PRODUCT INSTRUCTION MANUAL

ColoScape™ Colorectal Cancer Detection Test

For qPCR Assays
ColoScape™ V2.0

CATALOG NUMBER	COMPANY	INTENDED USE
DC-30-0004 (24 Reactions)	DiaCarta, Inc. 4385 Hopyard Rd., Suite 100 Pleasanton, CA 94588 United States P: +1 510-878-6662 F: + 1 510-735-8636 E: information@diacarta.com	Research Use Only

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

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PART 1. INTENDED USE AND ASSAY SUMMARY

1.1. Intended Use

ColoScape™ Colorectal Cancer Detection Test is a multiplex real-time PCR based *in vitro* diagnostic assay for qualitative and simultaneous detection of colorectal cancer associated mutations and methylations. The mutation genes include *APC* (codons 1309, 1367, 1450 and 876), *BRAF* (codon 600), *CTNNB1* (codons 41 and 45), *KRAS* (codons 12 and 13), *NRAS* (codons 12 and 13), *PIK3CA* (codon 545), *SMAD4* (codon 361) and *TP53* (codons 175, 248 and 273). In addition, the methylation genes include *MYO1G*, *KCNQ5*, *C9ORF50*, *FLI1*, *CLIP4*, *ZNF132,* and *TWIST1*.

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 and methylations in the targeted regions but does not specify the exact nature of the mutation and methylation. ColoScape™ Colorectal Cancer Mutation & Methylation Detection kit is for use in diagnostic procedures.

1.2. Assay Summary

ColoScape™ Colorectal Cancer Detection Test qPCR kit leverages DiaCarta's proprietary QClamp® XNA technology (see below) enabling the detection of small amount of gene mutations or gene methylation within the background of a majority of normal (wild type) DNA. The assay uses XNA clamps to specifically bind to and block the wild-type (normal) target gene sequence and selectively amplify the mutant DNA target sequence. Within six reactions, 8 genes, 16 codon and 61 mutations and 7 genes methylation can be detected within 4 hours. The mutational and methylation status of a sample was determined by Cq (quantification cycle) value. If the sample Cq is < cut-off, the mutation or methylation is detected. If the sample Cq is ⩾ cut-off, the mutation or methylation is not detected.

1.3. QClamp® Technology for Mutation and Methylation Detection

The ColoScape™ Colorectal Cancer Detection Test is based on xenonucleic acid (XNA) mediated PCR clamping technology. XNA is a synthetic DNA analog in which the phosphodiester backbone has been replaced by a 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 or methylation 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 and methylation DNA. DNAXNA oligomers are not recognized by DNA polymerases and cannot be utilized as primers in subsequent real-time PCR reactions.
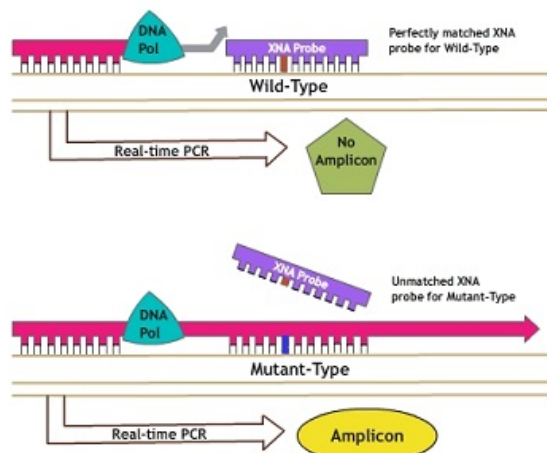

Figure 1. Principle of the ColoScape™ Mutation & Methylation Test in Targeted Genes

1.4. Gene Mutations, Methylation 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 proteins. 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 MAPK/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 wildtype 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.

Tumor protein p53 (*TP53*) is a gene that codes for a tumor suppressor protein, cellular tumor antigen p53. The protein regulates expression of genes involved in cell cycle arrest, apoptosis, senescence, DNA repair, and changes in metabolism. TP53 is the most frequently mutated gene in cancers, including ovarian, colon, and esophageal cancers. It is also significantly mutated in many other cancer types. *TP53* is altered in 68% of colorectal carcinoma patients.

NRAS (neuroblastoma RAS viral (v-ras) oncogene homolog) encodes for the GTPase NRas protein, one of three human RAS proteins. RAS proteins are small GTPases that are central mediators downstream of growth factor receptor signaling and therefore critical for cell proliferation, survival, and differentiation. NRAS is implicated in the pathogenesis of several cancers. *NRAS* is altered in 5% of colorectal adenocarcinoma patients.

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) is a gene that encodes the protein phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform, a subunit of the PI3K protein. Phosphatidyl 3-kinases (PI3K) are a family of lipid kinases involved in many cellular processes, including cell growth, proliferation, differentiation, motility, and survival. Mutant PIK3CA has been implicated in the pathogenesis of several cancers, including colon cancer, glioma, gastric cancer, breast cancer, endometrial cancer, and lung cancer. *PIK3CA* is altered in 20.75% of colon adenocarcinoma patients.

SMAD4 mutation is present in 3% of AACR GENIE cases, with colon adenocarcinoma, pancreatic adenocarcinoma, lung adenocarcinoma, colorectal adenocarcinoma, and rectal adenocarcinoma having the greatest prevalence.

Besides the expression changes caused by mutations, there are also epigenetic changes, such as DNA methylation, which impact the CRC related genes. Methylation within the promoter regions of tumor suppressor genes causes their silencing, and methylation within the gene itself can induce mutational events. These mechanisms may play a fundamental role in precipitating the development of a large and diverse number of human cancers. One of the most common ways to detect DNA methylation changes is using bisulfite conversion of DNA. Treating DNA with bisulfite leads to the deamination of cytosines to uracil, while methylated cytosines remain unchanged.

Myosin 1G(*MYO1G*) is a gene coding the plasma membrane-associated class I myosin. Myosins play essential roles in chromosomal and centrosomal amplification. MYO1G regulates cell elasticity, migration, exocytosis, and endocytosis in B-lymphocytes. Recent studies showed that MYO1G methylation could be a prognostic factor for colorectal cancer.

Potassium Voltage-Gated Channel Subfamily Q Member 5 (*KCNQ5*) is a member of KCNQ gene family that is differentially expressed in subregions of the brain and in skeletal muscle. KCNQ5 and KCNQ3 work together to form heterometric channels. Recent study shows that *KCNQ5* has significantly different methylation patterns between CRC and normal tissues.

Chromosome 9 open reading frame 50 is a protein that in humans is encoded by the *C9orf50* gene. Studies shows that Methylated C9orf50 fragments were blank in plasma from healthy individuals and positive >70% of plasma from CRC patients.

FLI1 gene encodes a transcription factor containing an ETS DNA-binding domain. *FLI1* gene function as a tumor suppressor in CRC and hypermethylation of *FLI1* gene could cause the loss of *FLI1* gene expression in CRC.

CAP-Gly domain containing linker protein family member 4 (CLIP4) is involved in plus-end blinding of microtubule, immune response-related biological process, cell migration and cancer metastases. Methylated *CLIP4* gene plays an important role in CRC development.

The zinc-finger protein (ZNF) families are involved in transcriptional regulation, ubiquitin-mediated protein degradation, signal transduction, and other cellular processes. *ZNF132* genes were significantly hypermethylated and downregulated in CRC compared to normal tissues.

