INSTRUCTION FOR USE

ColoScape™ Liquid 1.1 Version Kit

Colorectal Cancer Mutation Detection Test

For Real-Time PCR Assays

CATALOG NUMBER	MANUFACTURER	INTENDED USE
DC-30-0003 (24 Reactions)	DiaCarta, Inc. 2600 Hilltop Drive Richmond, CA 94806 United States P: +1 510-878-6662 F: + 1 510-735-8636 E: information@diacarta.com	Research-Use-Only (RUO)

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

Instruction Version: Rev.0, Date of Revision: July 2021

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Part 1. Intended Use

ColoScape™ is a multiplex real-time PCR based *in vitro* diagnostic assay for qualitative and simultaneous detection of colorectal cancer associated mutations in genes including APC (codons 1309, 1367, 1450 and 876), KRAS (codons 12 and 13), BRAF (codon 600), and CTNNB1 (codons 41 and 45). The assay is performed on DNA extracted from FFPE (Formalin-fixed paraffin embedded) or plasma. The detection kit identifies the presence or absence of mutations in the targeted regions but does not specify the exact nature of the mutation. 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

Genes	Exon	Amino Acid Change	Nucleotide Change	Cosmic No.
KRAS	2	G12>A	c.35G>C	COSM522
		G12>R	c.34G>C	COSM518
		G12>D	c.35G>A	COSM521
		G12>C	c.34G>T	COSM516
		G12>S	c.34G>A	COSM517
		G12>V	c.35G>T	COSM520
		G13>D	c.38G>A	COSM532
		G13>C	c.37G>T	COSM527
		G13>R	c.37G>C	COSM529
APC	15	R876*	c.2626C>T	COSM18852
		E1309fs*	c.3921_3925delAAAAG	COSM18764
		Q1367*	c.4099C>T	COSM13121
		R1450*	C.4348C>T	COSM13127
CTNNB1	3	p.T41A	c.121A>G	COSM5664
		p.T41I	c. 122C>T	COSM5676
		p.S45P	c.133T>C	COSM5663
		P.S45F	c.134C>T	COSM5667
		P.S45del	C133-135delTCT	COSM6128
BRAF	15	p.V600E	c.1799T>A	COSM476

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

Part 2. Gene Mutations and Colorectal Cancer (CRC)

Complex signaling 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 these cellular processes, 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.

Part 3. 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 novel synthetic 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 is a complete match to the 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.

