNA	1 vial	84 µL	Purple	-25°C to -15°C
	c769_c770insASV XNA	1007324	c769_c770insASV XNA	1 vial	84 µL	Blue	-25°C to -15°C
	c790 XNA	1007284	c790 XNA	1 vial	84 µL	Yellow	-25°C to -15°C
	c858 XNA	1007294	c858 XNA	1 vial	84 µL	Green	-25°C to -15°C
	c861 XNA	1007304	c861 XNA	1 vial	84 µL	Amber	-25°C to -15°C
	2X PCR Master Mix	1007354	PCR Reaction Premix	3 vials	980 µL	Clear	-25°C to -15°C

5.2. Materials Required but Not Provided in the Kit

A. Reagents for DNA Isolation

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

B. Consumables

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

C. Equipment

- Permanent marker
- Real time PCR instrument capable of TaqMan
- 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

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

Table 3. List of Instruments Validated with This Kit

Company	Model
Roche	LightCycler 480
Bio-Rad	CFX384
ABI	QuantStudio 5

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 -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 and 2) in a pre-amplification area and the controls (Box 3) in a postamplification (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 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, 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.

5.6. 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 6. Instructions for Use

6.1. DNA Isolation

Human genomic DNA must be extracted from tissue or blood, plasma, or fixed paraffin-embedded tissue prior to use. Several methods exist for DNA isolation. For consistency, we recommend using a commercial kit, such as Qiagen DNA extraction kit (QIAamp DNA FFPE Tissue Kit, cat No. 56404, for paraffin embedded specimens; DNeasy Blood & Tissue kit, Cat. No. 69504 or 69506, for tissue and blood specimens; for cfDNA extraction from plasma use Qiagen cfDNA extraction kits, such as Cat# 55114 or beads-based cfDNA extraction kit (Cat# 55204). Follow the DNA isolation procedure according to manufacturer's protocol.

This QClamp assay requires a total of 35- 70 ng of DNA per sample (5-10ng/reaction). After DNA isolation, measure the concentration using fluorometric analysis (i.e., Qubit, cat#Q33216) and dilute to 2.5- 5 ng/µl. 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.

6.2. Preparation of Reagents

Thaw all primer and probe mixes, XNAs, Positive Control, WT Negative Control, 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. QClamp Assay Components and Reaction Volume

Components	Volume/Reaction
2X PCR Master mix	5 µl
Primer and Probe Mix	1 µl
XNA	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 5 below
- If needed, the sample volume can be increased to 3 µl by eliminating 1 µl water

6.3. Preparation of Assay Mixes

Table 5. Preparation of Assay Mixes

	Volume of 2X PCR Master	Volume of Primer	Volume of XNA
c719 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
Ex19del Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
c768 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
Ex20insASV Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
c790 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
c858 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)
c861 Mix	5 µl x (n+1)	1µl x (n+1)	1 µl x (n+1)

Notes:

- n = number of reactions (DNA samples plus 3 controls). Prepare enough for 1 extra sample (n +1) to allow for sufficient overage for the PCR set.
- For accuracy, do not pipette less than 8 µl.

Assay mixes should be prepared just prior to use. Label a micro centrifuge tube (not provided) for each reaction mix available, as shown in Table 5. Please disregard any assay mixes listed that are not part of your kit.

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 EGFR Mixed Positive Control (PC), EGFR Negative Control (NC), and a No Template Control (NTC) should be included in each run. Negative Controls use wild-type DNA as the template. Wild-type DNA should have no mutations; therefore, the XNA probes will bind strongly, blocking the polymerase from making amplicons. However, the Internal Control assay (HEX) with the Negative Control should make amplicons efficiently, providing another way to monitor performance of the primers, polymerase, and sample.

A set of positive controls must also be run with each reaction mix, every time the assay is run. The Positive Control contains one mutant template for each reaction mix. Positive controls contain mutations; therefore, XNA probes will not bind, allowing amplification of the mutant template. Positive controls must show the appropriate values for the reaction to be valid. A set of no template control (tube NTC) is run with each reaction mix every time when 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.