Twist Family BHLH Transcription Factor 1 (*TWIST1*) encodes transcription factor that plays an important role in embryonic development. Hypermethylated and overexpressed *TWIST1* gene was found in multiple human cancers including CRC.

1.5. Gene Mutations and Methylation Covered in the ColoScape™ Colorectal Cancer Detection Test qPCR Kit

The following mutations in eight genes commonly found in colorectal cancer and detected by this kit are listed in Table 1. The kit detects total 8 genes, 16 codons and 61 mutations for colorectal cancer. Methylation targeted genes include *MYO1G, KCNQ5, C9ORF50, FLI1, CLIP4, ZNF132, and TWIST1* in this kit.

Table 1. List of Mutations and Cosmic Identities Found in ColoScape™ Colorectal Cancer Detection Test Targeted Genes

Genes	Exon	Nucleotide Change	Amino Acid Change	Cosmic No.
KRAS	2	c.35G>C	p. G12A	COSM522
		c.34G>C	p. G12R	COSM518
		c.35G>A	p. G12D	COSM521
		c.34G>T	p. G12C	COSM516
		c.34G>A	p. G12S	COSM517
		c.35G>T	p. G12V	COSM520
		c.38G>A	p. G13D	COSM532
		c.37G>T	p. G13C	COSM527
		c.37G>C	p. G13R	COSM529
APC	15	c.2626C>T	R876*	COSM18852
		c.3921_3925delAAAAG	E1309fs*	COSM18764
		c.4099C>T	Q1367*	COSM13121
		C.4348C>T	R1450*	COSM13127
CTNNB1	3	c.121A>G	p.T41A	COSM5664
		c. 122C>T	p.T41I	COSM5676

		c.133T>C	p.S45P	COSM5663
		c.134C>T	p.S45F	COSM5667
		C133-135delTCT	p.S45del	COSM6128
BRAF	15	c.1799T>A	p.V600E	COSM476
NRAS	2	c.35G>C	p.G12A	COSM565
		c.34G>T	p.G12C	COSM562
		c.34G>A	p.G12S	COSM563
		c.35G>T	p.G12V	COSM566
		c.34G>C	p.G12R	COSM561
		c.35G>A	p.G12D *	COSM564
		c.37G>A	p.G13S	COSM571
		c.38G>A	p.G13D	COSM573
		c.37G>T	p.G13C	COSM570
		c.38G>C	p.G13A	COSM575
		c.37G>C	p.G13R	COSM569
		c.38G>T	p.G13V	COSM574
TP53	5	c.523C>T	p.R175C	COSM43680
		c.523C>G	p.R175G	COSM10870
		c.524G>C	p.R175P	COSM45416
		c.524G>A	p.R175H *	COSM10648
		c.523C>A	p.R175S	COSM43931
		c.524G>T	p.R175L	COSM10718
	7	c.743G>A	p.R248Q *	COSM10662
		c.743G>C	p.R248P	COSM11491
		c.743G>T	p.R248L	COSM6549
		c.744G>A	p.R248=	COSM45084
		c.743_744delinsAA	p.R248Q	COSM44908
		c.742C>G	p.R248G	COSM11564
		c.742C>T	p.R248W	COSM10656
		c.741_742delinsTT	p.R248W	COSM6545
	8	c.817C>A	p.R273S	COSM43909
		c.818G>A	p.R273H *	COSM10660
		c.817C>G	p.R273G	COSM43843
		c.818G>C	p.R273P	COSM43896
		c.817C>T	p.R273C	COSM10659
		c.818G>T	p.R273L	COSM10779
PIK3CA	9	c.1634A>T	p.E545V	COSM27155
		c.1634A>C	p.E545A	COSM12458
		c.1635G>T	p.E545D	COSM765
		c.1634A>G	p.E545G	COSM764
		c.1635G>C	p.E545D	COSM27374
		c.1633G>A	p.E545K	COSM763
SMAD4	9	c.1081C>A	p.R361S	COSM14151
		c.1081C>G	p.R361G	COSM1158192

| | | c.1082G>A | p.R361H | COSM14122 |
| | | c.1081C>T | p.R361C | COSM14140 |

* The probe is specifically designed for only this mutation.

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. TERMINOLOGY, KIT COMPONENTS, INSTRUMENTS, AND HANDLING PRECAUTIONS

2.1. Terminology

2.1.1. Controls

- Negative Control (NC), Positive Control (PC), and No Template Control (NTC) must be included in each assay run.

2.1.2. Negative Control (NC)

- Commercially available wild type (no target mutations) fragmented human genomic DNA as the template.
- Efficient binding by XNA clamps and thereby suppressing wild type allele amplification.

2.1.3. Positive Control (PC)

- A mix of synthetic reference mutant templates for each target of the assay at certain allelic frequency in wild type fragmented human genomic DNA.
- Not binding to the XNA clamps, allowing amplification of the mutant template.
- Positive controls must show the appropriate values (See Table 8a to 8c) in both targets (FAM or HEX or TexasRed) and reference (CY5) channels for the run to be valid.

2.1.4. No template Control (NTC)

- Nuclease free water is used in place of template
- No amplification should be observed in all channels, assuring the absence of contamination during assay set-up.

2.1.5. Reference Gene (Internal Control)

Housekeeping gene ACTB is used as a reference to assess the quantity and quality of amplifiable DNA and to demonstrate if the reagents are working correctly.

When assessed using the CY5 channel, this reference gene as internal 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.

2.1.6. Cq Value

The qPCR instrument generates a quantitation cycle value (Cq) 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*).

2.2. Kit Components

The ColoScape™ qPCR kit uses liquid reaction reagents (**Table 2**).
Note: We provide enough positive and negative control materials to run the assay four times. Users should freeze these vials at -20°C after each use.

