# ColoScape™ Colorectal Cancer Mutation Detection Kit

## Instructions For Use

Catalog # DC-30-0024E, DC-30-0006E

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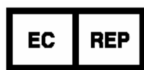

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# Table of Contents


1. Intended Use ..... 3
2. Gene Mutations and Colorectal Cancer (CRC) ..... 4
3. QClamp® Technology for Mutation Detection ..... 4
4. Reagents and Instruments ..... 6
    - Package Contents ..... 6
    - Materials Required but Not Provided in the Kit ..... 7
    - Instruments ..... 7
    - Handling and Storage ..... 7
    - General Considerations ..... 8
    - Warnings and Precautions ..... 8
5. Instructions for Use ..... 9
    - DNA Isolation ..... 9
    - Preparation of Reagents ..... 9
    - Preparation of Assay Mixes ..... 9
    - Suggested Run Layout (96-well plate, 384-well plate, tube strips, or tubes) ..... 10
    - Instrument Set-Up ..... 11
    - Roche Light cycler 96 and Roche Light cycler 480, BioRad CFX 384 ..... 11
    - Assessment of Real-Time PCR Results ..... 11
    - Data Analysis for Light Cycler 480 ..... 11
    - Judging Validity of Sample Data Based on Internal Control Results ..... 12
    - Scoring Mutational Status ..... 12
    - Differentiating KRAS c12/KRAS c13 Mutational Status ..... 13
6. Assay Performance Characteristics ..... 13
    - Analytical Performance ..... 13
    - Analytical Accuracy and Comparison to Reference Method ..... 14
    - Precision and Reproducibility ..... 14
    - Analytic Sensitivity (LOD) ..... 17
    - Analytic Specificity ..... 18
    - Cut-Offs ..... 19
    - Limit of Blank ..... 19
    - Interfering Substances ..... 19
    - Multiple Freeze-Thaw Cycles ..... 19
    - Shelf-Life ..... 19
    - Clinical performance of the assay ..... 19
7. Symbols Used in Packaging ..... 20
8. References ..... 21

# Intended Use

ColoScape™ is a real-time PCR based *in vitro* diagnostic assay for qualitative detection of colorectal cancer associated biomarkers including *APC (codons 1309, 1367, 1450), KRAS (codons 12 and 13), BRAF (codon 600) and CTNNB1 (codons 41 and 45)* . The detection kit identifies the presence or absence of mutations in the targeted regions but does not specify the exact nature of the mutation. The detection kits are designed to detect any mutation at or near the stated codon site without specifying the exact nucleotide change. ColoScape™ Colorectal Cancer Mutation Detection kit is for use in diagnostic procedures.

*Table 1. List of Mutations and Cosmic Identities Found in Coloscape targeted genes*

KRAS

| Exon | Amino Acid Change | Nucleotide change | Cosmic No. |
|---|---|---|---|
| 2 | G12>A | c.35G>C | 522 |
| | G12>R | c.34G>C | 518 |
| | G12>D | c.35G>A | 521 |
| | G12>C | c.34G>T | 516 |
| | G12>S | c.34G>A | 517 |
| | G12>V | c.35G>T | 520 |
| | G13>D | c.38G>A | 532 |
| | G13>C | c.37G>T | 527 |
| | G13>R | c.37G>C | 529 |

APC

| Exon | Amino Acid Change | Nucleotide change | Cosmic No. |
|---|---|---|---|
| 15 | E1309fs* | c.3921_3925delAAAAG | COSM18764 |
| | Q1367* | c.4099C>T | COSM13121 |
| | R1450* | C.4348C>T | COSM13127 |

CTNNB1

| Exon | Amino Acid Change | Nucleotide change | Cosmic No. |
|---|---|---|---|
| 3 | p.T41A | c.121A>G | COSM5664 |
| | p.T41I | c. 122C>T | |
| | p.S45P | c.133T>C | COSM5663 |
| | P.S45F | c.134C>T | |
| | P.S45del | C133-135delTCT | |

BRAF

| Exon | Amino Acid Change | Nucleotide change | Cosmic No. |
|---|---|---|---|
| 15 | p.V600E | c.1799T>A | COSM476 |
| | p.V600K | c.1798_1799GT>AA | COSM473 |
| | p.V600R | c.1798_1799GT>AG | COSM474 |
| | p.V600D | c.1799_1800TG>AT | COSM477 |

*Table 1* shows a list of mutations commonly found in the targeted gene that can be detected by the kit. The kit is to be used by trained laboratory professionals within a laboratory environment.

## Gene Mutations and Colorectal Cancer (CRC)

Complex signal pathways are involved in the colorectal cancer pathogenesis such as the WNT and RAS /RAF/MAPK pathways. Genetic and epigenetic changes in the pathway components have been studied extensively in relation to their roles in the initiation and development of CRC. KRAS mutations are found in several cancers including colorectal, lung, thyroid, and pancreatic cancers and cholangiocarcinoma. More than 90% KRAS mutations are located within codons 12 and 13 of exon 2, which may lead to abnormal growth signaling by the p21- ras protein. These alterations in cell growth and division may trigger cancer development as signaling is excessive. KRAS mutations have also been detected in many colorectal cancer patients.