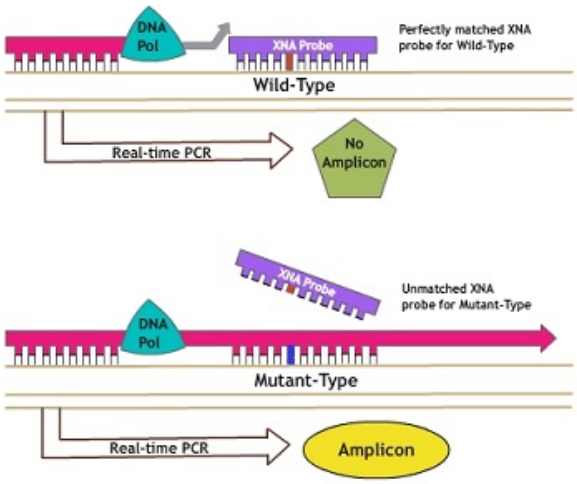

Figure 1. Principle of the QClamp® ColoScape™ mutation test in targeted genes

Part 4. Reagents and Instruments

4.1. Package Contents

Table 2. Package contents

Box	Vial No.	Cap Color	Name of Component	Description	Volume, 24 Rection kit
Box 1	1	Amber	ColoScape Mutation Panel A Primer Probe Mix	APC c1309 and c1367 Primers and probes (PPM)	1 X 62 µl
	2	Blue	ColoScape Mutation Panel A XNA	APC c1309 and c1367 XNA	1 X 28 µl
	3	Amber	ColoScape Mutation Panel B Primer/Probe Mix	APC c1450/876, KRAS c12 and CTNNB1 c41 Primers and probes	1 X 62 µl
	4	Yellow	ColoScape Mutation Panel B XNA	APC C1450/876, KRAS c12 and CTNNB1 c41 XNA	1 X 28 µl
	5	Amber	ColoScape Mutation Panel C Primer/Probe Mix	BRAF V600, KRAS 13 and CTNNB1 c45 (PPM) Primer/probe	1 X 62 µl
	6	Natural	ColoScape Mutation Panel C XNA	BRAF V600, KRAS 13 and CTNNB1 c45 XNA	1 X 28 µl
	7	Natural	2X ColoScape™ qPCR Master Mix	PCR Reaction Premix	1 X 1008 µl
Box 2	8	Green	ColoScape™ Negative Control	Wild-type human genomic DNA	1 X 48 µl uL
	9	Red	ColoScape™ Positive Control 1	APC c1309, c1367, c1450, CTNNB1 c41, CTNNB1 c45, KRAS c12, KRAS c13 and BRAF c600 mutant templates	1 X 48 µl
	10	Red	ColoScape™ Positive Control 2	APC c876 mutant template	1 X 48 µl
	11	Natural	Non template control	Nuclease-Free Water	1 X 76 µl

4.2. Materials Required but Not Provided with the Kit

A. Reagents for DNA Isolation

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

B. Consumables

- White 0.2 ml DNase-free PCR tubes or plates (384 well or 96 well) of the type recommended by the instrument manufacturer
- Nuclease-free, low-binding micro centrifuge tubes
- Nuclease-free pipet tips with aerosol barriers

C. 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

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

4.3. 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	Light cycler 480 II
Bio-Rad	CFX384
ABI	QuantStudio 5
ABI	7500 Fast Dx

4.4. Handling and Storage

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

The kit should be stored at -20 °C immediately upon receipt at -15°C to -25°C in a constant-temperature freezer and must be 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.

4.5. General Considerations

Effective use of real-time PCR tests requires good laboratory practices, including maintenance of equipment that is dedicated to molecular biology. Use nuclease-free lab ware (pipettes, pipette tips, reaction vials) and wear gloves when performing the assay. Use aerosol-resistant pipette tips for all pipetting steps to avoid cross contamination of the samples and reagents.

Prepare the assay mixes in designated pre-amplification areas using only equipment dedicated to this application. Add template DNA in a separate area (preferably a separate room). Use extreme caution to prevent DNase contamination that could result in degradation of the template DNA, or PCR carryover contamination, which could result in a false positive signal.

Reagents supplied are formulated specifically for use with this kit. Make no substitutions to ensure optimal performance of the kit. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data.

4.6. Warnings and Precautions

- Use extreme caution to prevent contamination of PCR reactions with the positive and wild type DNA controls provided.
- Minimize exposure of the 2X PCR Master mix to room temperature for optimal amplification.
- Avoid over exposure of 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 the 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 to ensure optimal performance of the kit. Further dilution of the reagents or alteration of incubation time and temperature may result in erroneous or discordant data.

Part 5. Instructions for Use

5.1. DNA Isolation

Human genomic DNA must be extracted from fixed paraffin-embedded tissue, tissue, or plasma prior to use. Several methods exist for DNA isolation. For consistency, we recommend using a commercial kit as follows:

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

Follow the DNA isolation procedure according to manufacturer's protocol. This QClamp assay requires a total of 30 ng of DNA per sample (10ng/reaction). After DNA isolation, measure the concentration using fluorometric analysis (i.e., Qubit) and dilute to 5 ng/µl. If using spectrophotometric analysis, make sure the A260/A230 value is greater than 2.0 and A260/A280 value is between 1.8 and 2.0.

5.2. Preparation of Reagents

A 21-sample 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 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 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. 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**

Table 4 shows the component volumes for each 10 µl reaction.

Note: For accuracy, 2x PCR Master mix, primers, and XNA should be pre-mixed into assay mixes as described in Table 5 below

5.3. 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 Non-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 the components needed for PCR except the sample.

Table 5. Preparation of assay mixes

	Volume of 2X PCR Master Mix	Volume of Primer and Probe Mix	Volume of XNA
ColoScape Mutation Panel A Mix	5 µl x (n+1)	2 µl x (n+1)	1 µl x (n+1)
ColoScape Mutation Panel B Mix	5 µl x (n+1)	2 µl x (n+1)	1 µl x (n+1)
ColoScape Mutation Panel C Mix	5 µl x (n+1)	2 µl x (n+1)	1 µl x (n+1)

Note: 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. You may want to consider increasing volume of mix to (n+2) when processing larger number of samples.