Table 2. Package Contents

Box #	Part #	Cap Color	Kit Component Name	Content	24 rxn kit
1	1011641	Blue	ColoScape Mutation Panel A Primer Probe Mix	APC E1309 and Q1367 Primers and probes (PPM)	1 × 48 µl
	1011651	Green	ColoScape Mutation Panel B Primer/Probe Mix	APC R1450/876, KRAS G12 and CTNNB1 T41 PPM	1 × 48 µl
	1011661	Yellow	ColoScape Mutation Panel C Primer Probe Mix	BRAF V600, KRAS G13 and CTNNB1 S45 PPM	1 × 48 µl
	1011671	Violet	ColoScape Mutation Panel D Primer probe Mix	TP53 c273, NRAS G12 and TP53 R175 PPM	1 × 48 µl
	1011681	Amber	ColoScape Mutation Panel E Primer Probe Mix	TP53 c248, SMAD4 R361 and PIK3CA E545 PPM	1 × 48 µl
	1011691	Natural	2X ColoScape™ qPCR Master Mix	PCR Reaction Premix	2 × 1200 µl
	1011701	Natural	No Template Control (NTC)	Nuclease-Free Water	1 × 1400 µl
2	1011711	Blue	ColoScape Mutation Panel A XNA	APC E1309 and Q1367 XNA	1 × 48 µl
	1011721	Green	ColoScape Mutation Panel B XNA	APC R1450/876, KRAS G12 and CTNNB1 T41 XNA	1 × 48 µl
	1011731	Yellow	ColoScape Mutation Panel C XNA	BRAF V600, KRAS G13 and CTNNB1 S45 XNA	1 × 48 µl
	1011741	Violet	ColoScape Mutation Panel D XNA	TP53 c273, NRAS c12 and TP53 R175 XNA	1 × 48 µl
	1011751	Amber	ColoScape Mutation Panel E XNA	TP53 c248, SMAD4 R361 and PIK3CA E545 XNA	1 × 48 µl
	1011771	Red	ColoScape™ Positive Control 1	APC E1309, Q1367, R1450, CTNNB1 T41, CTNNB1 S45, KRAS G12, KRAS G13 and BRAF	1 × 80 µl
	1011781	Orange	ColoScape™ Positive Control 2	APC R876, TP53 R273, NRAS G12 and TP53 R175, TP53 R248, SMAD4 R361 and PIK3CA	1 × 80 µl
	1011761	Natural	ColoScape™ Negative Control	Wild-type human diploid DNA	1 × 80 ul
3	1011841	Orange	ColoScape Methylation Panel F Primer Probe Mix	MYO1G, KCNQ5, C9orf50, FLI1, CLIP4, ZNF132 and Twitst1 PPM	1 × 96 µl
	1011801	Orange	ColoScape Methylation Panel F XNA	MYO1G, KCNQ5, C9orf50, FLI1, CLIP4, ZNF132 and Twitst1 XNA	1 × 48 µl
	1011791	Natural	2X ColoScape™ qPCR Master Mix	PCR Reaction Premix	1 × 480 µl
	1011811	Natural	ColoScape™ Methylation Negative Control	Fragmented unmethylated human genomic DNA	1 × 30 µl
	1011821	Red	ColoScape™ Methylation Positive Control	MYO1G, KCNQ5, C9orf50, FLI1, CLIP4, ZNF132 and Twitst1 methylated templates	1 × 30 µl
	1011831	Natural	No Template Control (NTC)	Nuclease-Free Water	1 × 900 µl

2.3. 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 MinElute ccfDNA Mini Kit (QIAGEN, Cat. No. 55204) or equivalent
- EZ DNA Methylation-Lightning Kit (ZYMO Research, Cat. No. D5030 or D5031) 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.

2.4. Instruments

The assays have been developed and validated on the instruments shown in Table 3. Instrument platforms not listed in Table 3 should be validated by the individual labs. Guidance for validation can be obtained from DiaCarta upon request.

Table 3. List of Instruments Validated with this Kit.

Company	Model
Roche	LightCycler 480 II (LC480II)
Bio-Rad	CFX384
ABI	QuantStudio 5
ABI	7500 Fast Dx

2.5. 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, please contact DiaCarta or the local distributors as soon as possible.

The kit should be stored 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 in a pre-amplification area and the controls 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.

2.6. 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.

2.7. 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 in order 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 3. INSTRUCTIONS FOR USE

3.1. DNA Isolation

3.1.1 cfDNA from Plasma

We recommend that apply Streck Cell-Free DNA BCT® (Cat. No. 230469, 230470, 230471) for whole blood collection. Two tubes of 10 mL whole blood (total 20 mL) are collected.

- Step 1. To separate plasma, centrifuge whole blood at 1,600 x g for 10 minutes at room temperature.
- Step 2. Remove the upper plasma layer and transfer to a new conical tube (not provided).
- Step 3. Centrifuge the plasma at 16,000 x g for 10 minutes.
- Step 4. Isolate cell-free DNA per kit manufacturer instructions.

FREEZING AND THAWING PLASMA

- To Freeze: For long-term storage, after spinning, collect and transfer the upper plasma layer to a cryogenic tube (not provided) and freeze at -80°C.
- To Thaw: Thaw cryogenic tubes at appropriate temperature as specified in your protocol. Note: If cryoprecipitates form in the plasma, vortex the tube for 30 seconds after thawing. Do not centrifuge the plasma.

These two kits from Qiagen are recommended for cfDNA isolation

- QIAamp MinElute ccfDNA Kit (QIAGEN, Cat. No. 55204)
- QIAamp Circulating Nucleic Acid Kit (QIAGEN, Cat. No. 55114)

Follow the DNA isolation procedure according to manufacturer's protocol:

https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/cell-free-dna/qiaamp-minelute-ccfdna-kits/

Elution is performed using Ultra-clean Water. The elution volume can be 25 µL for each sample and re-elute one more time: (1) Carefully pipet 25 µL of Ultra-clean Water into the center of the membrane. Close the lid and incubate at room temperature (RT) for 1 minute. Centrifuge at full speed (20,000 × g; 14,000 rpm) for 1 minute to elute the nucleic acids. (2) Re-apply the eluate to the column for re-elution. Place the QIAamp UCP MinElute Column in the same elution tube. Aspirate the eluate in the 1.5 ml elution tube from above step and reload it onto the center of the membrane. Close the lid and incubate 1 minute at RT. Centrifuge 1 minute at full speed (20,000 × g; 14,000 rpm) [Figure 2a]. Therefore, (3) You will have one tube with 25 µL cfDNA for DNA mutation test and methylation test (combine to 50 µL if two whole bloods tubes [20 mL] per patient).
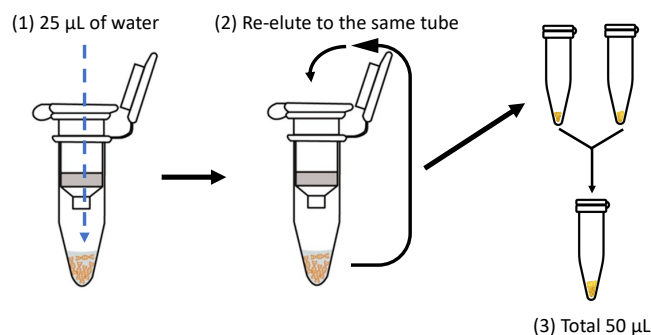

(1) 25 µL of water   (2) Re-elute to the same tube

(3) Total 50 µL

Figure 2a. Workflow of cfDNA Elution Process Using a Column-Based Extraction Method

As a backup, additional 10 µL Ultra-clean Water can be added into the membrane of column and aspirate the eluate in a new elution tube, close the lid, and incubate 1 min at RT. Centrifuge 1 min at full speed (20,000 × g; 14,000 rpm). Therefore, you will have another tube with 10 µL cfDNA (less DNA) to add up enough cfDNA for the test (combine to 20 µL if two whole bloods tubes [20 mL] per patient) (see **Figure 2b**).
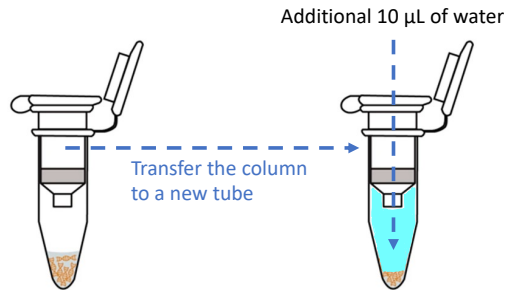

Figure 2b. Workflow of cfDNA Elution Process Using a Column-Based Extraction Method

This assay requires minimum 30 ng of cfDNA per sample (5 ng/reaction, 6 reactions). After cfDNA isolation, measure the concentration using fluorometric analysis (i.e., Qubit) and make sure cfDNA per reaction has 5-10 ng/8 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.