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

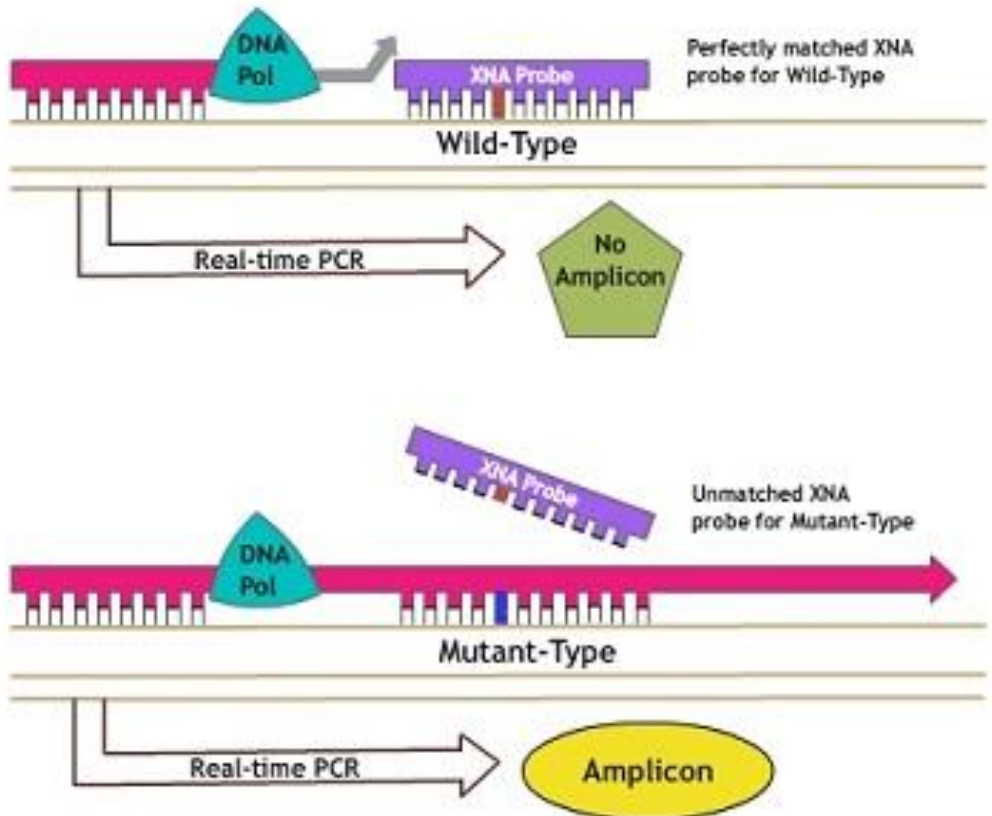
The adenomatous polyposis coli (APC) gene is a key tumor suppressor gene and APC mutation has been found in most colon cancers. The gene encodes a multi-domain protein that binds to various proteins, including -catenin, axin, CtBP, Asefs, IQGAP1, EB1 and microtubules. Most (~60%) cancer-linked APC mutations occur in a region referred to as the mutation cluster region (MCR) and result in C-terminal truncation of the protein. Mutations in the tumor suppressor gene APC result in the accumulation of catenin which activates the Wnt signaling pathway, leading to tumorigenesis. APC also plays roles in other fundamental cellular processes including cell adhesion and migration, organization of the actin and microtubule networks, spindle formation and chromosome segregation. Mutations in APC cause deregulation of theses cellular process, leading to the initiation and expansion of colon cancer. APC has been used as a biomarker for early colon cancer detection.

The β-catenin gene (*CTNNB1*) is also an important component of the Wnt pathway. Mutations in the serine or threonine phosphorylation sites in the regulatory domain (exon 3, codon 29-48) of the gene leads to accumulation of the gene product (β- catenin) which activates the Wnt pathway.

## QClamp® Technology for Mutation Detection

The QClamp® ColoScape™ mutation detection assay is based on xenonucleic acid (XNA) mediated PCR clamping technology. XNA is a synthetic DNA analog in which the phosphodiester backbone has been replaced by a repeat formed by units of (2-aminoethyl)-glycine. 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® Coloscape™ Mutation Test in targeted genes*



## Reagents and Instruments

### *Package Contents*

*Table 2. Package Contents.*

| Vial No. | Name of Component | Description | Volume, 24-test kit | Volume, 6-test kit |
|---|---|---|---|---|
| 1 | APC c1309 and c1367 Primer/Probe Mix | APC c1309 and c1367 Primers and probe | 1 X 62 uL | 1 X 15 uL |
| 2 | APC c1309 and c1367 XNA | APC c1309and c1367 XNA | 1 X 28 uL | 1 X 7 uL |
| 3 | BCT c41 Primer/probe Mix | BCT c41 Primers and probe | 1 X 62 uL | 1 X 15 uL |
| 4 | BCT c41 XNA | BCT c41 XNA | 1 X 28 uL | 1 X 7 uL |
| 5 | APC c1450 Primer/Probe Mix | APC c1450 Primer and probe | 1 X 62 µL | 1 X 15 µL |
| 6 | APC C1450 XNA | APC C1450 XNA | 1 X 28 uL | 1 X 7 uL |
| 7 | BCT c45 Primer/probe Mix | BCT c45 Primers and probe | 1 X 62 uL | 1 X 15 uL |
| 8 | BCT c45 XNA | BCT c45 XNA | 1 X 28 uL | 1 X 7 uL |
| 9 | KRAS c12 Primer/Probe mix | KRAS c12 Primer/probe | 1 X 62 uL | 1 X 15 uL |
| 10 | KRAS c12 XNA | XNA for KRAS c 12 | 1 X 28 uL | 1 X 7 uL |
| 11 | KRAS c13 Primer/Probe mix | KRAS c13 Primer/probe | 1 X 62 uL | 1 X 15 uL |
| 12 | KRAS c13 XNA | XNA for KRAS c 13 | 1 X 28 uL | 1 X 7 uL |
| 13 | BRAF c600 Primer/Probe Mix | BRAF V600 Primers and probe | 1 X 62 uL | 1 X 15 uL |
| 14 | BRAF c600 XNA | BRAF V600 XNA | 1 X 28 uL | 1 X 7 uL |
| 15 | 2X Coloscape™ qPCR Master Mix | PCR Reaction Premix | 1013 uL | 266 uL |
| 16 | ColoScape™ Negative Control | Wild-type DNA | 1 X 56 uL | 1 X 28 uL |
| 17 | ColoScape™ Positive Control | APC c1309, c1367, c1450, BCT c41, BCT c45, KRAS c12, KRAS c13 BRAF c600 mutant templates | 1 X 56 uL | 1 X 28 uL |
| 18 | Non template control | Nuclease-Free Water | 1 X 76 uL | 1 X 56 uL |

