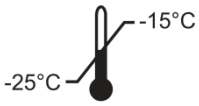
EntroGen
Automation • Reagents • Software

CE IVD **For In Vitro Diagnostic Use**



Store at -25ºC to -15ºC

REF **CTEGFR-48**

ctDNA EGFR Mutation Detection Kit

For Real-Time PCR

Kit for the detection of EGFR somatic mutations in circulating DNA in human plasma

For Use With:

- **Bio-Rad® CFX96**
- **QuantStudio™ 5**
- **Qiagen Rotor-Gene Q 5plex**

Instructions for use - version 2.6

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1. Intended Use

The ctDNA EGFR Mutation Analysis Kit is intended for the detection of EGFR exon 18, 19, 20, & 21 somatic mutations in cell-free, plasma derived, human genomic DNA (cf-DNA).

Warnings: The purchaser of this kit should follow the instructions in this manual. Any off-label use of this kit, and/or modification of the components will void EntroGen's support.

2. Reagents and Instruments

2.1 Package Contents

This product contains the following materials sufficient for 48 reactions. When testing at a capacity of 10 samples per plate (that is, a full plate, as illustrated in section 5.2), the provided reagents are sufficient for 40 samples, one positive control, and one no-template control, across all 7 reactions:

Name	No. of Tubes	Vol. per Tube (µl)	Cap
CT Mutation Detection Reaction Mix 1 (2X) For Reaction 1	1	800	
CT Mutation Detection Reaction Mix 2 (2X) For Reactions 2-7	4	1300	
Primer/Probe Mix	7*	158	
Positive Control Mix (PC)§	1	615	

* Each tube contains a distinct primer mix to amplify its designated mutations. The contents of these tubes should **not** be mixed.

§ The positive control mix contains a mixture of synthetic DNA sequences that correspond to each mutation detected by this kit and an internal control.

2.2 Handling and Storage

This product is shipped on frozen ice packs. The contents of the shipment should be stored at -25ºC to -15ºC non-frost-free freezer immediately upon receipt.

- Store all unopened components in original containers.
- Primer/probe mixes should be protected from light at all times to prevent photo bleaching of the fluorescent dyes.
- Centrifuge the tubes before opening.
- The expiration date of each component is printed on each tube label. This product will maintain its performance through this date. Its performance is not guaranteed after the expiration date.

A brief summary of the required storage and handling conditions necessary to ensure optimal stability for the kit:

CT Mutation Detection Reaction Mix 1 and 2 (2X): Store frozen between -25ºC to -15ºC. Thaw reaction mixes one vial at a time and store between 2ºC to 8ºC for up to 30 days. Do not freeze-thaw this reagent more than 3 times, as repeated freeze-thaw will adversely affect the efficiency of the enzyme. If a single vial of this reagent is expected to be used longer than 30 days, small volume aliquots are recommended after the first thaw.

Primer/Probe Mix: Store frozen between -25ºC to -15ºC. Once thawed, primer/probe mix will remain stable for up to 30 days when stored between 2ºC to 8ºC. If primer/probe mix is expected to be used longer than 30 days, small volume aliquots are recommended after first thaw for long term storage between -25ºC to -15ºC.

Positive Control Mix: Store between 2ºC to 8ºC once thawed.

2.3 Product Stability

This product and its components will maintain performance through the expiration date printed on the labels of each tube given that the storage and handling conditions described above are properly followed.

2.4 Quality Control

The components of this product are manufactured under GMP (Good Manufacturing Practice) standard. GMP requires extensive validation and documentation of manufacturing procedures to ensure highest production quality.

Each batch is tested on a Bio-Rad CFX96 or QuantStudio™ 5 instrument. Certificate of Analysis for each batch is available at www.entrogen.com/pi-download/.

2.5 Warnings and Precautions

PCR amplification is extremely sensitive to cross-over contamination. It is very important to carry out the pre-amplification steps (i.e. DNA isolation, PCR reaction preparation) in separate areas, ideally separate rooms with isolated air venting systems. It is especially important that the PCR reaction preparation is carried out in a room with positive air pressure (or laminar flow hood).

Additional precautions are necessary when handling DNA and PCR reagents to avoid contamination between samples/reagents:

- Wipe down the work area, pipettes, and equipment to be used near the work area with surface decontaminant (20% bleach or equivalent) to eliminate DNA and DNase followed by 70% ethanol.
- Vortex each tube before use.
- Spin down all tubes in picofuge before opening.
- Open each microcentrifuge tube carefully after vortexing/mixing and avoid touching the inside of the lid.
- Use aerosol-resistant (filtered) tips for all pipetting steps to avoid cross-contamination.
- Change pipette tips between all liquid transfers.
- Always wear gloves when handling DNA/reagents and change gloves between the pre-amplification and post-amplification steps.
- The flow of tubes, racks, pipettes and other equipment used should be from pre-amplification to post-amplification, and never backwards.

3. Required equipment & materials

3.1 Reagents

This kit does not contain reagents for protein digestion (Proteinase K) or the extraction of cell-free DNA. Cell-free DNA can be extracted using EntroGen's Cell-Free DNA Isolation Kit (Cat. No. CFDNA-50). See section 5.1 for more information regarding cfDNA isolation.

This assay requires the use of nuclease-free water (PCR grade).

3.2 Equipment

Equipment required to perform this assay are:

- Real-Time PCR instrument
  - Validated on:
    - QuantStudio™ 5
    - Bio-Rad CFX96
  - Compatible instruments require additional validation by user
- Disposable powder-free gloves
- Adjustable pipettes
- Sterile filtered pipette tips
- Vortex mixer
- Picofuge for 0.2 ml and 2.0 ml microcentrifuge tubes
- 96-well PCR plates
- Optical sealing film for PCR plates
- Centrifuge for PCR plates
- 0.2 ml (optional) and 2.0 ml microcentrifuge tubes
- Qubit reagents and instrument (or equivalent)
- 70% ethanol
- 20% chlorine bleach (or equivalent)

4. Product Description

4.1 Introduction

Epidermal growth factor receptor (EGFR) is a membrane protein that plays a central role in transmitting signals that promote cell growth and proliferation. EGFR tyrosine kinase (TK) domain activates several downstream effectors that lead to activation of the Ras-Raf-MAPK pathway [1]. Overexpression and oncogenic mutations that constitutively activate the TK domain of EGFR have been found in various solid tumors. Additionally, excessive activation of EGFR has been shown to be associated with advanced stages of cancer and a poor prognosis [2].