3.1.2. Genomic DNA from FFPE

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

3.1.3. DNA Bisulfite Conversion

After DNA isolation, take 5 to 10 ng of DNA and process bisulfite conversion. For consistency, we recommend using a commercial kit as follows:

- EZ DNA Methylation-Lightning Kit (ZYMO Research, Cat. No. D5030 or D5031) or equivalent

Follow the DNA methylation protocols to bisulfite convert cfDNA:

https://www.zymoresearch.com/collections/ez-dna-methylation-lightning-kits/products/ez-dna-methylation-lightning-kit

1) Mix lightning conversion reagent with cfDNA samples (as well as ColoScape™ Methylation Negative Control) and do the conversion following the steps:
   a. 98°C for 8 minutes
   b. 54°C for 60 minutes
   c. 4°C storage for up to 20 hours
2) Use M-Binding Buffer to collect bisulfite converted cfDNA in to Zymo-spin IC column. (5 minutes)
3) Use M-Wash Buffer and L-Desulphonation Buffer to wash converted cfDNA. (15~20 minutes, no more than 20 minutes)
4) Use ssRNA in Nanodrop to quantify Bisulfite-converted DNA.

3.2. Preparation of Reagents

- 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.

- Thaw all primer/probe mixes, XNAs, Positive Control, Negative Control, Nuclease-Free Water, and 2X PCR master mix provided at room temperature for a minimum of 30 minutes
- Heat XNA Mixes at 65°C until assay mixes setup.
- Vortex all components except the PCR Master Mix and Primer/ probe Mixes for 5 seconds and perform a quick spin.
- Gently invert the vials of qPCR Master Mix and Primer/probe Mixes several times
- Pre-mix the qPCR Master Mix, Primer/Probe Mixes and XNA Mixes into assay mixes with proper volumes according to the number of samples for qPCR detection
- The PCR reactions are set up in a total volume of 20 µl/reaction, following the guidelines in the **Tables 4a** & **4b**:

Table 4a. ColoScape™ Colorectal Cancer Mutation Assay Components and Reaction Volume

Components	Volume/Reaction
2X PCR Master mix	10 µl
Primer and probe Mix	1 µl
XNA	1 µl
DNA sample or Controls	Sample: x µl (5 – 10 ng equivalent), Controls: 2 µl
Total volume	20 µl

Table 4b ColoScape™ Colorectal Cancer Methylation Assay Components and Reaction Volume

Components	Volume/Reaction	PC/NC/Reaction
2X PCR Master mix	10 µl	10 µl
Primer and probe Mix	2 µl	2 µl
XNA	1 µl	1 µl
DNA sample or Controls	x µl (5 – 10 ng equivalent)	1 µl + 6 µl water
Total volume	20 µl	20 µl

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

3.3. Preparation of Assay Mixes

3.3.1. Mutation Assay

Assay mixes should be prepared just prior to use. Label a micro centrifuge tube (not provided) for each reaction mix, as shown in Table 5a. For each control and mutation detection reaction, prepare sufficient working assay mixes for the DNA samples, two Positive Controls, one Nuclease-Free Water for No Template Control (NTC), and one WT Negative Control, according to the volumes in Table 5a. 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.

Table 5a. Preparation of Mutation Assay Mixes

Kit Component Name	Volume of 2X PCR Master Mix	Volume of Primer and Probe Mix	Volume of XNA
ColoScape Mutation Panel A Mix	10 µl x (n+1)	1 µl x (n+1)	1 µl x (n+1)
ColoScape Mutation Panel B Mix	10 µl x (n+1)	1 µl x (n+1)	1 µl x (n+1)
ColoScape Mutation Panel C Mix	10 µl x (n+1)	1 µl x (n+1)	1 µl x (n+1)
ColoScape Mutation Panel D Mix	10 µl x (n+1)	1 µl x (n+1)	1 µl x (n+1)
ColoScape Mutation Panel E Mix	10 µl x (n+1)	1 µl x (n+1)	1 µl x (n+1)

Note: n = number of reactions (DNA samples plus 4 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 (Mutation): Negative Control (NC), Positive Control (PC) and No Template Control (NTC) must be run with each reaction mix, every time the assay is run.

3.3.2. Methylation Assay

Assay mixes should be prepared just prior to use. Label a micro centrifuge tube (not provided) for each reaction mix, as shown in **Table 5b**. For each control and methylation 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 unmethylated Negative Control, according to the volumes in Table 5b. 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.

Table 5b. Preparation of Methylation Assay Mixes

Kit Component Name	Volume of 2X PCR Master Mix	Volume of Primer and Probe Mix	Volume of XNA
ColoScape Methylation Panel F Mix	10 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 (Methylation): Negative Control, Positive Control and No Template Control must be run with each reaction mix, every time the assay is run.

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

3.4.1. Mutation Assay

Please always use white plates, strips, or tubes. In pre-amplification area, add 12 µl of the appropriate assay mix to the plate or tubes. In designated template area, add 8 µl of template to each well.

Table 6a. Suggested Plate Layout (Mutation Assay)

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

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

Table 6a is a suggested plate set-up for a single experiment analyzing 2 unknown samples. When all reagents have been added to the plate, tightly seal the plate to prevent evaporation. Spin at 1,000 rpm for 1 minute to collect all the reagents. Place in the real-time PCR instrument immediately.

3.4.2. Methylation Assay

Please always use white plates, strips, or tubes. In pre-amplification area, add 13 µl of the appropriate assay mix to the plate or tubes. In designated template area, add 7 µl of template to each well.

Table 6b. Suggested Plate Layout (Methylation Assay)

	1	2	3	4	5	6
A	NTC Methylation Panel F Mix	PC Methylation Panel F Mix	NC Methylation Panel F Mix	S1 Methylation Panel F Mix	S2 Methylation Panel F Mix	S3 Methylation Panel F Mix
B						
C						
D						
E						
F						
G						
H						

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

Table 6b 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 1,000 rpm for 1 minute to collect all the reagents. Place in the real-time PCR instrument immediately.

3.5. Instrument Set-Up

3.5.1. Mutation Assay

Roche LightCycler 480 II, Bio-Rad CFX 384, ABI QuantStudio 5 and 7500 Dx
1) Selection of Detectors:
   i. Use 'ColoScape 4 Color' as the Detector on Roche LightCycler 480 II.
   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 LightCycler 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™ Colorectal Cancer Detection Test Mutation Gene Targets

ColoScape	Target Name	Reporter
Mutation Panel A	APC E1309	FAM
	APC Q1367	HEX or VIC
	ACTB	CY5
Mutation Panel B	APC R1450/R876	FAM
	KRAS G12	HEX or VIC
	CTNNB1 T41	CFR610 or Texas Red or ROX
	ACTB	CY5
Mutation Panel C	BRAF V600E	FAM
	KRAS G13	HEX or VIC
	CTNNB1 S45	CFR610 or Texas Red or ROX
	ACTB	CY5
Mutation Panel D	TP53 R273	FAM
	NRAS G12	HEX or VIC
	TP53 R175	CFR610 or Texas Red or ROX
	ACTB	CY5
Mutation Panel E	TP53 R248	FAM
	SMAD4 R361	HEX or VIC
	PIK3CA E545	CFR610 or Texas Red or ROX
	ACTB	CY5

Table 7b. Roche LightCycler 480 II and Bio-Rad CFX 384 Platforms Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s) for Roche instruments*	Cycles	Data Collection
Preincubation	95	300	4.4	1	OFF
Denaturation	95	20	4.4	50	OFF
XNA Annealing	70	40	2.2		OFF
Primer Annealing	64	30	1		OFF
Extension	72	30	1		All channels