ColoScape™ kit controls: Negative Control, Positive Control and Non template control must be run with each reaction mix, every time the assay is run.

Negative Control:
- Uses commercially available wild-type human genomic DNA as the template at 2.5ng/µl concentration.
- No target mutations, efficient binding by XNA clamps suppressing the target amplification.

Positive Control:
- A mix of synthetic reference mutant templates for each target of the assay at 5% allelic frequency in 2.5ng/µl WT human genomic DNA (hgDNA).
- XNA clamps 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.

Non- Template Control (NTC):
- Nuclease free water is used in the place of template
- No amplification should be observed in all channels, assuring the absence of contamination during assay set-up.

The Internal Control assay uses ACT-B housekeeping gene as a reference gene to assess the quality of amplifiable DNA and demonstrating if the reagents are working correctly. When assessed using the CY5 channel, this control should make amplicons efficiently for all samples and controls except NTC, providing another way to monitor performance of the primers, probes, polymerase, and sample DNA quality/quantity.

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

Please always use white plates, strips, or tubes. In pre-amplification area, add 8 µl of the appropriate assay mix to the plate or tubes. In designated template area, add 2 µl of template to each well

Table 6. Suggested plate layout

	1	2	3	4	5	6
A	NTC Mutation Panel A Mix	PC 1 Mutation Panel A Mix	PC 2 Mutation Panel A Mix	CC Mutation Panel A Mix 1367 Mix	S1 Mutation Panel A Mix 1367 Mix	S2 Mutation Panel A Mix 1367 Mix
B	NTC Mutation Panel B Mix	PC 1 Mutation Panel A Mix	PC 2 Mutation Panel A Mix	CC Mutation Panel B Mix 1367 Mix	S1 Mutation Panel B Mix 1367 Mix	S2 Mutation Panel B Mix 1367 Mix
C	NTC Mutation Panel C Mix	PC 1 Mutation Panel A Mix	PC 2 Mutation Panel A Mix	CC Mutation Panel C Mix 1367 Mix	S1 Mutation Panel C Mix 1367 Mix	S2 Mutation Panel C Mix 1367 Mix
D						
E						
F						
G						

Note: PC: Positive Control, NTC: No-Template Control (water), CC: Negative Control (Wild-type DNA), S1-2: Samples 1-2.

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

5.5. Instrument Set-Up

Roche Light cycler 96 and Roche Light cycler 480 II, Bio-Rad CFX 384 and ABI QuantStudio 5

1) Selection of Detectors:
   i. Use ‘ColoScape 4 Color *’ as the Detector on Roche light cycler
   ii. Select ‘All Channel’ as detection format on Bio-Rad CFX384
   iii. For ABI QuantStudio 5 and ABI 7500 Dx, assign individual mutation target in each mutation panel as ‘FAM’ “VIC” “ROX” according to Table 7a respectively, and select all Targets and assign to CY5 as internal control. Make sure to turn off ROX as passive reference dye.

2) Setup the cycling parameters as shown in Table 7b or Table 7c
3) Start the run

Note: Color compensation needs to be performed on Roche Light Cycler 480 II before running the assay. There is a separate protocol for color compensation. In Detection Formats, select custom filter combinations and name it as ColoScape 4 Color or other names for the custom detection format for the assay.

Table 7a. Assigning Reporter to ColoScape V1.1 Gene Targets

ColoScape	Target Name	Reporter
Mutation Panel A	APC 1309	FAM
	APC1367	HEX or VIC
Mutation Panel B	APC 1450/876	FAM
	KRAS 12	HEX or VIC
	CTNNB1 41	CFR610 or Texas Red or ROX
Mutation Panel C	BRAFV600E	FAM
	KRAS 13	HEX or VIC
	CTNNB1 45	CFR610 or Texas Red or ROX