## *Materials Required but Not Provided in the Kit*

**Reagents for DNA Isolation**

QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) or equivalent

**Consumables**

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

**Equipment**

- Permanent marker
- Real time PCR instrument
- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR master mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA
- Micro centrifuge
- Bench top centrifuge* with rotor for 1.5 ml tubes
- Vortexer
- PCR rack
- Reagent reservoir
- Distilled water

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

## *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 | Light cycler 96 |
| Roche | Light cycler 480 |
| Bio-rad | CFX384 |

## *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 1and 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.

## *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.

## *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 temperaturefor 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.

# Instructions for Use

## *DNA Isolation*

Human genomic DNA must be extracted from fixed paraffin-embedded tissue, frozen tissue or plasma 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, QIAamp Circulating Nucleic Acid Kit, cat. No. 55114 for plasma). Follow the genomic DNA isolation procedure according to manufacturer's protocol. Sufficient amounts of DNA can be isolated from FFPE blocks or fresh frozen sections as well as plasma (approx. 2-10 µg).

This ColoScape™ assay requires a total of 22.5 – 35 ng of DNA per sample (2.5-5 ng/reaction). After DNA isolation, measure the concentration using fluorometric analysis (i.e. Qubit) and dilute to 1.25-2.5 ng/µl. If using spectrophotometric analysis, make sure the A260/A230 value is greater than 2.0 and A260/A280 value between 1.8 and 2.0.

## *Preparation of Reagents*

A 24-test kit contains enough control material for 3 runs. Thaw all primer and probe mixes, XNAs, Positive Control, WT Negative Control, Nuclease-Free Water and 2X PCR mastermix provided. Thaw all reaction mixes at room temperature for a minimum of 30 minutes. Vortex all components except the PCR Master Mix and Primer and 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 any precipitate in 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.
*Table 4* shows the component volumes for each 10 ul reaction.

*Table 4. QClamp Taqman Assay Components and Reaction Volume.*

| Components | Volume/Reaction |
|---|---|
| 2X PCR Master mix | 5 µl |
| Primer and probe Mix | 2 µl |
| XNA | 1 µl |
| DNA sample or Controls | 2 µl |
| Total volume | 10 µl |

For accuracy, 2x PCR Mastermix, primers and XNA should be pre-mixed into assay mixes as described in *Table 5* below

## *Preparation of Assay Mixes*

Assay mixes should be prepared just prior to use. Label a micro centrifuge tube (not provided) for each reaction mix, as shown in *Table 5.* For each control and mutation detection reaction, prepare sufficient working assay mixes for the DNA samples, one Positive Control, one Nuclease-Free Water for No-Template Control (NTC), and one WT Negative Control, according to the volumes in *Table 5*. Include reagents for 1 extra sample to allow sufficient overage for the PCR set up. The assay mixes contain all of the components needed for PCR except the sample.

Each sample requires one reaction for each mutation site detected by the kit and an Internal Control assay. The External Control assay uses ACTB as reference gene to determine if an appropriate level of amplifiable DNA is present in the sample.

*Table 5. Preparation of Assay Mixes.*

| | Volume of 2X PCR Master Mix | Volume of Primer and probe Mix | Volume of XNA* |
|---|---|---|---|
| APC 1309 and 1367 Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |
| APC 1450 Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |
| BCT 41 Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |
| BCT 45Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |
| KRAS11 Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |
| KRAS 13 Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |
| BRAF Mix | 5 µl x (n+1) | 2 µl x (n+1) | 1 µl x (n+1) |

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 the Internal Control Mix, use 1µl of water provided in the kit in place of XNA.For accuracy, do not pipette less than 10 µl.

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 ColoScape™ Positive Control (PC), ColoScape™ 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 External Control Mix 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 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.

## *Suggested Run Layout (96-well plate, 384-well plate, tube strips, or tubes)*

Add 8 ul of the appropriate assay mix to the plate or tubes. Add 2 ul of template.

*Table 6. Suggested Plate Layout.*

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | NTC APC1309, 1367 Mix | PC APC 1309, 1367 Mix | CC APC 1309, 1367 Mix | S1 APC 1309, 1367 Mix | S2 APC 1309, 1367 Mix | S3 APC 1309, 1367 Mix |
| B | NTC APC 1450 Mix | PC APC 1450 Mix | CC APC 1450 Mix | S1 APC 1450 Mix | S1 APC 1450 Mix | S3 APC 1450 Mix |
| C | NTC BCT41 Mix | PC BCT41Mix | CC BCT41 Mix | S1 BCT41 Mix | S2 BCT41 Mix | S3 BCT41Mix |
| D | NTC BCT45 Mix | PC BCT45 Mix | CC BCT45 Mix | S1 BCT45 Mix | S2 BCT45 Mix | S3 BCT45 Mix |
| E | NTC KRAS 12 Mix | PC KRAS 12 Mix | CC KRAS 12 Mix | S1 KRAS 12 Mix | S2 KRAS 12 Mix | S3 KRAS 12 Mix |
| F | NTC KRAS 13 Mix | PC KRAS 13Mix | CC KRAS 13Mix | S1 KRAS 13 Mix | S2 KRAS 13 Mix | S3 KRAS 13 Mix |
| G | NTC BRAF 600 Mix | PC BRAF 600 Mix | CC BRAF 600 Mix | S1 BRAF 600 Mix | S2 BRAF 600 Mix | S3 BRAF 600 Mix |

PC: Positive Control, NTC: No-Template Control (water), CC: Negative Control (Wild-type DNA), S1-3: Samples 1-3.