6.4. Suggested Run Layout (96-well plate, 384-well plate, tube strips, or tubes)

Add 8µl of the appropriate assay mix to the plate or tubes to ensure that each reaction has a total of 10µl.

Table 6. Suggested Plate Layout

	1	2	3	4	5	6
A	NTC	PC	NC	S1	S2	S3
	c719 Mix	c719 Mix	c719 Mix	c719 Mix	c719 Mix	c719 Mix
B	NTC	PC	NC	S1	S2	S3
	Ex19del Mix	Ex19del Mix	Ex19del Mix	Ex19del Mix	Ex19del Mix	Ex19del Mix
C	NTC	PC	NC	S1	S2	S3
	c768 Mix	c768 Mix	c768 Mix	c768 Mix	c768 Mix	c768 Mix
D	NTC	PC	NC	S1	S2	S3
	Ex20insASV Mix	Ex20insASV Mix	Ex20insASV Mix	Ex20insASV Mix	Ex20insASV Mix	Ex20insASV Mix
E	NTC	PC	NC	S1	S2	S3
	c790 Mix	c790 Mix	c790 Mix	c790 Mix	c790 Mix	c790 Mix
F	NTC	PC	NC	S1	S2	S3
	c858 Mix	c858 Mix	c858 Mix	c858 Mix	c858 Mix	c858 Mix
G	NTC	PC	NC	S1	S2	S3
	c861 Mix	c861 Mix	c861 Mix	c861 Mix	c861 Mix	c861 Mix

Notes:

- PC: Positive Control (mixed mutant DNA), NTC: No-Template Control (water), NC: Negative Control (Wild-type DNA), S1-3: Samples 1-3.
- When all reagents have been added to the plate, tightly seal the plate to prevent evaporation. Spin at 2000rpm for 1 minute to collect all the reagents. Place in the real-time PCR instrument immediately.

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

6.5. Instrument Set-Up

These parameters apply to BioRad CFX384, Roche LC480, ABI QuantStudio 5 Instruments. For Bio Rad CFX384, the entire run is on default and no manual adjustment should be performed. Choose all channels when prompted.

For Applied Biosystems Platforms select NONE for the Passive Reference Dye. For Roche LC480 the ramp rates should be adjusted according to the below table.

Table 7. Cycling Parameters

Step	Temperature	Time	Ramp Rates for Roche	Cycles	Data
PreIncubation	95	300	4	1	OFF
Denaturation	95	20	1.55	X45	OFF
XNA Annealing	70	40	1.75		OFF
Primer	60	30	1.2		OFF
Extension	72	30	1.4		FAM and HEX

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 stronger the PCR reaction it represents (**please see MIQE Guidelines under References for more information).

Negative Controls use wild-type DNA as the template. Wild-type DNA contains none of the targeted mutations, therefore the XNA probes will bind strongly, inhibiting the target amplification. In contrast, the Internal Control Assay assessed with HEX channel should make amplicons efficiently for the Negative Control, providing another way to monitor performance of the primers, polymerase, and sample DNA quality/quantity. The Positive Control contains one mutant template for each assay mix. Positive controls contain mutations; therefore, XNA probes will not bind, allowing amplification of the mutant template. Positive controls must show the appropriate values in both HEX and FAM channels for the run to be valid.

A set of no-template control (NTC) is run with each assay mix every time the assay is run. Nuclease-Free Water is used in the place of template. The NTC serves as a negative control and assesses potential contamination during assay set-up.

7.1. Data Analysis

For Bio Rad CFX384, select FAM and Hex channels, set the RFU threshold at 100 for both channels and export the data table.

For the Light Cycler 480, open the LightCycler480 SW 1.5.1.61, choose Dual Color Hydrolysis Probe and select Abs Quant/Fit Points for All Samples algorithm and set the threshold at 0.7 for both channels to analyze the run file data.