Table 7b. Roche Light Cycler and Bio-Rad CFX 384 platforms cycling parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s) for Roche Instruments *	Cycles	Data Collection
Pre-Incubation	95	300	4.4	1	OFF
Denaturation	95	20	4.4	X50	OFF
XNA Annealing	70	40	2.2		OFF
Primer Annealing	64	30	1		OFF
Extension	72	30	1		FAM and HEX

*On Bio-Rad CFX 384, use the default ramp rate

Table 7c. ABI QuantStudio 5 cycling parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s)	Cycles	Data Collection
Pre-Incubation	95	300	1.6	1	OFF
Denaturation	95	20	1.6	X50	OFF
XNA Annealing	70	40	1.6		OFF
Primer Annealing	66	30	1		OFF
Extension	72	30	1		FAM and VIC

Part 6. Assessment of Real-Time PCR Results

The real-time PCR instrument generates a cycle threshold (Cq, also called as Ct) 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 and the higher initial template concentration (**please see MIQE Guidelines under References for more information).

6.1. Data Analysis for Light Cycler 480 II

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

6.2. Data Analysis for Bio-Rad CFX384

For the BioRad CFX384, open the qPCR run file using BioRad CFX manager. In the Log scale view, adjust the threshold to 100±20 for CY5 (Internal control). For each of the target mutation, adjust the threshold according to Table 8. Export the Cq data to excel. Exact threshold setting may be different for individual instruments.

Table 8. Bio-Rad CFX384 recommended threshold

Target	Recommended Threshold
ACT-B (internal control)	100± 20
APC c1309	250 ± 50
APC c1367	100 ± 20
APC c1450	280 ± 60
CTNNB1 c41	100 ± 20
CTNNB1 c45	100 ± 20
KRAS c12	120 ± 20
KARS c13	200 ± 40
BRAF c600	100 ± 20

6.3. Data Analysis for ABI QuantStudio 5 and ABI 7500 Dx

For the ABI Quant Studio 5 instrument and ABI 7500 Dx, adjust the threshold according to Table 9a and Table 9b respectively. Exact threshold setting may be different for individual instruments.

Export the Cq data to excel. 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

Table 9a. ABI QuantStudio 5 recommended threshold

Target	Recommended Threshold
ACT-B (internal control)	5000 ± 500
APC c1309	50000 ± 5000
APC 1367	7500 ± 7500
APC c1450	50000 ± 5000
CTNNB1 c41	15000 ± 1500
CTNNB1 c45	10000 ± 1000
KRAS c12	5000 ± 500
KARS c13	5000 ± 500
BRAF c600	8000 ± 800

Table 9b. ABI 7500 Dx recommended threshold

Target	Threshold Setting
ACTB	5000±500
APC 1309	100000±10000
APC 1367	14000±1400
APC 1450	30000±3000
CTNNB1 41	40000±4000
CTNNB1 45	10000±1000
KRAS 12	6000±600
KRAS 13	3000±300
BRAF	20000±2000

6.4. Evaluation of Controls

Verify that no amplification is observed in the non-template controls (NTC) for each of the reaction mixes. Cq should be Undetermined. For each control or sample, calculate the difference in Cq value between the mutation assay and the External Control Assay as follows: Cq difference (ΔCq) = Mutation Assay Cq - Internal 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 10a and Table 10b.

Table 10a. Acceptable values for positive controls and negative controls on Roche Light Cycler 480 II and Bio-Rad CFX384

Assay	Positive	Negative
Internal Control	25 < Cq < 31	25< Cq <31
APC 1309	ΔCq ≤ 7.3	ΔCq> 20.5
APC1367	ΔCq ≤ 5.8	ΔCq> 20.5
APC 1450/876	ΔCq ≤ 4.5	ΔCq> 7.5
CTNNB1 41	ΔCq ≤ 2.3	ΔCq> 6.9
CTNNB1 45	ΔCq ≤ 6.0	ΔCq> 7.6
KRAS12	ΔCq ≤ 5.1	ΔCq> 11.1
KRAS 13	ΔCq ≤ 3.3	ΔCq> 8.8
BRAF V600	ΔCq ≤ 4.4	ΔCq > 20.1

Table 10b. Acceptable values for positive controls and negative controls on ABI QuantStudio 5 and 7500 Fast Dx