In a significant number of cases, tumor tissue is not available in sufficient amount for accurate molecular testing. Recent studies have demonstrated the utility of circulating tumor DNA (ctDNA) from plasma as an alternative source of genomic material for detection of sensitizing and resistance mutations in lung cancer [3].

4.2 About this assay

The ctDNA EGFR Mutation Analysis Kit is an ultra-sensitive assay to detect somatic mutations in exons 18, 19, 20, and 21 of EGFR in seven reactions. The assay works by amplifying mutant-specific sequences in samples that contain a mixture of mutant and wild-type DNA and relies on fluorescent probes for detection. The mutations detected by this kit are listed in Table A in the Appendix. A summary of mutations detected in each reaction is shown in the table below.

RXN	Mutation	Reporter
1	Exon 19 Deletions	CY5
	T790M	FAM
	L858R	ROX
2	C797S	FAM
3	L861Q	FAM
4	S768I	FAM
5	G719X	FAM
6	Ex20InsGGT/CAC	FAM
7	Ex20Ins9 (c.2300_2308dup)	FAM
All	Internal control	VIC

Additional information about the mutations detected:

- T790M with and without the presence of the neighboring SNP, (rs1050171 (c. G2361A, p. Q787Q), SNP Prevalence: 0.43 [4])
- G719X - detects G719A, G719S, G719D and G719C but does not distinguish between them
- Exon 20 Insertions - detects insCAC and insGGT but does not distinguish between them
- C797S – detects c.2389T>A and c.2390G>C, but does not distinguish between them

This kit contains primer mixtures for the detection of the aforementioned EGFR mutations, as well as an internal control gene. The internal control primers amplify beta-2 microglobulin (B2M) and are used to determine the condition of reagents and whether the reaction contains sufficient amount of amplifiable DNA.

4.3 Limitations of the Assay

- Successful detection of target mutations is dependent on sample quality and the amount of amplifiable DNA available. Samples that are overloaded or underloaded must be repeated.
- The performance of this assay has not been established in the presence of rare polymorphisms.
- This assay is intended for use with cell-free DNA only. It is unknown if other sample types are suitable for testing with this assay.

- Whole blood must be immediately processed for cell-free DNA isolation. Storage of blood prior to isolation will result in the formation of clots that will significantly reduce cell-free DNA yield.
- Reagent carry-over from isolation may interfere with amplification efficiency.
- This assay is intended to be used by laboratory personnel trained in molecular biology techniques.
- Adherence to this protocol is required for optimal results. Deviations from protocol may result in a loss of performance.
- Combination of reagents from different kit lots has not been evaluated. The performance characteristics of this modification have not been established.

5. Instructions for Use

5.1 DNA isolation

Several methods exist for DNA isolation. For consistency, we recommend using EntroGen's Cell-Free DNA Isolation Kit (Cat. No. CFDNA-50).

Follow the cf-DNA isolation procedure according to the protocol. This assay requires approximately 1.5-15 ng cf-DNA/reaction.

Streck Cell-Free DNA BCT Tube(s): Plasma from blood collected with Streck Cell-Free DNA BCT Tube(s) must go through a Proteinase K treatment prior to Cell-Free DNA isolation to ensure optimal yields. Forgoing Proteinase K treatment may decrease yields by 50%.

After DNA isolation, measure the concentration using fluorometric (e.g., Qubit) or qPCR analysis and dilute it to between 0.5 and 3 ng/µl if necessary. If the concentration is below 0.5 ng/µl, adjust the water volume during reaction setup. DNA extraction from plasma gives low yields. Typical yields are expected to be within 0.2 - 3 ng/µl. Greater yields may be obtained when extracting from higher volumes of plasma (>5 ml), but high concentrations can also be indicative of gDNA contamination.

Note: This DNA concentration is based on fluorometric analysis of isolated cf-DNA with Qubit Fluorometer. Some cf-DNA isolation kits use carrier RNA to enhance the binding of nucleic acids to the silica membrane. Carrier RNA may contribute to the UV absorbance readings on spectrophotometers such as Nanodrop. Therefore, each lab should evaluate optimal DNA concentration for the assay. For more information, refer to the isolation kit product manual.

5.2 Reagent preparation (for all instruments)

- Thaw the primer/probe mix tubes, positive control mixes, and 2X mutation detection reaction mixes, on ice.
    - Each primer/probe mix tube in the kit contains enough reagents to perform the test on 48 samples (including controls).
- Vortex and spin the tubes for about 5 seconds in picofuge at room temperature.

Note: It is recommended to keep reagents, controls, and samples on ice.

The PCR reactions are setup in a total volume of 30 µl/sample. Reaction mixes for multiple samples (as well as control samples) should be pre-mixed as a master mix with 5% excess volume to compensate for pipetting errors. 5 µl of the positive control should be tested with each reaction on every plate.

The following reagents go into each 30 µl reaction:

Components	Volume (µl)
CT Mutation Detection Reaction Mix 1 (2X) OR CT Mutation Detection Reaction Mix 2 (2X)	15
Primer Mix	3
cf-DNA sample (1.5-15 ng)	5*
Nuclease-free water	7**

*Sample volume may range between 1 µl and 12 µl.

**Water volume should be adjusted according to the sample volume to complete a 30 µl reaction.

1. Prepare the following master mixes using the formulas below to calculate the appropriate volumes (with 5% overage). Prepare a separate master mix for the positive controls and NTC if your sample volume is not 5 µl. The water volume should be 7 µl for the positive control and NTC master mix, but should be adjusted to complete a 30 µl reaction if your sample volume is not 5 µl.

   For Reaction 1:

Reagents	Formula*
CT Mutation Detection Reaction Mix 1 (2X)	N x 15 µl x 1.05
Reaction 1 Primer Mix	N x 3 µl x 1.05
Nuclease-free water	N x 7 µl* x 1.05

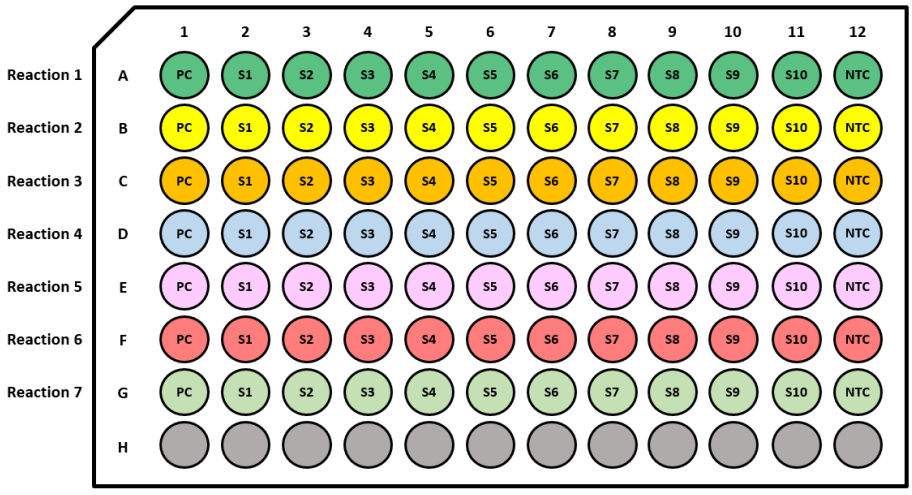
   N = number of samples to be run (including controls).
   * Water volume in this formula is calculated based on a 5 µl sample input per reaction.