*Table 6* is a suggested plate set-up for a single experiment analyzing 3 unknown samples. Please disregard any assay mixes listed below that are not part of your kit.

When all reagents have been added to the plate, tightly seal the plate to prevent evaporation. Spin at 1000rpm for 1 minute to collect all the reagents. Place in the real-time PCR instrument immediately.

## *Instrument Set-Up*

### *Roche Light cycler 96 and Roche Light cycler 480, BioRad CFX 384*

1. Use FAM/HEX as the Detector
2. Setup the cycling parameters as shown in the table below
3. Start the run.

*Table 7. Roche Light Cycler Platforms Cycling Parameters.*

| Step | Temperature (°C) | Time (Seconds) | Cycles | Data Collection |
|---|---|---|---|---|
| PreIncubation | 95 | 300 | 1 | OFF |
| Denaturation | 95 | 20 | X50 | OFF |
| XNA Annealing | 70 | 40 | | OFF |
| Primer Annealing | 64 | 30 | | OFF |
| Extension | 72 | 30 | | FAM and HEX |

## *Assessment of Real-Time PCR Results*

The real-time PCR instrument generates a cycle threshold (Ct) value for each sample. Ct 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.

## *Data Analysis for Light Cycler 480*

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

**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 External Control Assay as follows:

Cq difference (ΔCq) = Mutation Assay Cq - External Control Assay Cq

**Negative and Positive Controls**

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

*Table 8. Acceptable Values for Positive Controls and Negative Controls.*

| Assay | Positive Control | Negative Control |
|---|---|---|
| Internal Control | $25 < Cq < 31$ | $25 < Cq < 31$ |
| APC 1309/1367 | $\Delta Cq \leqslant 3$ | $\Delta Cq > 17$ |
| APC 1450 | $\Delta Cq \leqslant 4.5$ | $\Delta Cq > 7$ |
| BCT 41 | $\Delta Cq \leqslant -0.5$ | $\Delta Cq > 7.3$ |
| BCT 45 | $\Delta Cq \leqslant 1.5$ | $\Delta Cq > 9.2$ |
| KRAS12 | $\Delta Cq \leqslant 5$ | $\Delta Cq > 10.4$ |
| KRAS 13 | $\Delta Cq \leqslant 4.5$ | $\Delta Cq > 9$ |
| BRAF V600 | $\Delta Cq \leqslant 4$ | $\Delta Cq > 8.3$ |

## *Judging Validity of Sample Data Based on Internal Control Results*

The Cq value of the Internal Control Mix serve as an indication of the purity and concentration of DNA in each well. Thus, the validity of the test can be decided by the Cq value of the Internal Control mix. Cq values of any sample with Internal Control Mix should be in the range of $25<Cq<31$. If the Cq values fall outside this range, the test results should be considered invalid. The experiment should be repeated following the recommendations in Table 9.

*Table 9. Acceptable Internal Control Cq Ranges for Samples.*

| Validity | Cq Value of Internal Control Mix | Descriptions and Recommendations |
|---|---|---|
| Optimal | $25 < Cq < 31$ | The amplification and amount of DNA sample were optimal. |
| Invalid | $Cq < 25$ | Possibility of a false positive is high. Repeat the PCR reaction with less DNA. |
| Invalid | $Cq \geq 31$ | Not enough DNA or DNA not pure. The amplification is not optimal. Check DNA amount and purity. Repeat the experiment with more DNA or a new DNA prep may be required |

## *Scoring Mutational Status*

If a Cq value is Undetermined, assign a Cq of 50 and proceed to analysis.

The table below should be used to determine mutational status.

*Table 10. Scoring Mutational Status for Roche LC480 system*

| Mutation | APC c1309/1367 | APC c1450 | BCT c41 | BCT c45 | KRAS c12 | KRAS c13 | BRAF c600 |
|---|---|---|---|---|---|---|---|
| Positive: | <16.4 | < 7.6 | < 8.40 | < 9.5 | < 10.8 | < 10.9 | < 8.5 |
| Negative | ≥16.4 | ≥ 7.6 | ≥ 8.40 | ≥ 9.5 | ≥ 10.8 | ≥ 10.9 | ≥ 8.5 |

*Table 11. Scoring Mutational Status for BioRad CFX384 system*

| Mutation | APC c1309/1367 | APC c1450 | BCT c41 | BCT c45 | KRAS c12 | KRAS c13 | BRAF c600 |
|---|---|---|---|---|---|---|---|
| Positive: | <10 | < 7 | < 8.1 | < 10.5 | < 10.8 | < 9.4 | < 8.2 |
| Negative | ≥10 | ≥ 7 | ≥ 8.1 | ≥ 10.5 | ≥ 10.8 | ≥ 9.4 | ≥ 8.2 |

### *Differentiating KRAS c12/KRAS c13 Mutational Status*

The KRAS c12 reaction mix detects both KRAS c12 and KRAS c13 mutations, whereas the KRAS c13 reaction mix detects only KRAS c13 mutations. Therefore, in order to differentiate between KRAS c12 and KRAS c13 Mutations a combination of results from the 2 mixes should be used, as described in *Table 12* below.