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

Table 7c. ABI QuantStudio 5 and ABI 7500 Dx Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s)	Cycles	Data Collection
Preincubation	95	300	1.6	1	OFF
Denaturation	95	20	1.6	50	OFF
XNA Annealing	70	40	1.6		OFF
Primer Annealing	66	30	1		OFF
Extension	72	30	1		All Channels

The typical qPCR run files using the ABI 7500 Fast Dx are shown in Figure 3 (for the controls PC, NC, and NTC), Figure 4 (the mutation assay, positive sample), Figure 5 (the mutation assay, negative sample), and Figures 4 & 5 (Act-B, the reference gene control).
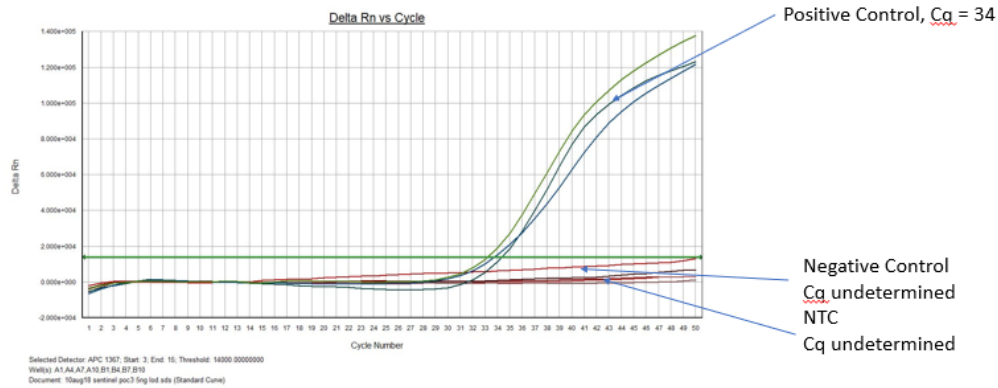

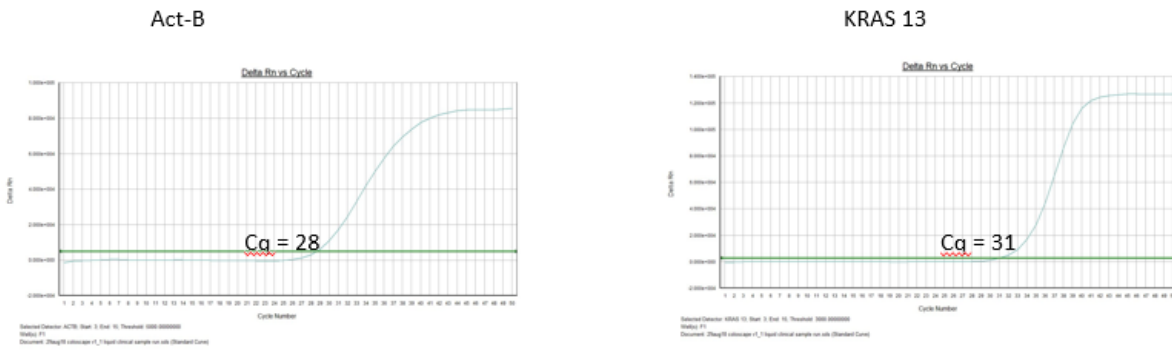**Figure 3. The APC 1367 Amplification Plot Showing PC, NC, and NTC**

Figure 4. A Run File Showing a KRAS G13 Positive Clinical Sample
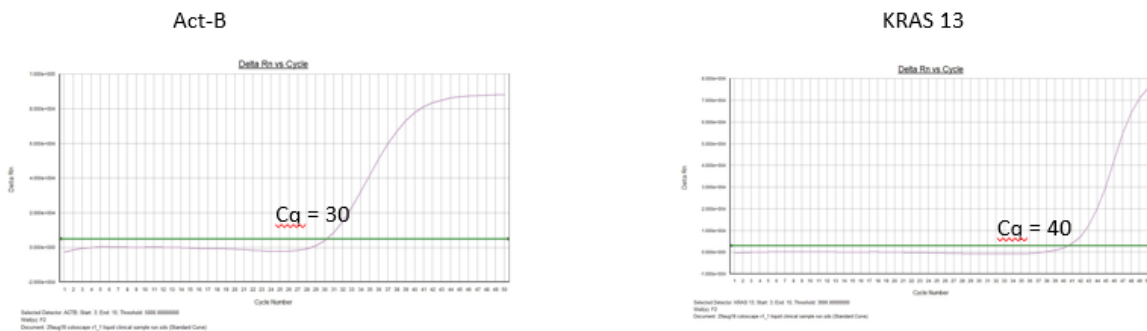

Figure 5. A Run File Showing KRAS G13 Negative Clinical Sample

3.5.2. Methylation Assay

Roche LightCycler 480 II, Bio-Rad CFX 384 and ABI QuantStudio 5

1) Selection of Detectors:
   i. Use 'ColoScape 4 Color' as the Detector on Roche LightCycler.
   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 7d** 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 7e** or **7f.**
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 7d. Assigning Reporter to ColoScape™ Colorectal Cancer Detection Test Methylation Gene Targets

ColoScape™	Target Name	Reporter
	Panel1	FAM
Methylation Panel F	Panel2	HEX or VIC
	Internal Control (IC)	CY5

Table 7e. Roche LightCycler 480 II and Bio-Rad CFX 384 Platforms Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s) for Roche Instruments *	Cycles	Data Collection
Preincubation	95	300	4.4	1	OFF
Denaturation	95	15	4.4		OFF
XNA Annealing	70	40	2.2	50	OFF
Primer Annealing	59	30	1		All channels

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

Table 7f. ABI QuantStudio 5 and ABI 7500 Dx Cycling Parameters

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s)	Cycles	Data Collection
Preincubation	95	300	1.6	1	OFF
Denaturation	95	15	1.6		OFF
XNA Annealing	70	40	1.6	50	OFF
Primer Annealing	59	30	1		All channels

PART 4. DATA ANALYSIS

4.1. 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).

4.1.1. Data Analysis for Roche LightCycler 480 II

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

4.1.2. 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. Recommended Threshold for Bio-Rad CFX384

Target	Recommended Threshold
ACT-B (internal control)	100 ± 20
APC E1309	250 ± 50
APC Q1367	100 ± 20
APC R1450	280 ± 60
CTNNB1 T41	100 ± 20
CTNNB1 S45	100 ± 20
KRAS G12	120 ± 20
KARS G13	200 ± 40
BRAF V600	100 ± 20
TP53 R273	120 ± 20
NRAS G12	200 ± 30
TP53 R175	100 ± 20
TP53 R248	120 ± 20
SMAD4 R361	200 ± 30
PIK3CA E545	100 ± 20
Methylation Panel 1	100 ± 20
Methylation Panel 2	100 ± 20
Methylation Internal Control	100 ± 20

4.1.3. 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 Tables 9a and 9b respectively. Exact threshold setting may be different for individual instruments. Export the Cq data to excel.