   For Reactions 2-7:

Reagents	Formula*
CT Mutation Detection Reaction Mix 2 (2X)	N x 15 µl x 1.05
Reactions 2-7 Primer Mixes	N x 3 µl x 1.05
Nuclease-free water	N x 7 µl* x 1.05

   N = number of samples to be run (including controls).
   *Water volume in this formula is calculated based on a 5 µl sample input per reaction.

2. Mix by gentle vortexing and centrifuge for 5 seconds in picofuge at room temperature.
3. Dispense 25 µl of the master mix per well. Adjust the master mix volume if sample volume is not 5 µl.
4. Dispense 25 µl of positive control and NTC master mix per control well.
5. Add 1-12 µl of cf-DNA sample to each corresponding well and mix by pipetting up and down several times
6. Add 5 µl of the positive control and water (NTC) to the corresponding control wells and mix by pipetting up and down several times.
7. Seal the plate with optical sealing film (for plates) or caps (for tubes).
8. Briefly centrifuge the plate to collect reaction at the bottom of the wells and to ensure that there are no bubbles in the bottom of the wells.



Note: Seven reactions are setup for each sample. A single 96-well PCR plate can accommodate up to 10 unknown samples, one positive control mix, and one no template control mix. The recommended plate layout is shown above.

5.3 Instrument Setup

Bio-Rad CFX96 (CFX Manager Software version 3.1 and above)

1. In the Startup Wizard select **Create New Experiment** and make sure **CFX96** is selected in the drop-down list. Click OK.
2. In the **Protocol** tab of the Experiment Setup window select **Create New** and enter the following cycling parameters:

Step	Temperature	Time
1	95ºC	10min
2	95ºC	15sec
3	58ºC	30sec
	+ Plate Read	
4	GOTO 2 39 more times	
	END	

3. When finished, click OK to save the protocol template. Note the location of the saved template file to use for subsequent runs. Click **Next** to go to the setup window.
4. Change the Sample Volume to 30 µl.
5. Set the Fluorophores to All Channels.
6. Click **Next**, then click **Start Run** to begin the run.

ABI QuantStudio™ 5 (Software version 2.0 and above)

1. In the QuantStudio Design and Analysis software, select create new experiment.
2. In the **Properties** tab:
   a. Select **"96-Well 0.2 mL block"** under Block
   b. Select **"Comparative Ct (ΔΔCt)"** under Experiment type
   c. Select **"TaqMan® Reagents"** under Chemistry
   d. Select **"Fast"** under Run mode
3. In the **Method** tab:
   a. Change the volume to 30 µl
   b. Change the cooling ramp rate to **2.38ºC/sec** and all other ramp rates to **3ºC/sec** as shown below
   c. Enter the following cycling parameters:

Step	Temperature	Time
Hold stage		
	Ramp rate 3ºC/sec	
1	95ºC	10min
PCR stage		
	Ramp rate 3ºC/sec	
1	95ºC	15sec
	Ramp rate 2.38ºC/sec	
2	58ºC	30sec
	+ Plate Read	
Cycle PCR stage 40x		
END		

4. In the **Plate** tab:
    a. Set passive reference to "**None**".
    b. Click the **Advanced Setup** tab and add the following targets under the Targets field.

Target Name	Reporter	Quencher
RXN 1 Ex19Del	CY5	NFQ-MGB
RXN 1 T790M	FAM	NFQ-MGB
RXN 1 L858R	ROX	NFQ-MGB
RXN 1 IC	VIC	NFQ-MGB
RXN 2 C797S	FAM	NFQ-MGB
RXN 2 IC	VIC	NFQ-MGB
RXN 3 L861Q	FAM	NFQ-MGB
RXN 3 IC	VIC	NFQ-MGB
RXN 4 S768I	FAM	NFQ-MGB
RXN 4 IC	VIC	NFQ-MGB
RXN 5 G719X	FAM	NFQ-MGB
RXN 5 IC	VIC	NFQ-MGB
RXN 6 Ex20InsGGT/CAC	FAM	NFQ-MGB
RXN 6 IC	VIC	NFQ-MGB
RXN 7 Ex20Ins9	FAM	NFQ-MGB
RXN 7 IC	VIC	NFQ-MGB

5. Under the Samples field, assign sample IDs to the desired wells.
6. Click Save to save the run file.
7. Open the run file on the QuantStudio™ 5 instrument.
8. Make sure the reaction volume is set to 30 µl and Data Collection is turned on at the 58°C step.
9. Load the plate onto the instrument and click START RUN.

Rotor-Gene Q (5plex) (Software version 2.1.0 and above)

1. Select File>New, click on the Advanced tab in the dialog box, select Two Step and click New.
2. Select 72-well rotor, check the "Locking Ring Attached" box, click Next, then skip wizard.
3. Go to View>Run Settings, enter 30 µl for Reaction volume and click OK.
4. Go to View>Profile Editor.
5. Click on Hold and enter 95ºC for Hold Temperature and 10 minutes for Hold Time.
6. Click on Cycling, enter "This cycle repeats" 40 time(s), then enter the following Timed Step parameters by clicking on each step in the panel on the right:

Step	Temperature (°C)	Time	Data Acquisition
Hold	95ºC	10 min	Not Acquiring
Timed Step	95ºC	15 Sec	Not Acquiring
Timed Step	58ºC	30 Sec	Acquiring to Cycling A on Green, Yellow, Red, Orange

7. Click OK.

Optional: Save these run settings for future use by selecting the **Save As** button at the top of the dialog box.

8. Go to **Gain**, enter the following settings:

Note: The suggested gain settings may need to be adjusted due to instrument-to-instrument variability.