For ABI Quant Studio 5, under Properties select TaqMan and under Plate, select Fam for Target 1 and Vic for Target 2. For the Passive reference select None. Set the deltaRn for both targets at 10,000 and proceed to the data analysis.

The thresholds can be adjusted as needed for each run separately for each assay based on the levels of noise.

7.1.1. No-Template Controls

Verify that no amplification is observed in the no-template controls (NTC) for each of the reaction mixes. Cq should be Undetermined. 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.

7.1.2. Analysis of Negative and Positive Controls

For the assay to be valid, the Negative Control and Positive Control must meet the criteria in Table 8a-c.

Table 8a. Acceptable Values for Positive Controls and Negative Controls (BioRad CFX384)

	Positive Control	Negative Control
c719	Cq ≤ 40	Cq > 40
Ex19del	Cq ≤ 40	Cq > 40
c768	ΔCq ≤ 6.5	ΔCq > 14
Ex20insASV	ΔCq ≤ 7	ΔCq > 10
c790	Cq ≤ 40	Cq > 40
c858	Cq ≤ 40	Cq > 40
c861	Cq ≤ 40	Cq > 40

Table 8b. Acceptable Values for Positive Controls and Negative Controls (Roche LC480)

	Positive Control Acceptable Values	Negative Control Acceptable Values
c719	Cq ≤ 40	Cq > 40
Ex19del	Cq ≤ 40	Cq > 40
c768	ΔCq ⩽ 5	ΔCq > 14
Ex20insASV	ΔCq ⩽ 5.5	ΔCq > 12
c790	Cq ≤ 40	Cq > 40
c858	Cq ≤ 40	Cq > 40
c861	Cq ≤ 40	Cq > 40

Table 8c. Acceptable Values for Positive Controls and Negative Controls (ABI QuantStudio 5)

	Positive Control Acceptable Values	Negative Control Acceptable Values
c719	Cq ≤ 40	Cq > 40
Ex19del	Cq ≤ 40	Cq > 40
c768	ΔCq ⩽ 5.5	ΔCq > 14
Ex20insASV	ΔCq ⩽ 7	ΔCq > 10
c790	Cq ≤ 40	Cq > 40
c858	Cq ≤ 40	Cq > 40
c861	Cq ≤ 40	Cq > 40

7.1.3. Judging Validity of Sample Data Based on Internal Control Test Results (HEX)

The Cq value of the Internal Control Mix can serve as an indication of the purity and concentration of DNA. Thus, the validity of the test can be decided by the Cq value of the Int Control mix. Cq values of any sample with Internal Control Mix should be in the range of 24-32. 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 9.

Table 9. Acceptable Cq Ranges for Samples with Internal Control Mix

Validity	Cq Value of Int Control Mix	Descriptions and Recommendations
Optimal	24 < Cq < 32	The amplification and amount of DNA sample were optimal.
Invalid	Cq ≤24	Possibility of a false positive is high. Repeat the PCR reaction with less DNA.
Invalid	Cq ≥ 32	Not enough DNA or DNA not pure. The amplification is not optimal. Check DNA amount and purity. Repeat the experiment with more DNA or a new DNA prep may be required.

7.1.4. Scoring Mutational Status and Cut-Offs

If a Cq value is Undetermined, assign a Cq of 45 and proceed to analysis. The table below should be used to determine mutational status. Along with studies for analytical accuracy, both reference gDNA and DNA extracted from FFPE and cfDNA were tested to establish cut-offs for the assay. Cutoffs are presented in Table 10.