Table 9a. Recommended Threshold for ABI QuantStudio 5

Target	Recommended Threshold
ACT-B	5000 ± 500
APC E1309	50000 ± 5000
APC Q1367	7500 ± 7500
APC R1450/R876	50000 ± 5000
CTNNB1 T41	15000 ± 1500
CTNNB1 S45	10000 ± 1000
KRAS G12	5000 ± 500
KRAS G13	5000 ± 500
BRAF V600	8000 ± 800
NRAS G12	5000 ± 1000
TP53 R175	10000 ± 1000
TP53 R273	10000 ± 1000
PIK3CA E545	40000 ± 4000
SMAD4 R361	10000 ± 1000
TP53 R248	20000 ± 1000
Methylation Panel 1	5000 ± 500
Methylation Panel 2	10000 ± 1000
Methylation Internal Control	10000 ± 1000

Table 9b. Recommended Threshold for ABI 7500 Dx

Target	Threshold Setting
ACTB	5000±500
APC E1309	100000±10000
APC Q1367	14000±1400
APC R1450/R876	30000±3000
CTNNB1 T41	40000±4000
CTNNB1 S45	10000±1000
KRAS G12	6000±600
KRAS G13	3000±300
BRAF V600	20000±2000
NRAS G12	10000 ± 500
TP53 R175	10000 ± 1000
TP53 R273	10000 ± 1000
PIK3CA E545	40000 ± 4000
SMAD4 R361	10000 ± 1000
TP53 R248	10000 ± 2000
Methylation Panel 1	40000±4000
Methylation Panel 2	40000±4000
Methylation Internal	10000 ± 500

4.1.4. Evaluation of Controls

Verify that no amplification is observed in the No Template Controls (NTC) for each of the reaction mixes. Cq should be Undetermined.

Negative and Positive Controls: For the assay to be valid, the Negative Control (NC) and Positive Control (PC) must meet the criteria in Table 10.

Table 10. Acceptable Values for Positive Controls and Negative Controls

Assay	Positive Control (PC)	Negative Control (NC)
Internal Control (ACTB)	23.0 ≤ Cq < 33.0	23.0 ≤ Cq <33.0
APC E1309	Cq ≤ 35.0	Cq > 43.0
APC Q1367	Cq ≤ 35.0	Cq > 43.0
APC R1450/R876	Cq ≤ 35.0	Cq> 39.0
CTNNB1 T41	Cq ≤ 35.0	Cq> 40.0
CTNNB1 S45	Cq ≤ 35.0	Cq> 39.5
KRAS G12	Cq ≤ 35.0	Cq> 40.0
KRAS G13	Cq ≤ 35.0	Cq> 40.0
BRAF V600	Cq ≤ 35.0	Cq> 43.0
NRAS G12	Cq ≤ 35.0	Cq > 35.5
TP53 R175	Cq ≤ 35.0	Cq > 36.0
TP53 R273	Cq ≤ 35.0	Cq > 39.0
PIK3CA E545	Cq ≤ 35.0	Cq > 38.0
SMAD4 R361	Cq ≤ 35.0	Cq > 40.0
TP53 R248	Cq ≤ 35.0	Cq > 38.0
Panel 1	Cq < 40.0	Cq ≥ 40.0
Panel 2	Cq < 40.0	Cq ≥ 40.0
Methylation IC	20.0 < Cq < 38.0	20.0 < Cq <38.0

4.1.5. 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. In mutation test, Cq values of any sample with Internal Control Mix should be in the range of 25 ≤ Cq < 31 (Roche LightCycler 480 II and Bio-Rad CFX 384) or 25 ≤ Cq < 30 (ABI QuantStudio 5). In methylation test, Cq values of any sample with Internal Control Mix should be in the range of 25 ≤ Cq < 35. 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	Reaction	Descriptions and Recommendations	
Optimal	Mutation	23.0 ≤ Cq < 33.0	The amplification and amount of DNA sample were valid.
	Methylation	20.0 ≤ Cq < 38.0	
Invalid	Mutation	Cq < 23.0	Possibility of a false positive is high. Check DNA quality/quantity and repeat the PCR reaction with less DNA.
	Methylation	Cq < 20.0	
Invalid	Mutation	Cq ≥ 33.0	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.
	Methylation	Cq ≥ 38.0	

4.1.6. Scoring Mutational and Methylation Status

If a Cq value is 'Undetermined', assign a Cq of 50 and proceed to analysis. Tables 12 shows the Cq cutoff values and should be used to determine mutational status.

Note:
- If your sample Cq values of CY5 (Internal Control) are outside the range of 25 to 31, the results will be invalid.
- If No Template Control (NTC) has an amplification signal, the results will be invalid.
- When sample Cq values are greater than the NC Cq value for each target, the mutational status will be scored as "Negative" regardless of the Cq values.
- When sample Cq is smaller than or equal to the positive Cq for each target (**Table 12**), the mutational status will be scored as "positive."
- When sample Ct is greater than or equal to the negative Cq (**Table 12**), the result is determined as "negative."
- When sample Cq is between the positive Cq and the negative Cq, the result is undetermined. It is highly recommended to re-test the sample and send the PCR amplicons for Sanger sequencing. The sequences of primer for Sanger sequencing will be provided upon request; however, the amplicons from the following targets are not suitable for the sequencing: BRAF V600, PIK3CA E545, APC R876.

For the methylation analysis, Cq values of less than 40 will be scored as "positive."

Table 12. The Cut-Off Cq Values for Each Mutational Target

Panel	Target	Cutoff
A	APC E1309	≤ 43.0
	APC Q1367	≤ 43.5
B	APC R1450/R876	≤ 39.0
	CTNNB1 T41	≤ 39.9
	KRAS G12	≤ 39.0
C	BRAF V600	≤ 43.0
	CTNNB1 S45	≤ 39.2
	KRAS G13	≤ 40.0
D	NRAS G12	≤ 35.5
	TP53 R175	≤ 40.0
	TP53 R273	≤ 40.0
E	PIK3CA E545	≤ 42.0
	SMAD4 R361	≤ 41.5
	TP53 R248	≤ 39.0

4.1.7. Differentiating KRAS G12/KRAS G13 Mutational Status

The KRAS G12 reaction mix detects both KRAS G12 and KRAS G13 mutations, whereas the KRAS G13 reaction mix detects only KRAS G13 mutations. Therefore, in order to differentiate between KRAS G12 and KRAS G13 mutations a combination of results from the two mixes should be used as described in Table 13.

Table 13. Interpretation of G12/G13 Mutational Status

Reaction Mix	Result Based on Tables 11-12	Mutational Status
KRAS G12 Reaction Mix KRAS G13 Reaction Mix	Positive Negative	G12 Mutation
KRAS G12 Reaction Mix KRAS G13 Reaction Mix	Positive Positive	G13 Mutation
KRAS G12 Reaction Mix KRAS G13 Reaction Mix	Negative Positive	G13 Mutation

PART 5. ASSAY PERFORMANCE

The performance characteristics of ColoScape™ v2.0 kit were established on the Roche LightCycler 480, Bio-Rad CFX 384, ABI 7500 Dx and ABI QuantStudio 5 real-time PCR instruments. The studies were performed using genetically defined reference standards (genomic DNA) 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 digital PCR. Additional samples consisted of cancer patient tissue, plasma samples and normal healthy donor DNA from tissue and plasma.

5.1. 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 digital PCR. 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.