Detector	Gain
Green	6.67
Yellow	10
Orange	5
Red	5

9. Load the strip tubes into the rotor.
10. Go to Run>Start Run, then click on the Start button to begin the run.

Note: The Edit Samples dialog box opens once the run begins. Input sample information while the instrument is running or after the run has completed.

6. Data Analysis

Important: Threshold settings may require adjustment based on the fluorescence detection sensitivity of each thermocycler. Use the settings listed below as a starting point.

6.1 Instrument Specific Analysis Steps

Bio-Rad CFX96 (CFX Manager Software version 3.1 and above)
Analyze the data in the real-time PCR instrument software using the following criteria:

- Ct Determination Mode: Single Threshold
- Baseline Setting: Baseline Subtracted Curve Fit
- Baseline and Threshold: User Defined according to the table below:

Target	RXN	Baseline	Threshold
HEX (VIC)	1, 2	3-22	700
	3, 4, 6, 7	3-22	450
	5	3-22	400
FAM	1	3-22	400
	2-7	3-22	300
ROX	1	3-22	400
CY5	1	3-22	300

ABI QuantStudio™ 5 (Software version 2.0 and above)
Analyze the data in the real-time PCR instrument software using the following criteria:

- Baseline and Threshold: User Defined according to the table below:

Target	RXN	Baseline	Threshold
HEX (VIC)	1, 2	3-22	55,000
	3, 4, 6, 7	3-22	27,500
	5	3-22	20,000
FAM	1	3-22	65,000
	2-7	3-22	40,000
ROX	1	3-22	35,000
CY5	1	3-22	30,000

Rotor-Gene Q (5plex) (Software version 2.1.0 and above)
Analyze the data in the real-time PCR instrument software using the following criteria:

- Dynamic Tube: On
- Slope Correct: On
- Outlier removal: 5%

Set the Thresholds as follows:

VIC -Yellow	FAM - Green	CY5 - Red	ROX – Orange
0.1	0.15	0.05	0.05

6.2 Results Interpretation

Bio-Rad CFX96 (CFX Manager Software version 3.1 and above)

1. Select the positive control wells and check detector for a signal. PC should have similar VIC Ct values for each reaction.
2. Select the negative control wells and confirm there is either no signal or the Ct value is greater than 38.5 for each detector.
3. Select the reaction wells of each unknown sample and check the signal for each detector. Use the Ct table below to interpret results.

Target Ct is:	VIC (HEX) Ct is:	Result
Ct = any	Ct <28	Overloaded. Rerun with less DNA.
38.5< Ct; Ct=N/A	28≤ Ct ≤ 31.5	Negative sample. No need to rerun.
Ct ≤38.5	28≤ Ct ≤ 31.5	Positive for target channel: Ex19Del (Cy5), L858R (ROX), all others (FAM).
Ct = any	Ct >31.5; Ct=N/A	Underloaded. Rerun with more DNA.

ABI QuantStudio™ 5 (Software version 2.0 and above)

1. Select the positive control wells and check detector for a signal. PC should have similar VIC Ct values for each reaction.
2. Select the negative control wells and confirm there is either no signal or the Ct value is greater than 38.5 for each detector.
3. Select the reaction wells of each unknown sample and check the signal for each detector. Use the Ct table below to interpret results.

Target Ct is:	VIC (HEX) Ct is:	Result
Ct = any	Ct <28	Overloaded. Rerun with less DNA.
38.5< Ct; Ct=Undetermined	28≤ Ct ≤ 31.5	Negative sample. No need to rerun.
Ct ≤38.5	28≤ Ct ≤ 31.5	Positive for target channel: Ex19Del (Cy5), L858R (ROX), all others (FAM).
Ct = any	Ct >31.5; Ct= Undetermined	Underloaded. Rerun with more DNA.

Rotor-Gene Q (5plex) (Software version 2.1.0 and above)

1. Select the positive control wells and check detector for a signal. PC should have similar VIC Ct values for each reaction.
2. Select the negative control wells and confirm there is either no signal or the Ct value is greater than 38.5 for each detector.
3. Select the reaction wells of each unknown sample and check the signal for each detector. Use the Ct table below to interpret results.

Target Ct is:	VIC (Yellow) Ct is:	Result
Ct = any	Ct <23.5	Overloaded. Rerun with less DNA.
38.5< Ct; Ct=0	23.5 ≤ Ct ≤ 27	Negative sample. No need to rerun.
Ct ≤38.5	23.5 ≤ Ct ≤ 27	Positive for target channel: Ex19Del (Cy5-Red), L858R (ROX-Orange), all others (FAM-Green).
Ct = any	Ct >27; Ct=0	Underloaded. Rerun with more DNA.

7. Performance Validation

7.1 Precision

The assay's reproducibility was determined by analyzing synthetic DNA representing the target mutations, at an allelic frequency of 2X LoD in the background of wildtype DNA at the minimum input of the assay (at least 20 replicates per target mutation). Samples were tested across three reagent lots, by two different operators, on non-consecutive days. This study was performed on Bio-Rad CFX96 and ABI QuantStudio™ 5.

Bio-Rad CFX96 (CFX Manager Software version 3.1):

Reaction	Mutation	Mutant Copies	No. of Repeats	Average VIC Ct	SD	Average Target Ct	SD
RXN 1	T790M (with Q787Q SNP)	25	27	31.22	0.51	35.69	0.69
	T790M	50	22	31.28	0.41	34.10	0.35
	Exon 19del	25	20	30.99	0.17	34.41	0.36
	L858R	50	28	31.29	0.38	35.15	0.50
RXN 2	C797S T>A	50	30	31.25	0.24	35.19	0.59
	C797S G>C	50	22	31.11	0.22	34.20	0.54
RXN 3	L861Q	25	22	31.00	0.28	35.08	0.32
RXN 4	S768I	100	20	31.55	0.50	34.93	0.65
RXN 5	G719A	50	23	30.84	0.30	34.24	0.35
	G719C	50	28	30.91	0.28	35.07	0.65
	G719D	100	23	30.89	0.30	34.09	0.33
	G719S	100	24	30.98	0.34	34.03	0.31
RXN 6	Exon 20 ins GGT	50	24	31.36	0.44	34.19	0.36
	Exon 20 ins CAC	25	26	31.39	0.51	35.52	0.54
RXN 7	Exon 20 ins 9	50	20	31.27	0.41	36.10	0.77

ABI QuantStudio™ 5 (Software version 2.0):