Table 10. Scoring Mutational Status and cut-offs

Instrument	Call		EGFR Target Codon						
			c719	Ex19del	c768	Ex20insASV	c790	c858	c861
BioRad CFX384	Positive:		⩽ 40	⩽ 40	ΔCq ⩽ 14.9	ΔCq ⩽ 15.0	⩽ 40	⩽ 40	⩽ 40
	Negative:		> 40	> 40	ΔCq > 14.9	ΔCq > 15.0	> 40	> 40	> 40
Roche LC480	Positive:		⩽ 40	⩽ 40	ΔCq ⩽ 14.6	ΔCq ⩽ 12.2	⩽ 40	⩽ 40	⩽ 40
	Negative:	Cq	> 40	> 40	ΔCq > 14.6	ΔCq > 12.2	> 40	> 40	> 40
ABI QuantStudio 5	Positive:		⩽ 40	⩽ 40	ΔCq ⩽ 17.0	ΔCq ⩽ 16.3	⩽ 40	⩽ 40	⩽ 40
	Negative:		> 40	> 40	ΔCq > 17.0	ΔCq > 16.3	> 40	> 40	> 40

Part 8. Assay Performance Characteristics

The performance characteristics of this product were established on the BioRadCFX384, ABI QuantStudio 5, Roche Light Cycler 480 real-time PCR instruments.

8.1. Analytical Performance

The specific performance characteristics of the QClamp® EGFR Mutations Detection Test were determined by studies involving EGFR-defined genomic DNA reference standards and FFPE standards of the cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England). These samples have been characterized genetically as containing heterozygous mutations in the coding sequence of the EGFR gene at exons 18, 19, 20 and 21. These standards were also spiked in the cfDNA extracted from a normal human plasma to determine the performance characteristics of the QClamp® EGFR Mutations Detection Test.

8.2. Analytical Sensitivity (LOD)

The specific performance characteristics of the QClamp® EGFR assay were determined by studies involving EGFR -defined genomic DNA reference standards and FFPE standards from cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England). These samples have been characterized genetically as containing heterozygous mutations in the coding sequence of the EGFR gene.

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 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 7.5, 5 and 2.5ng/well. Mutant allele concentrations tested were 1%, 0.5%, 0.25%, and 0.1% VAF. Results demonstrate effective clamping of wild type, providing reproducible detection of mutations at concentrations as low as 0.1% VAF for some assays run in multiple replicates.

DNA isolated from reference FFPE DNA with known mutational status, patient FFPE and plasma samples were used to determine the limit of detection of this assay. Serial dilutions of DNA extracted from FFPE reference materials with known mutations were made using wild-type DNA from samples to yield various percentages of mutant allele over wild-type background. LOD for FFPE and cfDNA is about 0.5% VAF (Table 11-13).

Based on the data from the tables below, recommended DNA input is 5-10ng/well to achieve 0.5% VAF sensitivity (LOD). Optimal DNA sample input is between 24 and 32 Cq of the Internal Control reaction.

Table 11. LOD Summary: Reference gDNA Samples (Bio Rad CFX384)

DNA input	Assay	1%	0.50%	0.25%	0.13%	WT
2.5 ng	c719	100%	100%	100%	0%	0%
	Ex19del	100%	100%	66%	66%	0%
	S768I	100%	100%	66%	33%	0%
	Ex20ins	100%	100%	100%	100%	0%
	T790M	100%	66%	33%	33%	0%
	L858R	100%	33%	66%	33%	0%
	c861	100%	66%	0%	0%	0%
5 ng	c719	100%	100%	100%	33%	0%
	Ex19del	100%	100%	100%	66%	0%
	S768I	100%	100%	100%	100%	0%
	Ex20ins	100%	100%	100%	100%	0%
	T790M	100%	100%	33%	0%	0%
	L858R	100%	100%	100%	66%	0%
	c861	100%	100%	0%	0%	0%
7.5 ng	c719	100%	100%	100%	0%	0%
	Ex19del	100%	100%	100%	66%	0%
	S768I	100%	100%	100%	100%	0%
	Ex20ins	100%	100%	100%	100%	0%
	T790M	100%	100%	100%	0%	0%
	L858R	100%	100%	100%	66%	0%
	c861	100%	100%	0%	33%	0%

Table 12. LOD Summary: Reference FFPE DNA Samples (Bio Rad CFX384)