Assay	Positive	Negative
Internal Control	25 < Cq < 31	25< Cq <31
APC 1309	ΔCq ≤ 4	ΔCq>18.8
APC 1367	ΔCq ≤ 7.2	ΔCq>18.8
APC 1450/876	ΔCq ≤ 4.4	ΔCq>6.9
CTNNB1 41	ΔCq ≤ 2.7	ΔCq>6
CTNNB1 45	ΔCq ≤ 5.7	ΔCq>7.1
KRAS12	ΔCq ≤ 4.6	ΔCq>14.6
KRAS 13	ΔCq ≤ 3.0	ΔCq>6.9
BRAF V600	ΔCq ≤ 4.9	ΔCq >19.5

6.5. Evaluating 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 (Roche Light cycler 480 II and Bio-Rad CFX 384) or 25<Cq<30 (ABI QuantStudio 5). 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 11.

Table 11. Acceptable internal control Cq ranges for samples

Validity	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 ≥ 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.

6.6. Scoring Mutational Status

If a Cq value is Undetermined, assign a Cq of 50 and proceed to analysis. The tables below show the ΔCq cutoff values and should be used to determine mutational status.

Note:

- If the Cq value of FAM is >40, the mutational status will be scored as "Negative" regardless of the ΔCq values.
- If the ΔCq value is greater than the cutoff value but less than "cutoff + 1", it is highly recommended to re-test the sample and send the PCR amplicons for Sanger sequencing when possible. The sequences of primer for Sanger sequencing will be provided upon request.

Table 12. Scoring mutational status for Roche Light Cycler 480 II

Sample type	Mutation	APC c1309/1367	APC c1450/876	CTNNB1 c41	CTNNB1 c45	KRAS c12	KRAS c13	BRAF c600
FFPE	Positive	< 20.9	< 9.2	< 8.8	< 8.8	< 9.1	< 8.1	< 20.7
	Negative	≥ 20.9	≥ 9.2	≥ 8.8	≥ 8.8	≥ 9.1	≥ 8.1	≥ 20.7

Table 13. Scoring mutational status for Bio-Rad CFX384

Sample type	Mutation	APC c1309/1367	APC c1450/876	CTNNB1 c41	CTNNB1 c45	KRAS c12	KRAS c13	BRAF c600
FFPE	Positive	<20.3	< 7.8	< 6.5	< 8.0	< 10.7	<8.1	<20.3
	Negative	≥20.3	≥ 7.8	≥ 6.5	≥ 8.0	≥ 10.7	≥8.1	≥ 20.3

Table 14. Scoring mutational status for ABI QuantStudio 5

Sample type	Mutation	APC c1309/1367	APC c1450/876	CTNNB1 c41	CTNNB1 c45	KRAS c12	KRAS c13	BRAF c600
FFPE	Positive	<17.7	< 6.8	< 5.1	< 6.4	< 14.6	<6.9	< 18.6
	Negative	≥17.7	≥ 6.8	≥ 5.1	≥ 6.4	≥ 14.6	≥6.9	≥ 18.6
Plasma cfDNA	Positive	Ct<40	< 7.8	< 12.8	< 7.1	Ct<40	< 12.5	Ct<40
	Negative	Ct≥40	≥ 7.8	≥ 12.8	≥ 7.1	Ct≥40	≥ 12.5	Ct≥40

Note: Values in the table are ΔCt values except those indicated as Ct values

Table 15. Scoring mutational status for ABI 7500 Fast Dx

Sample type	Mutation	APC c1309/136767	APC c1450/876	CTNNB1 c41	CTNNB1 c45	KRAS c12	KRAS c13	BRAF c600
FFPE	Positive	<20.3	< 9.6	< 7.9	< 7.0	< 12.3	<7.1	< 20.5
	Negative	≥20.3	≥ 9.6	≥ 7.9	≥ 7.0	≥ 12.3	≥7.1	≥ 20.5
Plasma cfDNA	Positive	Ct<40	< 10.7	< 10.1	<10.4	Ct< 11,1	<9.1	Ct< 40
	Negative	Ct≥40	≥ 10.7	≥ 10.1	≥ 10.4	Ct≥ 11.1	≥9.1	Ct≥ 40

Note: Values in the table are ΔCt values except those indicated as Ct values

6.7. 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, to differentiate between KRAS c12 and KRAS c13 mutations a combination of results from the two mixes should be used as described in Table 14 below.