Reaction	Mutation	Mutant Copies	No. of Repeats	Average VIC Ct	SD	Average Target Ct	SD
RXN 1	T790M (with Q787Q SNP)	25	20	30.91	0.18	34.92	0.60
	T790M	50	20	30.95	0.16	33.39	0.38
	Exon 19del	25	20	30.98	0.19	33.56	0.51
	L858R	50	20	31.18	0.40	33.59	0.59
RXN 2	C797S T>A	50	20	31.26	0.23	33.73	0.66
	C797S G>C	50	20	31.19	0.17	33.30	0.52
RXN 3	L861Q	25	20	31.24	0.31	35.06	0.67
RXN 4	S768I	150	22	31.47	0.26	35.01	0.44
RXN 5	G719A	50	22	31.12	0.24	33.51	0.43
	G719C	100	22	31.12	0.27	33.74	0.51
	G719D	100	22	31.16	0.27	34.83	0.85
	G719S	100	22	31.38	0.32	34.65	0.37
RXN 6	Exon 20 ins GGT	50	20	31.55	0.28	33.66	0.47
	Exon 20 ins CAC	50	20	31.49	0.24	33.63	0.51
RXN 7	Exon 20 ins 9	50	20	31.67	0.33	35.45	0.63

7.2 Limit of Detection

Limit of detection was assessed using synthetic mutant controls at a low copy number in the background of either WT cfDNA or fragmented DNA (to resemble the fragmentation pattern of cfDNA), at the assay's minimum and maximum inputs (corresponding to a VIC Ct of 31.5 and 28, respectively). At least 4 sample preparations were tested across three reagent lots. At least 21 replicates were tested per target mutation at each DNA input. This study was performed on Bio-Rad CFX96 and ABI QuantStudio™ 5.

Bio-Rad CFX96 (CFX Manager Software version 3.1):

Reaction	Mutation	Minimum input (31.5 IC Ct)					Maximum input (28 IC Ct)				
		Mutant Copies	Average VIC Ct	SD	Average Target Ct	SD	Mutant Copies	Average VIC Ct	SD	Average Target Ct	SD
RXN 1	T790M (with Q787Q SNP)	**10**	31.82	0.27	36.88	0.72	**25**	28.04	0.26	36.42	0.91
	T790M	**25**	31.56	0.19	35.08	0.47	**25**	28.10	0.24	36.05	0.86
	Exon 19 del	**10***	31.45	0.17	36.76	0.99	**25***	28.10	0.20	35.66	1.04
	L858R	**25**	31.23	0.21	36.32	0.44	**25**	28.05	0.29	37.31	0.65
RXN 2	C797S T>A	**25**	31.73	0.18	36.82	0.56	**50**	28.39	0.17	37.46	0.52
	C797S G>C	**25**	31.85	0.27	36.20	0.67	**25**	28.28	0.29	37.08	0.68
RXN 3	L861Q	**10**	31.40	0.18	36.60	0.75	**10**	27.90	0.25	36.78	0.48
RXN 4	S768I	**50**	31.59	0.15	37.12	0.47	**50**	27.97	0.26	36.82	0.37
RXN 5	G719A	**25**	31.50	0.31	35.24	0.65	**50**	27.87	0.24	36.08	1.21
	G719C	**25**	31.51	0.26	36.39	0.52	**100**	27.90	0.21	36.18	1.14
	G719D	**50**	31.58	0.29	35.89	0.44	**200**	27.92	0.19	35.06	0.84
	G719S	**50**	31.29	0.23	36.83	0.97	**100**	27.91	0.28	35.37	0.78
RXN 6	Exon 20 ins GGT	**25**	31.73	0.25	35.74	0.44	**25**	27.88	0.31	35.92	0.90
	Exon 20 ins CAC	**10**	31.54	0.25	36.79	0.82	**25**	27.90	0.30	35.45	0.75
RXN 7	Exon 20 ins 9	**75**	31.26	0.18	37.14	0.36	**75**	28.25	0.10	37.38	0.33

** The limit of detection (LOD) for EGFR exon 19 deletion variant c.2252_2276delinsA (p.T751_I759delinsN) is 75 copies per reaction under the specified assay conditions*

ABI QuantStudio™ 5 (Software version 2.0):

Reaction	Mutation	Minimum input (31.5 IC Ct)					Maximum input (28 IC Ct)				
		Mutant Copies	Average VIC Ct	SD	Average Target Ct	SD	Mutant Copies	Average VIC Ct	SD	Average Target Ct	SD
RXN 1	T790M (with Q787Q SNP)	**10**	31.84	0.29	37.48	0.60	**25**	28.08	0.15	35.99	1.06
	T790M	**25**	31.84	0.24	35.77	0.89	**25**	28.09	0.18	34.97	0.70
	Exon 19 del	**10***	31.52	0.15	36.70	0.80	**10***	28.05	0.19	35.73	0.81
	L858R	**25**	31.49	0.41	35.72	1.12	**25**	28.23	0.37	35.24	0.99
RXN 2	C797S T>A	**25**	31.55	0.44	36.31	1.00	**50**	28.42	0.29	34.90	0.95
	C797S G>C	**25**	31.72	0.16	34.90	0.62	**25**	28.30	0.42	35.30	0.79
RXN 3	L861Q	**10**	31.72	0.23	36.90	0.71	**10**	28.07	0.36	36.56	0.65
RXN 4	S768I	**75**	31.96	0.33	37.29	0.73	**75**	28.22	0.39	36.87	0.65
RXN 5	G719A	**25**	32.75	1.75	34.55	0.75	**100**	28.01	0.31	34.38	1.57
	G719C	**50**	31.72	0.38	35.53	0.66	**350**	28.14	0.14	33.44	1.31
	G719D	**50**	31.75	0.34	36.25	0.83	**300**	28.10	0.27	35.40	1.11
	G719S	**50**	31.81	0.38	36.36	0.56	**200**	28.19	0.42	35.94	1.10
RXN 6	Exon 20 ins GGT	**25**	31.56	0.59	34.88	0.85	**25**	28.40	0.40	35.36	0.60
	Exon 20 ins CAC	**25**	31.88	0.20	35.69	0.59	**25**	28.31	0.30	35.13	0.52
RXN 7	Exon 20 ins 9	**50**	31.53	0.24	36.90	0.59	**75**	28.52	0.20	35.74	0.53

** The limit of detection (LOD) for EGFR exon 19 deletion variant c.2252_2276delinsA (p.T751_I759delinsN) is 75 copies per reaction under the specified assay conditions*

7.3 Accuracy

Accuracy was assessed with cfDNA or cell-line standards with at least one representative mutation for each reaction, in duplicate. Samples were diluted to target approximately 2X LoD* at a total DNA input based on VIC Ct of ~30. This study was performed on Bio-Rad CFX96 (CFX Manager Software version 3.1). Results were reproducible on the ABI QuantStudio™ 5.