*Table 12. Interpretation of G12/G13 Mutational Status*

| Reaction Mix | Result Based on Tables 10, 11 | Mutational Status |
|---|---|---|
| KRAS c12 Reaction Mix<br>KRAS c13 Reaction Mix | Positive<br>Negative | G12 Mutation |
| KRAS c12 Reaction Mix<br>KRAS c13 Reaction Mix | Positive<br>Positive | G13 Mutation |
| KRAS c12 Reaction Mix<br>KRAS c13 Reaction Mix | Negative<br>Positive | G13 Mutation |

## Assay Performance Characteristics

The performance characteristics of this product were established on the, Roche LightCycler 96, Roche LightCycler 480 and BioRad CFX 384 real-time PCR instruments.

### *Analytical Performance*

The specific performance characteristics of the ColoScape™ kit were determined by studies involving APC, Beta Catenin (CTNNB1),KRAS and BRAF-defined genomic DNA reference samples  and FFPE samples of the cell lines with defined mutations obtained from Horizon Diagnostics (Cambridge, England) and ATCC. These samples have been characterized genetically as containing heterozygous or homozygous mutations in the coding sequence of the respective target regions. These single nucleotide polymorphisms in the target regions of the four genes in the assay have been confirmed by genomic DNA sequencing.  Additional samples consisted of formalin-fixed, paraffin-embedded (FFPE) reference and patient tissue samples, as well as wild-type DNA (no KRAS mutations).

## *Analytical Accuracy and Comparison to Reference Method*

QClamp analytical accuracy is verified and validated through testing of well-characterized samples with known mutations verified by sequencing. Three studies were done to demonstrate concordance in mutation status of FFPE samples tested with the ColoScape™ Mutation Test kit relative to sequencing. A set of samples were chosen for evaluation based on mutation status. Samples were chosen blindly to test with the ColoScape™ CRC mutation Test kit for comparison to the mutation status returned from sequencing. The results demonstrated a 100% match between sequencing and the ColoScape™ Mutation Test kit. A total of 200+ samples were tested in the accuracy and reproducibility studies, 64 of them represent unique FFPE patient samples. 8 gDNA mixed positive controls and 3 FFPE controls were tested multiple times. In samples with sufficient amount of DNA positive samples were correctly detected in >97% of tests, negative samples were detected correctly in >96% cases of samples.

## *Precision and Reproducibility*

Precision of the ColoScape™ kit was determined with defined analyte levels of gDNA of known mutational status and allelic frequencies. To establish lot-to-lot variation, a reproducibility study was performed using two different kit lots. Each lot was tested on one wild-type control and two reference samples containing each mutation at 5% and 1% allelic frequency in nine replicates. Tests were performed on three separate dates. Inter-assay %CV was established with results from two different users at two different instruments performing the assay using the same lot of reagents, with tests run one to two times a day for three days. Intra-assay %CV was established through performance of kit on reference samples run in replicates of nine separately for two different qPCR instrument types. All testing was done using sequence-verified samples from Horizon Diagnostics and ATCC.

*Table 13. Summary of Reproducibility Results*

| Variation | %CV |
|---|---|
| Intra-assay | ≤ 6% |
| Inter-assay | ≤ 8% |
| Lot-to-Lot Variation | ≤ 5% |
| Operator Variability | ≤ 3% |

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

*Table 14. Summary of Intra-assay Reproducibility Results on LC480.*

| | WT | | | 5% mutant | | | 1% mutant | | |
|---|---|---|---|---|---|---|---|---|---|
| Target | Average Cq | SD | %CV | Average Cq | SD | %CV | Average Cq | SD | %CV |
| APC1309 | 50 | 0 | 0.0% | 30.2 | 0.2 | 0.6% | 32.6 | 0.2 | 0.6% |
| APC1367 | 50 | 0 | 0.0% | 30.1 | 0.1 | 0.4% | 32.6 | 0.3 | 1.0% |
| APC1450 | 42.3 | 4.3 | 10.1% | 31.4 | 1.6 | 5.2% | 34.6 | 0.3 | 0.8% |
| BTC 41 | 39.8 | 2.2 | 5.5% | 27.9 | 0.1 | 0.4% | 30.4 | 0.3 | 0.8% |
| BTC 45 | 39.6 | 1.3 | 3.3% | 30.4 | 0.2 | 0.7% | 32.9 | 0.6 | 1.7% |
| KRAS 12 | 42.2 | 2 | 4.7% | 32.2 | 0.4 | 1.1% | 35.6 | 0.9 | 2.5% |
| KRAS 13 | 42.1 | 1.2 | 2.9% | 33.3 | 0.3 | 0.9% | 36.6 | 0.6 | 1.7% |
| BRAF 600 | 39.1 | 0.9 | 2.3% | 31.9 | 0.2 | 0.7% | 35 | 0.6 | 1.6% |
| Internal Control | 28.7 | 0.8 | 0.3% | 28.9 | 0.8 | 2.1% | 28.6 | 0.8 | 2.1% |