5.2. 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 14. Summary of Reproducibility Results

Variation	%CV
Intra-assay	≤ 10%
Inter-assay	≤ 10%
Lot-to-Lot Variation	≤ 5%
Operator Variability	≤ 5%

Table 15. 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 (IC)	29.19	0.42	1.43%	29.15	0.39	1.35%
Methylation Panel1	34.04	0.97	2.90%	35.56	3.49	9.80%
Methylation Pane2	34.22	0.33	1.00%	34.90	0.29	0.90%
Methylation IC	32.41	0.26	0.80%	32.26	0.24	0.80%
	5% Mutant			0.5% Mutant		
NRAS 12	28.63	0.15	0.01	32.75	0.10	0.00
TP53 175	29.56	0.08	0.00	33.32	0.09	0.00
TP53 273	29.35	0.12	0.00	32.89	0.14	0.00
PIK3CA 545	30.91	0.12	0.00	34.37	0.03	0.00
SMAD4 361	28.03	0.06	0.00	31.47	0.06	0.00
TP53 248	30.91	0.16	0.01	35.64	1.75	0.05
Internal Control (IC)	26.58	0.08	0.00	28.25	0.11	0.00

The intra-assay data demonstrated good reproducibility with low %CV (Table 15).

5.3. 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 wildtype DNA used for dilution was obtained from mutant-free FFPE and normal human plasma respectively. Mutant allelic frequencies tested were 1% VAF, 0.5% VAF and 0.1% VAF at 2.5 ng, 5ng 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 16a.

Table 16a. Mutant DNA Copy Numbers at Different Allelic Frequencies

Variant Allelic Frequency	Mutant DNA Copy Numbers at Different DNA Inputs		
	10 ng DNA	5 ng DNA	2.5 ng DNA
1.0%	28 copies	14 copies	7 copies
0.5%	14 copies	7 copies	3.5 copies
0.1%	2.8 copies	1.4 copies	0.7 copies

Table 16b. LOD Summary Determined Using Genomic DNA Reference Standards (LC480II)

Roche LC480II was used for this test.

Target Mutation	DNA Input, ng/well		
	VAF (%)	10 ng fragmented DNA	5 ng fragmented DNA
		% Correct Call	% Correct Call
APC E1309	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	20%	0%
APC Q1367	1% mutation	100%	100%
	0.5% mutation	100%	100%

	0.10% mutation	50%	10%
APC R1450/R876	1% mutation	100%/100%	100%/100%
	0.5% mutation	100%/100%	100%/100%
	0.10% mutation	60%/20%	60%
CTNNB1 T41	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	30%	10%
CTNNB1 S45	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	80%	40%
KRAS G12	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	80%	45%
KRAS G13	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 16c. LOD Summary Determined Using cfDNA Reference Standards

Roche LC480II was used for this test.

Target Mutation	cfDNA Input, ng/well		
	VAF%	10 ng cfDNA % Correct Call	5 ng cfDNA % Correct Call
APC E1309	1% mutation	100%	100%
	0.5% mutation	100%	95%
	0.10% mutation	65%	40%
APC Q1367	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	100%
APC R1450/R876	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	90%	90%
CTNNB1 T41	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	100%
CTNNB1 S45	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	90%	85%
KRAS G12	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.10% mutation	100%	95%
KRAS G13	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%
NRAS G12D	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.25% mutation	100%	100%
	0.1% mutation	95%	100%
TP53 R175H	1% mutation	100%	100%
	0.5% mutation	100%	100%

	0.25% mutation	100%	100%
	0.1% mutation	100%	100%
TP53 R273H	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.25% mutation	100%	75%
	0.1% mutation	100%	65%
PIK3CA E545K	1% mutation	100%	100%
	0.5% mutation	100%	80%
	0.25% mutation	100%	15%
	0.1% mutation	90%	n/a
SMAD4 R361C	1% mutation	100%	100%
	0.5% mutation	100%	100%
	0.25% mutation	100%	80%
	0.1% mutation	100%	55%
TP53 R248Q	1% mutation	95%	100%
	0.5% mutation	95%	90%
	0.25% mutation	100%	10%
	0.1% mutation	100%	n/a
Methylation Panel1	1% methylation	95%	
	0.5% methylation	95%	
Methylation Panel2	1% methylation	100%	
	0.5% methylation	100%	

Conclusion:

- For FFPE DNA samples, 0.5% variant allelic frequency and 0.5% methylation can be detected with 10 ng DNA input.
- For plasma cfDNA samples, 0.5% variant allelic frequency in all targets mutants and 0.5% methylation can be detected with 10 ng cfDNA input. Even at 0.1% VAF, APC Q1367, CTNNB1 T41, KRAS G12, BRAF V600, TP53 R175/R273/R248 and SMAD4R361 can be detectable.
- Therefore, the recommended DNA input is 10 ng/well to obtain high sensitivity.
- LOD for FFPE and cfDNA is 0.5% VAF.

5.4. Analytic Specificity

Analytical specificity of the kit was determined as the correct calling of the samples with no mutation at different concentrations of wildtype DNA template. There were no false positive calls for up to 320 ng of genomic DNA per well and up to 20 ng 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 17a. Analytic Specificity: Cross-Reactivity in the Mutation Assay

Panel	Target primers/probe	APC E1309	APC Q1367	APC R1450	APC R876	CTNNB1 T41	KRAS G12*	BRAF V600	CTNNB1 S45	KRAS G13	NRAS G12	TP53 R175	TP53 R273	PIK3CA E545	SMAD4 R361	TP53 R248
A	APC E1309	28.15	50.00	48.27	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
	APC Q1367	30.26	30.09	50.00	48.11	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
B	APC R1450/R876	45.24	46.21	27.87	27.36	43.81	47.03	35.55	42.11	46.89	47.51	44.09	50.00	47.99	46.60	46.38
	CTNNB1 T41	43.31	41.07	49.45	45.15	24.72	46.94	38.77	31.36	43.39	43.81	44.01	42.17	43.21	41.13	41.12
	KRAS G12	49.18	50.00	31.45	50.00	47.96	27.48	50.00	45.85	30.41	50.00	48.99	50.00	48.85	47.74	50.00
C	BRAF V600	50.00	50.00	50.00	50.00	47.16	50.00	24.05	48.64	50.00	50.00	50.00	50.00	50.00	50.00	50.00
	CTNNB1 S45	42.06	42.94	42.60	41.31	33.34	43.43	44.38	26.06	44.60	42.90	41.78	41.14	41.15	42.16	43.72
	KRAS G13	45.28	46.16	43.20	47.48	47.05	36.68	43.62	45.13	25.93	44.75	41.61	45.84	46.87	47.23	48.67
D	NRAS G12	42.63	40.25	43.14	42.70	40.92	41.36	40.69	41.64	42.06	27.72	42.71	40.04	42.10	40.47	39.14
	TP53 R175	50.00	46.28	48.76	50.00	50.00	50.00	50.00	50.00	50.00	46.15	28.85	48.29	50.00	44.43	50.00
	TP53 R273	50.00	46.66	45.76	50.00	50.00	50.00	50.00	46.82	50.00	48.95	50.00	28.42	50.00	47.57	45.68
E	PIK3CA E545	49.41	47.13	50.00	45.88	46.18	49.76	46.68	46.87	36.57	50.00	47.37	48.33	31.83	45.03	45.82
	SMAD4 R361	50.00	46.50	49.16	49.52	50.00	50.00	50.00	50.00	48.81	50.00	50.00	50.00	50.00	29.00	44.45
	TP53 R248	46.52	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	46.13	47.20	48.06	28.29

Average Cq values are shown. The data demonstrates that the ColoScape™ Kit can correctly identify several mutations within co-differentiated (Refer to **Table 13**).