		Reported Target Mutation (by reaction)								
		Rxn 1	**Rxn 2**	**Rxn 3**	**Rxn 4**	**Rxn 5**	**Rxn 6**	**Rxn 7***	**Negative**	**Total**
CTEGFR Results	**Positive**	9	3	2	2	2	1	1		**20**
	Negative							1	1	**2**
	Total	**9**	**3**	**2**	**2**	**2**	**1**	**2**	**1**	**22**

* Reaction 7 was tested below the assay's LoD due to the mutation's low allelic frequency in the tested samples.

All mutations within the assay's limit of detection were correctly called.

7.4 Cross-Reactivity

Cross reactivity of each of the seven reactions was examined using synthetic mutant controls representing the 15 target mutations, at 50% mutant allele frequency at the maximum input (VIC Ct of ~28), in duplicate. This study was performed on Bio-Rad CFX96 and ABI QuantStudio™ 5. *Note:* Blank cells show no cross reactivity. The assay's target mutations are highlighted in green and produce an earlier signal than any off-target amplification.

Bio-Rad CFX96 (CFX Manager Software version 3.1):

		Average Target Ct for Each RXN						
	Mutation	1	2	3	4	5	6	7
RXN 1	T790M (with Q787Q SNP)	26.47	-	-	-	-	-	-
	T790M	26.36	-	-	-	-	-	-
	Exon 19del	26.34	-	-	-	-	-	-
	L858R	26.93	-	-	-	-	-	-
RXN 2	C797S T>A	39.08	26.76	-	-	-	-	-
	C797S G>C	39.05	26.63	-	-	-	-	-
RXN 3	L861Q	-	-	26.46	-	-	-	-
RXN 4	S768I	-	-	-	28.30	-	-	-
RXN 5	G719A	-	-	-	-	26.10	-	-
	G719C	-	-	-	-	26.41	-	-
	G719D	-	-	-	-	27.17	-	-
	G719S	-	-	-	-	27.73	-	-
RXN 6	Exon 20 ins GGT	-	-	-	-	-	27.07	39.00
	Exon 20 ins CAC	-	-	-	-	-	27.06	-
RXN 7	Exon 20 ins 9	-	-	-	-	-	-	30.55

ABI QuantStudio™ 5 (Software version 2.0):

		Average Target Ct for each RXN						
	Mutation	1	2	3	4	5	6	7
RXN 1	T790M (with Q787Q SNP)	26.35	-	-	-	-	-	-
	T790M	26.01	-	-	-	-	-	-
	Exon 19del	25.99	-	-	-	-	-	-
	L858R	26.70	-	-	-	-	-	-
RXN 2	C797S T>A	37.67	26.63	-	-	-	-	-
	C797S G>C	37.63	25.94	-	-	-	-	-
RXN 3	L861Q	39.78	-	26.32	-	-	-	-
RXN 4	S768I	39.76	-	-	29.07	-	-	-
RXN 5	G719A	39.72	-	-	-	25.92	-	-
	G719C	39.81	-	-	-	26.77	-	-
	G719D	39.83	-	-	-	26.96	-	-
	G719S	-	-	-	-	28.00	-	-
RXN 6	Exon 20 ins GGT	39.79	-	-	-	-	26.82	39.95
	Exon 20 ins CAC	-	-	-	-	-	26.93	-
RXN 7	Exon 20 ins 9	-	-	-	-	-	-	30.47

7.5 Limit of Blank

Limit of Blank was evaluated using at least five unique WT cfDNA or fragmented gDNA samples as well as intact WT gDNA. Each sample was tested at four different inputs, ranging from half the minimum to twice the maximum assay's input (i.e., VIC Ct of approximately 32.5, 31.5, 28 and 27). In addition, molecular grade water was also tested to demonstrate no non-specific background amplification. Each sample type was tested in at least 15 replicates across three reagent lots, at least 60 total replicates across the four inputs. No non-specific amplification was detected with WT samples or water. This study was performed on Bio-Rad CFX96 and ABI QuantStudio™ 5.

8. Appendix

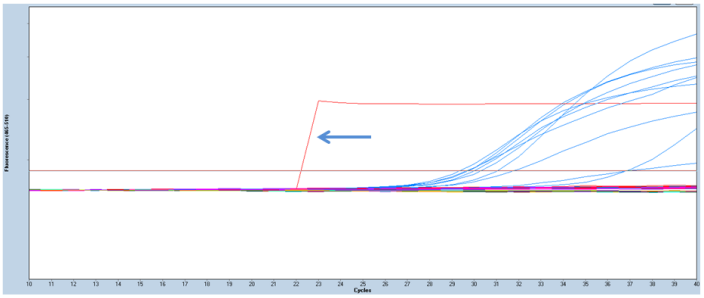
A. Mutations detected by this kit.
Note: These notations were last updated in September 2020 based on COSMIC v92