Table 16. Interpretation of G12/G13 mutational status

Reaction Mix	Result Based on Tables 11-13	Mutational Status
KRAS c12 Reaction Mix KRAS c13 Reaction Mix	Positive Negative	G12 Mutation
KRAS c12 Reaction Mix KRAS c13 Reaction Mix	Positive Positive	G13 Mutation
KRAS c12 Reaction Mix KRAS c13 Reaction Mix	Negative Positive	G13 Mutation

Part 7. Assay Performance Characteristics

The performance characteristics of ColoScape™ kit were established on the Roche LightCycler 96, Roche LightCycler 480, Bio-Rad CFX 384 and ABI QuantStudio 5 real-time PCR instruments. The studies were performed using genetically defined reference standards (genomic DNA and FFPE) from cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England) and cfDNA reference standards from SeraCare (Massachusetts, US). 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 have been confirmed by genomic DNA sequencing and/or ddPCR. Additional samples consisted of cancer patient tissue, plasma samples and normal healthy donor DNA from tissue and plasma.

7.1. Analytical Accuracy and Comparison to Reference Method

The analytical accuracy was verified and validated through testing of well-characterized samples with known mutations verified by NGS, Sanger sequencing or ddPCR. Studies were conducted to demonstrate concordance in mutation status of FFPE and plasma samples. The results demonstrated a 100% match between reference methods and the ColoScape™ kit.

7.2. Precision

Precision of the ColoScape™ kit was determined with defined analytical levels of genomic DNA with known mutational status and allelic frequencies.

- To establish lot-to-lot variation, a reproducibility study was performed using three different lots of kit. 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 on Roche LC480 and Bio-Rad CFX 384 instruments.
- Inter-assay %CV was established for same lot of reagents tested on the same instrument by the same user.
- Intra-assay %CV was established through performance of kit on reference samples run in replicates of nine.
- Operator variability was evaluated with one lot of reagents by two operators.

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

Table 17. Summary of reproducibility results

Variation	%CV
Intra-assay	≤ 3%
Inter-assay	≤ 5%
Lot-to-Lot Variation	≤ 4%
Operator Variability	≤ 3%

Table 18. Intra-assay reproducibility results on Roche LC480 II

Target	1% Mutant			0.5% Mutant		
	Average Cq	SD	%CV	Average Cq	SD	%CV
APC 1309/1367	33.57	0.71	2.11%	35.22	1.52	4.32%
APC 1450/876	32.27	0.31	0.96%	33.12	0.27	0.81%
CTNNB1 41	31.86	0.33	1.04%	32.49	0.3	0.93%
CTNNB1 45	31.54	0.32	1.03%	32.43	0.43	1.34%
KRAS 12	33.37	0.45	1.36%	33.79	0.91	2.69%
KRAS 13	34.33	0.59	1.71%	34.85	0.7	2.00%
BRAF 600	33.21	0.24	0.73%	34.06	0.78	2.30%
Internal Control	29.19	0.42	1.43%	29.15	0.39	1.35%

The intra-assay data demonstrated good reproducibility with low %CV (Table 18).

7.3. Analytic Sensitivity and Limit of Detection (LOD)

To determine the limit of detection (LOD) and analytical sensitivity of the kit, the studies were performed using serial dilutions of mutant DNA (reference FFPE and cfDNA) in wild-type background. The wild-type DNA used for dilution was obtained from mutant-free FFPE and normal human plasma respectively. Mutant allelic frequencies tested were 1%, 0.5% and 0.1% at 2.5, 5 and 10ng/reaction DNA input levels. The mutant copy numbers present in genomic DNA with 1%, 0.5% and 0.1% variant allelic frequency at different DNA input levels are shown in Table 19a.