*Table 15. Summary of Inter-assay Reproducibility Results on LC480.*

| Assay | Run 1 | Run 2 | Run 3 | Average | SD | CV% |
|---|---|---|---|---|---|---|
| | | | WT | | | |
| APC1309/1367 | 50.0 | 50.0 | 50.0 | 50.0 | 0.0 | 0.0% |
| APC1450 | 42.3 | 42.5 | 43.4 | 42.7 | 0.6 | 1.3% |
| BTC 41 | 39.8 | 40.0 | 39.7 | 39.8 | 0.1 | 0.3% |
| BTC 45 | 39.6 | 40.4 | 41.8 | 40.6 | 1.1 | 2.7% |
| KRAS 12 | 42.2 | 43.4 | 45.3 | 43.6 | 1.6 | 3.6% |
| KRAS 13 | 42.1 | 41.6 | 42.2 | 42.0 | 0.3 | 0.8% |
| BRAF 600 | 39.1 | 38.3 | 39.4 | 38.9 | 0.6 | 1.6% |
| Internal Control | 28.7 | 29.1 | 29.2 | 29.0 | 0.3 | 0.9% |
| | | | 5% mutation | | | |
| APC1309/1367 | 30.1 | 30.3 | 30.2 | 30.2 | 0.1 | 0.3% |
| APC1450 | 31.4 | 32.0 | 32.7 | 32.0 | 0.5 | 1.6% |
| BTC 41 | 27.9 | 28.2 | 27.6 | 27.9 | 0.3 | 0.9% |
| BTC 45 | 30.4 | 30.6 | 29.3 | 30.1 | 0.6 | 1.9% |
| KRAS 12 | 32.2 | 33.0 | 31.7 | 32.3 | 0.5 | 1.7% |
| KRAS 13 | 33.3 | 33.9 | 32.0 | 33.0 | 0.8 | 2.4% |
| BRAF 600 | 31.9 | 32.6 | 32.2 | 32.3 | 0.3 | 0.9% |
| Internal Control | 29.3 | 29.7 | 28.4 | 29.1 | 0.5 | 1.8% |
| | | | 1% mutation | | | |
| APC1309/1367 | 32.6 | 32.9 | 32.7 | 32.8 | 0.1 | 0.4% |
| APC1450 | 34.6 | 34.5 | 34.8 | 34.6 | 0.1 | 0.4% |
| BTC 41 | 30.4 | 30.6 | 30.3 | 30.4 | 0.1 | 0.4% |
| BTC 45 | 32.9 | 33.0 | 31.8 | 32.6 | 0.6 | 1.7% |
| KRAS 12 | 35.6 | 36.3 | 34.6 | 35.5 | 0.7 | 1.9% |
| KRAS 13 | 36.6 | 37.3 | 33.8 | 35.9 | 1.5 | 4.2% |
| BRAF 600 | 35.0 | 34.9 | 34.3 | 34.7 | 0.3 | 0.9% |
| Internal Control | 28.8 | 29.0 | 28.8 | 28.9 | 0.1 | 0.3% |

*Table 16. Summary of Lot-to Lot Reproducibility Results on Roche LC480*

| | | | WT | | |
|---|---|---|---|---|---|
| Assay | Lot 1 | Lot 2 | Average | St Deviation | %CV |
| APC1309 | 50.0 | 50.0 | 50.0 | 0.0 | 0.0% |
| APC1367 | 44.3 | 42.4 | 43.3 | 1.3 | 3.0% |
| APC1450 | 40.2 | 39.9 | 40.0 | 0.2 | 0.5% |
| BTC CD41 | 41.7 | 40.0 | 40.8 | 1.2 | 3.0% |
| BTC CD45 | 44.5 | 42.8 | 43.7 | 1.2 | 2.8% |
| KRAS CD12 | 42.8 | 41.9 | 42.3 | 0.7 | 1.6% |
| KRAS CD13 | 39.9 | 38.7 | 39.3 | 0.9 | 2.3% |
| BRAF V600 | 50.0 | 50.0 | 50.0 | 0.0 | 0.0% |

| | | | | | |
|---|---|---|---|---|---|
| Internal Control | 29.0 | 28.9 | 28.9 | 0.1 | 0.3% |
| | | | 5% Mutation | | |
| APC1309 | 30.6 | 30.2 | 30.4 | 0.3 | 1.0% |
| APC1367 | 33.1 | 31.7 | 32.4 | 1.0 | 3.2% |
| APC1450 | 27.6 | 28.1 | 27.8 | 0.3 | 1.2% |
| BTC CD41 | 29.4 | 30.5 | 29.9 | 0.8 | 2.6% |
| BTC CD45 | 32.3 | 32.6 | 32.5 | 0.2 | 0.7% |
| KRAS CD12 | 32.0 | 33.6 | 32.8 | 1.1 | 3.4% |
| KRAS CD13 | 32.2 | 32.3 | 32.2 | 0.0 | 0.1% |
| BRAF V600 | 30.6 | 30.2 | 30.4 | 0.3 | 1.0% |
| Internal Control | 28.8 | 29.5 | 29.1 | 0.5 | 1.6% |
| | | | 1% Mutation | | |
| APC1309 | 33.0 | 32.8 | 32.9 | 0.1 | 0.4% |
| APC1367 | 35.0 | 34.5 | 34.8 | 0.4 | 1.0% |
| APC1450 | 30.3 | 30.5 | 30.4 | 0.1 | 0.4% |
| BTC CD41 | 31.7 | 33.0 | 32.4 | 0.9 | 2.7% |
| BTC CD45 | 35.4 | 35.9 | 35.6 | 0.4 | 1.1% |
| KRAS CD12 | 34.5 | 37.0 | 35.7 | 1.8 | 5.0% |
| KRAS CD13 | 34.5 | 34.9 | 34.7 | 0.3 | 0.8% |
| BRAF V600 | 33.0 | 32.8 | 32.9 | 0.1 | 0.4% |
| Internal Control | 28.8 | 28.9 | 28.8 | 0.1 | 0.2% |