DNA input	5 ng						
Assay target	c719	Ex19del	S768I	Ex20ins	T790M	L858R	c861
1% VAF	100%	100%	100%	100%	100%	100%	100%
0.50% VAF	100%	100%	100%	100%	100%	100%	100%
0.25% VAF	50%	100%	100%	100%	100%	50%	100%
0.13%VAF	50%	100%	100%	100%	100%	0%	100%
WT	0%	0%	0%	0%	0%	0%	0%

Table 13a. LOD Summary: Reference cfDNA Samples (Bio Rad CFX384)

DNA input	5 ng						
Assay target	c719	Ex19del	S768I	Ex20ins	T790M	L858R	c861
1% VAF	100%	100%	100%	100%	100%	100%	100%
0.50% VAF	100%	100%	100%	100%	100%	100%	100%
0.10% VAF	66%	66%	66%	33%	0%	100%	66%
WT	0%	0%	0%	0%	0%	0%	0%

Table 13b. LOD Summary: Reference cfDNA Samples (Roche LC480)

DNA input	**5 ng**						
Assay target	c719	Ex19del	S768I	Ex20ins	T790M	L858R	c861
1% VAF	100%	100%	100%	100%	100%	100%	100%
0.50%VAF	100%	100%	100%	100%	100%	100%	100%
0.10%VAF	0%	33%	33%	66%	33%	66%	0%
WT	0%	0%	0%	0%	0%	0%	0%

Table 13c. LOD Summary: Reference cfDNA Samples (ABI QuantStudio 5)

DNA input	**5 ng**						
Assay target	c719	Ex19del	S768I	Ex20ins	T790M	L858R	c861
1% VAF	100%	100%	100%	100%	100%	100%	100%
0.50%VAF	100%	100%	100%	100%	100%	100%	100%
0.10%VAF	0%	33%	33%	66%	33%	66%	0%
WT	0%	0%	0%	0%	0%	0%	0%

8.3. Multiple Freeze-Thaw Cycles

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

8.4. Shelf-Life

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

Part 9. Symbols Used in Packaging

Table 14. Symbols Used in Packaging

Symbol	Definition
REF	Catalog Number
	Manufactured By
	Temperature Limitation
LOT	Batch Code
	Expiration Date
EC REP	Authorized Representative in the European Community
2012-11-25	Date Format (year-month-day)
2012-11	Date Format (year-month)

References

1. Wlliam Pao et al. 2005. Adenocarcinomas to Gefitinib or Erlotinib is associates with a second mutation in the EGFR kinase domain. Plos Medicine 2(3):1-11.
2. Herbst RS, 2004. Review of epidermal growth factor receptor biology. Int. J. Radiat. Oncol. Biol. Phys. 59 (2 Suppl): 21–6.
3. Lynch TJ, Bell DW, Sordella R, et al, 2004. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. 350 (21): 2129–39.
4. Oda K, Matsuoka Y, Funahashi A, Kitano H, 2005. A comprehensive pathway map of epidermal growth factor receptor signaling. Mol. Syst. Biol. 1: 2005.0010.
5. Ørum, Henrik., PCR Clamping. Current. Issues Mol. Biol. 2000; 2(1), 27-30.
6. PaoW, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H, 2005. Acquired resistance of Lung
7. Powell et. al., Detection of the hereditary hemochromatosis gene mutation by real-time fluorescence polymerase chain reaction and peptide nucleic acid clamping. Analytical Biochemistry 1998; 260: 142–8.
8. Seth D, Shaw K, Jazayeri J and Leedman PJ, 1999. Complex post-transcriptional regulation of EGF-receptor expression by EGF and TGF-α in human prostate cancer cells. Br J Cancer 80(5-6):657-69.
9. Zhang H, Berezov A, Wang Q, Zhang G, Drebin J, Murali R, Greene MI, 2007. ErbB receptors: from oncogenes to targeted cancer therapies. J. Clin. Invest. 117 (8): 2051–8.
10. ****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.