Table 19a. Mutant DNA copy numbers at different allelic frequencies

Variant Allelic Frequency	Mutant DNA Copy Numbers at Different DNA Inputs		
	10ng DNA	5ng DNA	2.5ng DNA
1%	28 copies	14 copies	7 copies
0.50%	14 copies	7 copies	3.5 copies
0.10%	2.8 copies	1.4 copies	0.7 copies

Table 19b. LOD summary determined using genomic DNA reference standards

Target Mutation	DNA Input, ng/well		
	VAF%	10 ng	5 ng
		% Correct Call	% Correct Call
APC 1309	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	20%	0%
APC 1367	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	50%	10%
APC 1450/876	1% mutation	100%/100%	100%/100%
	0.5% mutation	100%/100%	100%/100%
	0.10% mutation	60%/20%	60%
CTNNB1 41	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	30%	10%
CTNNB1 45	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	80%	40%
KRAS 12	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	80%	45%
KRAS 13	1% mutation	100%	100%
	0.5% mutation	100%	80%
	0.10% mutation	45%	45%
BRAF V600	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	15%

Table 19c. LOD summary determined using cfDNA reference standards

Target Mutation	DNA Input, ng/well		
	VAF%	10 ng	5 ng
		% Correct Call	% Correct Call
APC 1309	1% mutation	100%	100%
	0.5% mutation	100%	95%
	0.10% mutation	65%	40%
APC 1367	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	100%
APC 1450	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	90%	90%
CTNNB1 41	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	100%
CTNNB1 45	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	90%	85%
KRAS 12	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	95%
KRAS 13	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	90%	95%
BRAF V600	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	95%

Conclusion:

- All targets can be detected at 1% variant allelic frequency at 5 ng or 10 ng DNA input per PCR reaction.
- For FFPE DNA samples, 0.5% variant allelic frequency can be detected at 10 ng DNA input.
- For plasma cfDNA samples, 0.5% variant allelic frequency in all targets except APC 1309
- can be detected at 5 ng DNA input.
- To obtain high sensitivity, the recommended DNA input is a minimum of 10ng/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.

7.4. Analytic Specificity

Analytical specificity of the kit was determined as the correct calling of the samples with no mutation at different concentrations of WT template. There were no false positive calls for up to 320ng of gDNA per well and up to 20ng FFPE DNA.

Cross-reactivity of the assays within the kit was tested with one or more mutations present in a mixed positive control at 50% allelic frequency.

Table 20. Analytic specificity: cross-reactivity

Assay	Expected Mutations in Tested 50% Templates							
	APC 1309	APC 1367	APC 1450; KRAS 12	CTNNB1 41	CTNNB1 45; KRAS 13	KRAS 12	KRAS 13	BRAF 600; CTNNB1 45; KRAS 13
APC 1309	+	_	_	_	_	_	_	_
APC 1367	_	+	_	_	_	_	_	_
APC 1450	_	_	+	_	_	_	_	_
CTNNB1 41	_	_	_	+	_	_	_	_
CTNNB1 45	_	_	_	_	+	_	_	+
KRAS 12	_	_	+	_	*	+	*	*
KRAS 13	_	_	_	_	+	_	+	+
BRAF V600	_	_	_	_	_	_	_	+

The data demonstrates that the ColoScape™ Kit can correctly identify several mutations within one template. There is cross reactivity between KRAS12 and KRAS13, due to the proximity of the mutations, which can be differentiated (Refer to Table 16).

7.5. Cut-Offs

DNA from FFPE and plasma samples were used to establish cut-offs for the assay. Please refer to Tables 12-14.

7.6. 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.

7.7. 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 (same as used in FFPE preparation) 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 cut-offs. None of the potentially interfering substances evaluated at concentrations encountered in normal use impacted the ability of the kit to distinguish between mutation-positive and mutation-negative control samples.

7.8. 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.

7.9. Shelf-Life

Approximately 12 months after production of kit-see product labels for actual expiration date. Do not use expired reagents from the kit.

7.10. Clinical Performance of the Assay

Clinical sensitivity and specificity were 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 12-14.