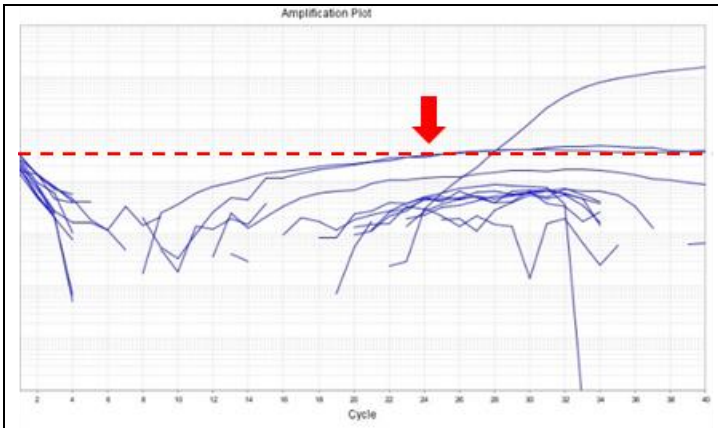
Nucleotide change	Amino acid change	Cosmic ID	HGVS nt	HGVS prot
Exon 18				
c.2155G>A	p.G719S	6252	LRG_304t1:c.2155G>A	LRG_304p1:p.(Gly719Ser)
c.2155G>T	p.G719C	6253	LRG_304t1:c.2155G>T	LRG_304p1:p.(Gly719Cys)
c.2156G>A	p.G719D	18425	LRG_304t1:c.2156G>A	LRG_304p1:p.(Gly719Asp)
c.2156G>C	p.G719A	6239	LRG_304t1:c.2156G>C	LRG_304p1:p.(Gly719Ala)
Exon 19				
c.2233_2247del15	p.K745_E749delKELRE	26038	LRG_304t1:c.2233_2247del15	LRG_304p1:p.(Lys745_Glu749del)
c.2234_2248del15	p.K745_A750delinsT	1190791	LRG_304t1:c.2234_2248del15	LRG_304p1:p.(Lys745_Ala750delinsThr)
c.2235_2246del12	p.E746_E749delELRE	28517	LRG_304t1:c.2235_2246del12	LRG_304p1:p.(Glu746_Glu749del)
c.2235_2248delinsAATTC	p.E746_A750delinsIP	13550	LRG_304t1:c.2235_2248delinsAATTC	LRG_304p1:p.(Glu746_Ala750delinsIlePro)
c.2235_2249del15	p.E746_A750delELREA	6223	LRG_304t1:c.2235_2249del15	LRG_304p1:p.(Glu746_Ala750del)
c.2235_2251delinsAATTC	p.E746_T751delinsIP	13552	LRG_304t1:c.2235_2251delinsAATTC	LRG_304p1:p.(Glu746_Thr751delinsIlePro)
c.2235_2252delinsAAT	p.E746_T751delinsI	13551	LRG_304t1:c.2235_2252delinsAAT	LRG_304p1:p.(Glu746_Thr751delinsIle)
c.2235_2255delinsAAT	p.E746_S752delinsI	12385	LRG_304t1:c.2235_2255delinsAAT	LRG_304p1:p.(Glu746_Ser752delinsIle)
c.2236_2248delinsAGAC	p.E746_A750delinsRP	12413	LRG_304t1:c.2236_2248delinsAGAC	LRG_304p1:p.(Glu746_Ala750delinsArgPro)
c.2236_2248delinsCAAC	p.E746_A750delinsQP	13557	LRG_304t1:c.2236_2248delinsCAAC	LRG_304p1:p.(Glu746_Ala750delinsGlnPro)
c.2236_2250del15	p.E746_A750delELREA	6225	LRG_304t1:c.2236_2250del15	LRG_304p1:p.(Glu746_Ala750del)
c.2236_2253del18	p.E746_T751delELREAT	12728	LRG_304t1:c.2236_2253del18	LRG_304p1:p.(Glu746_Thr751del)
c.2236_2256del21	p.E746_S752delELREATS	133189	LRG_304t1:c.2236_2256del21	LRG_304p1:p.(Glu746_Ser752del)
c.2237_2251delinsTTC	p.E746_T751delinsVP	18421	LRG_304t1:c.2237_2251delinsTTC	LRG_304p1:p.(Glu746_Thr751delinsValPro)
c.2237_2251del15	p.E746_T751delinsA	12678	LRG_304t1:c.2237_2251del15	LRG_304p1:p.(Glu746_Thr751delinsAla)
c.2237_2252delinsT	p.E746_T751delinsV	12386	LRG_304t1:c.2237_2252delinsT	LRG_304p1:p.(Glu746_Thr751delinsVal)
c.2237_2253delinsTC	p.E746_T751delinsV	133193	LRG_304t1:c.2237_2253delinsTC	LRG_304p1:p.(Glu746_Thr751delinsVal)
c.2237_2253delinsTTCCT	p.E746_T751delinsVP	52935	LRG_304t1:c.2237_2253delinsTTCCT	LRG_304p1:p.(Glu746_Thr751delinsValPro)

Nucleotide change	Amino acid change	Cosmic ID	HGVS nt	HGVS prot
c.2237_2253delinsTTGCT	p.E746_T751delinsVA	12416	LRG_304t1:c.2237_2253delinsTTGCT	LRG_304p1:p.(Glu746_Thr751delinsValAla)
c.2237_2254del18	p.E746_S752delinsA	12367	LRG_304t1:c.2237_2254del18	LRG_304p1:p.(Glu746_Ser752delinsAla)
c.2237_2255delinsT	p.E746_S752delinsV	12384	LRG_304t1:c.2237_2255delinsT	LRG_304p1:p.(Glu746_Ser752delinsVal)
c.2237_2256delinsTC	p.E746_S752delinsV	18426	LRG_304t1:c.2237_2256delinsTC	LRG_304p1:p.(Glu746_Ser752delinsVal)
c.2237_2257delinsTCT	p.E746_P753delinsVS	18427	LRG_304t1:c.2237_2257delinsTCT	LRG_304p1:p.(Glu746_Pro753delinsValSer)
c.2238_2248delinsGC	p.L747_A750delinsP	12422	LRG_304t1:c.2238_2248delinsGC	LRG_304p1:p.(Leu747_Ala750delinsPro)
c.2238_2252delinsGCA	p.L747_T751delinsQ	12419	LRG_304t1:c.2238_2252delinsGCA	LRG_304p1:p.(Leu747_Thr751delinsGln)
c.2238_2255del18	p.E746_S752delinsD	6220	LRG_304t1:c.2238_2255del18	LRG_304p1:p.(Glu746_Ser752delinsAsp)
c.2239_2247delTTAAGAGAA	p.L747_E749delLRE	6218	LRG_304t1:c.2239_2247delTTAAGAGAA	LRG_304p1:p.(Leu747_Glu749del)
c.2239_2248TTAAGAGAAG>C	p.L747_A750delinsP	12382	LRG_304t1:c.2239_2248delinsC	LRG_304p1:p.(Leu747_Ala750delinsPro)
c.2239_2251delinsC	p.L747_T751delinsP	12383	LRG_304t1:c.2239_2251delinsC	LRG_304p1:p.(Leu747_Thr751delinsPro)
c.2239_2252delinsCA	p.L747_T751delinsQ	12420	LRG_304t1:c.2239_2252delinsCA	LRG_304p1:p.(Leu747_Thr751delinsGln)
c.2239_2256delinsCAA	p.L747_S752delinsQ	12403	LRG_304t1:c.2239_2256delinsCAA	LRG_304p1:p.(Leu747_Ser752delinsGln)
c.2239_2256del18	p.L747_S752delLREATS	6255	LRG_304t1:c.2239_2256del18	LRG_304p1:p.(Leu747_Ser752del)
c.2239_2258delinsCA	p.L747_P753delinsQ	12387	LRG_304t1:c.2239_2258delinsCA	LRG_304p1:p.(Leu747_Pro753delinsGln)
c.2239_2262del24	p.L747_K754delLREATSPK	24970	LRG_304t1:c.2239_2262del24	LRG_304p1:p.(Leu747_Lys754del)
c.2240_2251del12	p.L747_T751delinsS	6210	LRG_304t1:c.2240_2251del12	LRG_304p1:p.(Leu747_Thr751delinsSer)
c.2240_2254del15	p.L747_T751delLREAT	12369	LRG_304t1:c.2240_2254del15	LRG_304p1:p.(Leu747_Thr751del)
c.2240_2257del18	p.L747_P753delinsS	12370	LRG_304t1:c.2240_2257del18	LRG_304p1:p.(Leu747_Pro753delinsSer)
c.2248_2273delinsCC	p.A750_E758delinsP	26440	LRG_304t1:c.2248_2273delinsCC	LRG_304p1:p.(Ala750_Glu758delinsPro)
c.2250_2264del15	p.T751_A755del	26718	LRG_304t1:c.2250_2264del15	LRG_304p1:p.(Thr751_Ala755del)
c.2252_2275del24	p.T751_E758delTSPKANKE	23634	LRG_304t1:c.2252_2275del24	LRG_304p1:p.(Thr751_Glu758del)
c.2252_2276delinsA	p.T751_I759delinsN	96856	LRG_304t1:c.2252_2276delinsA	LRG_304p1:p.(Thr751_Ile759delinsAsn)
c.2252_2277delinsAT	p.T751_I759delinsN	24270	LRG_304t1:c.2252_2277delinsAT	LRG_304p1:p.(Thr751_Ile759delinsAsn)