Table 17b. Analytic Specificity: Cross-Reactivity in the Methylation Assay

Target Primes/Probe	NTC	NTC	Methlated templates from PANEL 1			Methlated templates from PANEL 2		
PANEL 1	50.0	50.0	18.8	18.9	19.0	46.0	44.8	43.6
PANEL 2	50.0	50.0	50.0	46.3	50.0	23.7	24.1	23.6

Average Cq values are shown. The data demonstrates that the ColoScape™ Kit can correctly identify specific methylations in each panel.

5.5. Cut-Offs

cfDNA from plasma samples and custom standard ctDNAs (Anchor Molecular Inc) were used to establish cut-offs for the assay. Please refer to **Table 12**.

5.6. 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.

5.7. Interfering Substances

A study was performed to evaluate the impact of potentially interfering substances on the performance of the ColoScape™ v2.0 assay. Ethanol (EtOH), Plasma, Hemoglobin, EDTA, Triglyceride, Cholesterol, FFPE wax was spiked in the DNA samples at 2%, 5% and 10% concentration and tested in 5 replicates on BioRad CFX 384. The average Ct values were calculated for each sample. 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.

5.8. Multiple Freeze-Thaw Cycles

The effect of 1-8 freeze-thaw cycles were tested in ColoScape™ v2.0 Mutation Test kit reagents. The content of the ColoScape kit can be freeze-thawed for up to 8 freeze-thaw cycles without an adverse effect on the kit performance, indicating the kit is stable up to 8 freeze-thaw cycles.

Caution: Repeated freeze-thaw cycles may decrease the reliability of test results.

5.9. Shelf-Life

The ColoScape v2.0 kit is stable up to **12 months** under recommended storage conditions of -25°C to -15°C.
Approximately 12 months after production of kit-see product labels for actual expiration date. Do not use expired reagents from the kit.

5.10. 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.

A sample was considered positive if at least one of the target mutations tested positive based on the cutoffs presented in Table 12.

5.10.1. Clinical Performance for FFPE Samples

30 CRC FFPE samples were collected and tested with ColoScape v2.0 on the ABI Quant Studio 5 instrument. Scored for mutation status for ColoScape targeted mutations based on the established cut-off value for FFPE samples. The result indicated that all 30 samples were identified positive with at least one mutant (Table 18). All mutants were validated by Sanger Sequencing.

Table 18. Summary of ColoScape^{TM} v2.0 for FFPE Samples

ColoScape™ v2.0 Test	Clinical Status		Sensitivity (%)	Specificity (%)
	Positive	Negative		
Positive	30	0	100% (95% CI: 0.88-1.00)	N/A
Negative	0	0		
Total	30	0		

5.10.2. Clinical Performance for cfDNA Samples

To validate this kit's clinical performance with clinical samples, 28 CRC and 49 health donator individuals' whole blood were collected and isolated for cfDNA samples with Qiagen kit (CRC cohort). The samples were tested with ColoScape v2.0 Kit on the ABI Quant Studio 5 instrument. Twenty-four CRC samples were identified multiplex mutants and 4 samples were unidentified. All health samples have not mutants detectable. The result indicates that the clinical sensitivity for CRC is 85.7 % (95% CI: 0.66-0.95) and specificity 100% (95% CI: 0.91-1.00). (Table 19a). In addition, another cohort total 38 patients were tested including advanced adenoma (AA) samples which were identified by Colonoscopy. Three out of 5 AA samples are detected as positive. The result indicates that the clinical sensitivity for advanced adenoma is 60% (95% CI: 0.17-0.93) and specificity 91% (95% CI: 0.75-0.98).

Table 19a. Summary of ColoScape™ v2.0 for CRC Detection

ColoScape™ v2.0 Test	Clinical status		Sensitivity (%)	Specificity (%)
	Positive	Negative		
Positive	24	0	85.7 % (95% CI: 0.66-0.95)	100% (95% CI: 0.91-1.00)
Negative	4	49		
Total	28	49		

Table 19b. Summary of ColoScape™ v2.0 for Advanced Adenoma Detection

ColoScape™ v2.0 Test	Colonoscopy		Sensitivity (%)	Specificity (%)
	positive	negative		
positive	3	3	60% (95% CI: 0.17-0.93)	91 % (95%CI: 0.75-0.98)
negative	2	30		
Total	5	33		

PART 6. ASSAY TROUBLESHOOTING

Problem	Cause	Solution
Fluorescence signals in No Template Control (NTC), e.g., Cq = 42.12	The positive signal may be caused by contamination during setting-up of the PCR; or the signal is not true target amplification, but background curves generated by the software of the qPCR instrument.	Repeat the PCR with new reagents. Follow the general rules of GLP in a PCR lab. It is recommended to set up the qPCR reactions in a separate area, where no DNA is handled and with equipment designated for pre-PCR activities. Make sure the workspace and instruments are decontaminated regularly. Ignore the Ct value of NTC if the amplification curve looks not real but background noise.
The Clamping Control and Positive Control did not meet the criteria set for acceptable values of the ColoScape™ Colorectal cancer mutation detection kit. The assay is invalid.	Kit was not stored at the recommended conditions; or Kit shelf-life expired.	Check the kit label for storage conditions and expiration date and use a new kit if necessary.
The sample Cq value of CY5 (Internal control) is not in the range of 23.0 to 33.0 (20.0 to 38.0 for the methylation assay).	The amplification and amount of DNA input are not optimal.	Repeat the experiments following the recommendations described in Table of the ColoScape™ Colorectal Cancer Detection Kit Instruction Manual.
A sample that is positive for the mutation, but the Ct of internal control (CY5) is not in the range of 23.0 to 33.0 (20.0 to 38.0 for the methylation assay).	It is most likely due to the DNA input is not optimal.	If the sample Cq of CY5 is >33.0 (>38.0 for the methylation assay), the test result of the sample can still be considered valid. The positive signal with CY5 Cq >33.0 (>38.0 for the methylation assay) may indicate the mutation (or methylation) ratio of the tested sample is relatively high. If the sample Cq of CY5 is <23.0 (<20.0 for the methylation assay), the sample is overloaded, which can possibly lead to false positive result. Repeat the experiments following the recommendations described in Table of the ColoScape™ Colorectal Cancer Detection Kit Instruction Manual.
After analysis of CY5 channel, all wells show no Cq values being called.	Edge wells show high background fluorescence which prevents software from calling Cq values for sample wells.	All wells showing high background fluorescence must be deselected, threshold reset to a lower value and then reanalyzed using user defined threshold setting.

Sample Cq is between the positive Cq and the negative Cq.	It is most likely due to the DNA input is not optimal.	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.

PART 7. SYMBOLS USED IN PACKAGING

Table 20. **Symbols Used in Packaging**

Symbol	Definition
EC REP	Authorized Representative in the European Community
REF	Catalog Number
(manufacturer symbol)	Manufactured By
(thermometer symbol)	Temperature Limitation
LOT	Batch Code
(hourglass symbol)	Expiration Date
EC REP	Authorized Representative in the European Community
1011-11-17	Date Format (year-month-day)
1011-11	Date Format (year-month)

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