Table 21. Clinical Sensitivity and Specificity

Test	Types of Clinical Samples	Clinical Parameter	
I		Specificity	Sensitivity
	CRC (cfDNA)	N/A	100%
	CRC FFPE	N/A	90%
	Advanced Adenomas	95%	60%
II	FFPE	100%	93%
	cfDNA	100%	90%
III	cfDNA (Pre-cancer)	92%	70%
Average	FFPE	100%	92%
	cfDNA	96%	95%

Part 8. Symbols Used in Packaging

Table 22. Symbols used in packaging

Symbol	Definition
	Authorized Representative in the European Community
	Catalog Number
	Manufactured By
	Temperature Limitation
	Batch Code
	Expiration Date
	Authorized Representative in the European Community
1011-11-17	Date Format (year-month-day)
1011-11	Date Format (year-month)

References

1. Chang, Y. S., Yeh, K. T., Chang, T. J., Chai, C., Lu, H. C., Hsu, N. C., & Chang, J. G. (2009). Fast simultaneous detection of K-RAS mutations in colorectal cancer. *BMC Cancer*, *9*, 1-7.
2. Chan, T. L., Zhao, W., Leung, S. Y., & Yuen, S. T. (2003). BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. *Cancer Res*, *63*(16), 4878-81.
3. Dong, S. M., Traverso, G., Johnson, C., Geng K, Goodman SN, D'Allessio, L., Favis, R., Boynton, K., … Hibi, K. (2001). Detecting colorectal cancer in stool with the use of multiple genetic targets. *J Natl Cancer Inst.*, *93*(11), 858-65.
4. Fodde, R., Smits, R., & Clevers, H. (2001). APC, signal transductCqction and genetic instability in colorectCqctal cancer. *Nature Reviews Cancer*, *1*(1), 55-67.
5. Kobunai, T., Watanabe, T., Yamamoto, Y., & Eshima, K. (2010). The frequency of KRAS mutation detection in human colon carcinoma is influenced by the sensitivity of assay methodology: A comparison between direct sequencing and real-time PCR. Biochem Biophys Res Commun. *Biochem Biophys Res Commun.*, *395*(1), 158-162.
6. Kwon, M. J., Lee, S. E., Kang, S. Y., & Choi, Y. L. (2011). Frequency of KRAS, BRAF, and PIK3CA mutations in advanced colorectal cancers: Comparison of peptide nucleic acid-mediated PCR clamping and direct sequencing in formalin-fixed, paraffin-embedded tissue. *Pathol Res Pract*, *207*(12), 762-768.
7. KygerMichael J., E. M., Krevolin, M. D., & Powell, M. J. (1998). Detection of the Hereditary Hemochromatosis Gene Mutation by Real-Time Fluorescence Polymerase Chain Reaction and Peptide Nucleic Acid Clamping. *Analytical Biochemistry*, *260*(2), 142-148.
8. Lüchtenborg, M. I., Weijenberg, M. P., Wark, P. A., Saritas, A. M., Roemen, G. M., Van Muijen, G. N., & De Bruïne, A. P. (2005). Mutations in APC, CTNNB1 and K-ras genes and expression of hMLH1 in sporadic colorectal carcinomas from the Netherlands Cohort Study. *BMC Cancer*, *5*, 160.
9. Schneider, M., Scholtka, B., Gottschalk S, D, K, U., Faiss, S., Schatz, D., & Berghof-J€ager, K. (2011). Detection of up to 65% of precancerous lesions of the human colon and rectum by mutation analysis of apc, kras, b-raf and ctnnb1. *Cancers*, *3*, 91–105.
10. Scholtka, B., Schneider, M., Melcher, R., Katzenberger, T., Friedrich, D., & Berghof-J€ager, K. (2009). A gene marker panel covering the Wnt, and the Ras-Raf-MEK-MAPK signalling pathways allows to detectCqct gene mutations in 80% of early (UICC I) colon cancer stages in humans. *Cancer Epidemiol*, *33*, 123-129.
11. Smith, G., Carey, F. A., Beattie, J., Wilkie, M. J., Lightfoot, T. J., Coxhead, J., Garner, R. C. (2002). Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc Natl Acad Sci U S A*, *99*(14), 9433-8.
12. Sparks, A. B., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectCqctal cancer. *Cancer Res*, *58*(6), 1130-1134.
13. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., & Leppert, M. (1988). Genetic alterations during colorectal-tumor development. *N Engl J Med*, *319*, 525–32.
14. Steinberg, P.; Scholtka, B. Method for conducting non-invasive early detection of colon cancer and/or of colon cancer precursor cells. US Patent: 0,172,823 A1, 2007.