*Table 17. Summary of Lot-to Lot Reproducibility Results on BioRad CFX384*

| Assay | Lot 1 | Lot 2 | Average | St Deviation | %CV |
|---|---|---|---|---|---|
| | | | WT | | |
| APC1309 | 48.5 | 48.5 | 48.5 | 0.0 | 0.0% |
| APC1367 | 47.2 | 47.3 | 47.3 | 0.1 | 0.1% |
| APC1450 | 43.5 | 39.5 | 41.5 | 2.0 | 4.7% |
| BTC CD41 | 40.7 | 40.1 | 40.4 | 0.3 | 0.7% |
| BTC CD45 | 44.3 | 40.7 | 42.5 | 1.8 | 4.2% |
| KRAS CD12 | 45.4 | 38.5 | 41.9 | 3.4 | 8.2% |
| KRAS CD13 | 40.7 | 39.9 | 40.3 | 0.4 | 1.0% |
| BRAF V600 | 37.6 | 38.0 | 37.8 | 0.2 | 0.4% |
| Internal Control | 29.0 | 29.0 | 29.0 | 0.1 | 0.2% |
| | | | 5% Mutation | | |
| APC1309 | 27.5 | 28.1 | 27.8 | 0.3 | 1.0% |
| APC1367 | 27.6 | 27.9 | 27.7 | 0.2 | 0.6% |
| APC1450 | 29.8 | 29.6 | 29.7 | 0.1 | 0.2% |
| BTC CD41 | 26.2 | 26.5 | 26.4 | 0.2 | 0.7% |
| BTC CD45 | 29.3 | 30.2 | 29.8 | 0.4 | 1.4% |
| KRAS CD12 | 29.8 | 29.4 | 29.6 | 0.3 | 0.9% |
| KRAS CD13 | 30.1 | 31.6 | 30.8 | 0.8 | 2.5% |
| BRAF V600 | 31.0 | 31.2 | 31.1 | 0.2 | 0.5% |
| Internal Control | 28.8 | 26.8 | 26.8 | 0.3 | 1.3% |

| 1% Mutation | | | | | |
|---|---|---|---|---|---|
| APC1309 | 29.79 | 30.54 | 30.17 | 0.38 | 1.25% |
| APC1367 | 30.16 | 30.99 | 30.58 | 0.58 | 1.90% |
| APC1450 | 32.42 | 32.05 | 32.24 | 0.18 | 0.57% |
| BTC CD41 | 29.06 | 28.97 | 29.02 | 0.06 | 0.22% |
| BTC CD45 | 31.59 | 32.71 | 32.15 | 0.56 | 1.75% |
| KRAS CD12 | 32.33 | 31.78 | 32.06 | 0.39 | 1.21% |
| KRAS CD13 | 32.21 | 34.22 | 33.22 | 1.00 | 3.02% |
| BRAF V600 | 32.10 | 33.30 | 32.70 | 0.84 | 2.58% |
| Internal Control | 27.27 | 27.27 | 27.27 | 0.49 | 1.83% |

## *Analytic Sensitivity (LOD)*

### *Table 18. LOD Summary: Reference DNA Samples.*

| Target mutation | DNA Input, ng/well | | | |
|---|---|---|---|---|
| | | 10 | 5 | 2.5 |
| | | % Correct Call | % Correct Call | % Correct Call |
| APC 1309 | 1% mutation | 100% | 94% | 44% |
| | 0.5% mutation | 44% | 67% | 33% |
| | 0.10% mutation | 0% | 0% | 0% |
| APC 1367 | 1% mutation | 100% | 100% | 100% |
| | 0.5% mutation | 100% | 100% | 100% |
| | 0.10% mutation | 100% | 67% | 56% |
| APC 1450 | 1% mutation | 100% | 100% | 100% |
| | 0.5% mutation | 100% | 100% | 67% |
| | 0.10% mutation | 0% | 0% | 0% |
| BCT 41 | 1% mutation | 100% | 100% | 100% |
| | 0.5% mutation | 100% | 100% | 100% |
| | 0.10% mutation | 100% | 100% | 78% |
| BCT 45 | 1% mutation | 100% | 100% | 100% |
| | 0.5% mutation | 100% | 100% | 100% |
| | 0.10% mutation | 100% | 73% | 22% |
| KRAS 12 | 1% mutation | 100% | 100% | 100% |
| | 0.5% mutation | 100% | 100% | 67% |
| | 0.10% mutation | 87% | 33% | 24% |
| KRAS 13 | 1% mutation | 100% | 100% | 100% |
| | 0.5% mutation | 100% | 100% | 100% |
| | 0.10% mutation | 56% | 56% | 56% |
| BRAF V600 | 1% mutation | 100% | 100% | 78% |
| | 0.5% mutation | 100% | 100% | 67% |
| | 0.10% mutation | 44% | 56% | 56% |

To determine the limit of detection (LOD) for the kit, the ColoScape™ assay was run using a serial dilution of mutant DNA in wild-type background. Mutant samples were sequence verified by Horizon Diagnostics and ATCC. Mutant concentrations tested were 5, 1, 0.5 and 0.1%. Results demonstrated effective clamping of wild type, providing reproducible detection of mutations at concentrations as low as 0.1% for some targets.