Exon 20				
c.2369C>T	p.T790M	6240	LRG_304t1:c.2369C>T	LRG_304p1:p.(Thr790Met)
c.2303G>T	p.S768I	6241	LRG_304t1:c.2303G>T	LRG_304p1:p.(Ser768Ile)
c.2300_2308dup	p.A767_V769dup	12376	LRG_304t1:c.2300_2308dupCCAGCGTGG	LRG_304p1:p.(Ala767_Val769dup)
c.2310_2311insGGT	p.D770_N771insG	12378	LRG_304t1:c.2310_2311insGGT	LRG_304p1:p.(Asp770_Asn771insGly)
c.2317_2319insCAC	p.H773_V774insH	12377	LRG_304t1:c.2317_2319dupCAC	LRG_304p1:p.(His773dup)
c.2361G>A	p.Q787Q	1451600	LRG_304t1:c.2361G>A	LRG_304(p1):p.(=)
c.2389T>A	p.C797S	6493937	NM_005228.5:c.2389T>A	NM_005228.5(NP_005219.2):p.(Cys797Ser)
c.2390G>C	p.C797S	5945664	NM_005228.5:c.2390G>C	NM_005228.5(NP_005219.2):p.(Cys797Ser)
Exon 21				
c.2573T>G	p.L858R	6224	LRG_304t1:c.2573T>G	LRG_304p1:p.(Leu858Arg)
c.2582T>A	p.L861Q	6213	LRG_304t1:c.2582T>A	LRG_304p1:p.(Leu861Gln)

B. Troubleshooting

Problem:	Solution:
Internal control (VIC) signal for a sample has a Ct value greater than the upper Ct cut-off specified in section 6.	• If internal control signal appears after the cut-off or not at all, the reaction was under loaded (not enough DNA), rerun samples with more DNA. • If running a sample that is known to have PCR inhibitors (e.g., melanoma samples have melanin which is a PCR inhibitor), rerun sample with less DNA or dilute the sample in water 1:5 and re-run. • If increasing/decreasing DNA volume does not resolve the problem: 1. Isolate fresh DNA (if possible). 2. Ethanol precipitate and re-suspend in a lower volume of solvent. 3. Use a commercially available DNA concentrator.
Target and/or internal control signals are negative for the positive control (PC).	• Reagents may be degrading. Contact your supplier for more information. • To avoid future reagent failures, follow the storage and handling instructions in section 2.2 of the IFU.
Abnormal spike in amplification plot of a sample using an ABI instrument. 	• The abnormal spike line may be a result of bubbles in the well containing the sample. Centrifuge the plate before rerunning the sample. • The abnormal spike may be an instrument collection error. Re-run another plate to make sure this is not reproducible. If the spike is reproducible contact the instrument manufacturer.
Signal in NTC control.	• Indicates setup or gDNA contamination. Evaluate lab procedure. Refer to section 2.5 in the IFU for suggestions on minimizing bench contamination. • If target signal and/or internal control signal are present, the results may produce a false positive and a re-run is required.
Target signal crosses the threshold for NTC samples when running assay on ABI instrument.	• Ensure the manual thresholds suggested in the IFU are set. • Verify the signal is not true amplification by checking the signal in the multicomponent plot (ABI instruments). If the signal is horizontal, then this is not true amplification. • If signal in the multicomponent plot is linear and the signal crosses the threshold, adjust the baseline end cycle. Set the start cycle to: 3 and stop: earliest VIC Ct cycle (if signal still

	crosses threshold, increase stop cycle number by increments of 3 until signal no longer crosses threshold).
Wildtype sample has a target signal Ct value that falls within the parameters for a positive mutation status.	• If the internal control signal is within range, then the wildtype sample has contamination from a positive sample, gDNA, or the positive control. Re-run the assay with a new wild type sample. • It is also possible the wildtype sample is not a true wildtype.

9. References

1. Benvenuti S. et al., Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res. 2007 Mar 15; 67(6):2643-2648.
2. Salomon DS. Et al., Epidermal growth factor-related peptides and their receptors in human malignancies. Critical Reviews in Oncology/Haematology 1995; 19:183-232.
3. Fenizia F. et al., EGFR mutations in lung cancer: from tissue testing to liquid biopsy. Future Oncol. 2015; 11(11):1611-23. Doi: 10.2217/fon.15.23.
4. NCBI, dbSNP Short Genetic Variations, https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs1050171, accessed 31 January 2017.

10. Revision History

To receive notifications regarding IFU changes, please register to the EntroGen technical support panel at entrogen.com/support/register.

Last Change: April 2026	
Change	**Affected section(s)**
Updated LoD tables in section 7.2 to add a notation regarding exon 19 deletion variant c.2252_2276delinsA (p.T751_I759delinsN).	7.2
Updated table of listed exon 19 deletions to remove c.2253_2276del24 and c.2254_2277del24 variants.	Appendix

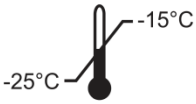
11. Symbols Used

The following symbols used on labels and packaging of this product conform to the harmonized standard EN980.

Catalog Number

Lot/Batch Number

Expiration Date



Storage Conditions

Manufactured by

Intended Use

European Union Authorized Representative

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