DNA isolated from patient FFPE and plasma samples and reference DNA with known mutational status were used to determine the limit of detections (analytical sensitivity) of this assay. Broad-range serial dilutions of DNA extracted from FFPE and plasma reference materials with known mutations controls were made using wild-type human DNA from FFPE samples to yield various percentages of mutant allele over wild-type background. The assay was performed in multiple replicates (9) near the LOD. For all tested DNA inputs from 10ng/well to 2.5ng/well all target mutations were detected with 100% correct calls at 5% allelic frequency. Table 18 below shows data on 1%, 0.5% and 0.1% mutation detection at various DNA input levels.

Based on the data from the tables below, recommended DNA input is 5ng/well. APC1367, 1450, BTC 41, 45 and KRAS12, 13 can be detected accurately at 0.5% allelic frequency with 2.5 ng/well.
Recommended input of FFPE should not be higher than 20 ng/well due to possible PCR inhibition. Optimal FFPE sample input is between 25 and 31 Cq of the Internal Control reaction.

## *Analytic Specificity*

Analytic specificity of the ColoScape™ test was determined as both the correct calling of the samples with no mutation at different concentrations of WT template and as cross-reactivity of the assays within the kit when one or more mutations are present.
Table 19 shows that all the assays in the kit are accurate in detecting the target mutations. Only in case of KRAS 13 mutations present both KRAS 12 and KRAS 13 signals were detected, but the pattern of lower Cq in KRAS 12 assay and higher Cq in KRAS 13 assay allows for discerning the two targets.
For known WT templates specificity of the assay at 5ng per well is over 97%. There were no false positive calls for up to 320ng of gDNA per well and up to 20ng FFPE DNA. Higher amounts of DNA input were not tested.
For the purposes of detecting colorectal cancer in a test sample, presence of any of the mutations detected by ColoScape™ kit is considered positive signal.

*Table 19. Analytic specificity: Cross-reactivity Summary*

| Assay | Expected mutations in tested 50% templates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | APC 1309 | APC 1367 | APC 1450, KRAS12 | BCT 41 | BCT 45, KRAS 13 | KRAS 12 | KRAS 13 | BRAF 600, BCT 45, KRAS 13 |
| APC 1309 | + | – | – | – | – | – | – | – |
| APC 1367 | – | + | – | – | – | – | – | – |
| APC 1450 | – | – | + | – | – | – | – | – |
| BCT 41 | – | – | – | + | – | – | – | – |
| BCT 45 | – | – | – | – | + | – | – | + |
| KRAS 12 | – | – | + | – | * | + | * | * |
| KRAS 13 | – | – | – | – | + | – | + | + |
| BRAF V600 | – | – | – | – | – | – | – | + |

* 6Cq higher FAM values than KRAS13

## *Cut-Offs*

Along with studies for analytical accuracy, both reference DNA and FFPE samples were tested to establish cut-offs for the assay. Cutoffs are presented in *Tables 9-11* of the manual.

## *Limit of Blank*

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

## *Interfering Substances*

A study was performed to evaluate the impact of potentially interfering substances on the performance of the ColoScape™ assay. Potentially interfering substances tested were paraffin (FFPE) and ethanol. The impact of each substance on resultant ΔCq and mutation status of test samples was determined via spiking experiments conducted at three different concentrations, 0.1%, 1% and 5%. DNA extracted from FFPE samples was tested and shown to be compatible with the assay within established cutoffs. None of the potentially interfering substances evaluated at concentrations encountered in normal use impacted the ability of the ColoScape™ to distinguish between mutation-positive and mutation-negative control samples.

## *Multiple Freeze-Thaw Cycles*

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

## *Shelf-Life*

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

## *Clinical performance of the assay*

Clinical sensitivity and specificity was tested on the samples extracted from FFPE and plasma of patients with different stages of CRC from normal to advanced adenomas (AA), to colorectal cancer stages 1 through 4.
A sample was considered positive if at least one of the target mutations tested positive based on the cutoffs presented in *Tables 9-11*. TP-true positive, FP- false positive, TN- True negative, FN - false negative, NC- no call/inconclusive.

*Table 20. Clinical sample testing summary*

| Types of Clinical Samples | | Calls | | | | | | Clinical parameter | |
|---|---|---|---|---|---|---|---|---|---|
| | | Number of samples | TP | FP | TN | FN | NC | specificity | sensitivity |
| clinical stage/sample type | CRC, including plasma and FFPE | 22 | 22 | 0 | 0 | 0 | 0 | N/A | 100% |
| | CRC, tissue | 10 | 9 | 0 | 0 | 1 | 0 | N/A | 90% |
| | Advanced Adenomas | 10 | 6 | 0 | 0 | 4 | 0 | N/A | 60% |
| | Non-malignant | 22 | 0 | 1 | 20 | 0 | 1 | 95% | N/A |
| | Non-malignant, FFPE only | 10 | 0 | 1 | 9 | 0 | 0 | 90% | N/A |
| Total | FFPE | 67 | 41 | 1 | 20 | 4 | 1 | 95% | 91% |
| | Plasma | 24 | 12 | 0 | 11 | 0 | 1 | 100% | 100% |
| | Excluding adenomas | 57 | 35 | 1 | 21 | 0 | 0 | 95% | 100% |
| | CRC FFPE | 35 | 35 | 0 | 0 | 0 | 0 | N/A | 100% |

## Symbols Used in Packaging

*Table 11. Symbols Used in Packaging*

| Symbol | Definition |
|---|---|
| IVD | In Vitro Diagnostic Device |
| CE | CE Mark |
| EC REP | Authorized Representative in the European Community |
| REF | Catalog Number |
| | Manufactured By |
| c | Temperature Limitation |
| LOT | Batch Code |
| | Expiration Date |
| EC REP | Authorized Representative in the European Community |
| 2016-11-16 | Date Format (year-month-day) |
| 2016-11 | Date Format (year-month) |

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