epi pro colon

Real-time PCR Kit

CE IVD

Instructions for Use

The instructions for use must be read carefully prior to use and followed precisely to achieve reliable results.

REF M5-01-002

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epigenomics

epi pro colon
Real-time PCR Kit

Content

Important Note

Important: Users should familiarize themselves with the instructions contained in this booklet and the components of the Epi *pro*Colon Real-time PCR Kit prior to use.

Meaning of Symbols:

	Consult Instructions for Use
	Order Number
	*In Vitro* Diagnostic Medical Device
	Lot Number
	Expiration Date
	Manufacturer
	Store at indicated Temperature
	Number of Tests

Intended Use

The Epi *pro*Colon real-time PCR Kit is an *in vitro* polymerase chain reaction (PCR) assay for the qualitative detection of SEPT9 gene methylation (^{m}SEPT9) in bisulfite converted DNA from human EDTA plasma samples. Presence of ^{m}SEPT9 is associated with, and may aid in, the detection of invasive colorectal adenocarcinoma.

Product Description

The ^{m}SEPT9 assay is based on detecting aberrant DNA methylation of the v2 region of the Septin 9 gene. Cytosine residues in the v2 region become methylated in colorectal cancer (CRC) tissue but not in normal colon mucosa. This aberrant methylation can be detected by

specific amplification of DNA shed into the blood stream by tumor cells. Detection of CRC DNA using the Septin 9 methylation biomarker (*m*SEPT9) has been demonstrated in multiple case control studies of plasma from CRC patients and colonoscopy-verified negative controls[1-4].

The product is an *in vitro* polymerase chain reaction (PCR) assay for the qualitative detection of SEPT9 gene methylation (*m*SEPT9). It uses bisulfite converted DNA as input material, which is derived from human EDTA plasma samples via a procedure described in the Instructions for Use of the Epi *pro*Colon Plasma DNA Preparation Kit (M5-01-001).

The product is designed as a duplex PCR with a simultaneous detection of the target *m*SEPT9 DNA and ACTB (ß-actin) DNA as an internal control assessing input DNA adequacy. ACTB PCR results are required to validate generated results.

The product must be used with external positive and negative controls available in the Epi *pro*Colon Work Flow Control Kit (M5-01-003). Plasma specimens used in the assay should only be processed with the Epi *pro*Colon Plasma DNA Preparation Kit (M5-01-001), which has been validated for use with the Epi *pro*Colon real-time PCR Kit.

Technological Principles

The principle of detection of methylated Septin 9 DNA is comprised of three steps, starting with a plasma sample that is prepared from whole blood using standard techniques. DNA methylation occurs predominantly on a CpG dinucleotide, a cytosine nucleotide followed by a guanine nucleotide. DNA contained within the plasma sample is extracted using magnetic bead-based nucleic acid extraction. The extracted DNA is then chemically converted to reveal the methylation status using sodium bisulfite. This chemical treatment converts unmethylated cytosines in the CpG context to uracils whereas methylated cytosines are not changed, resulting in a single base-pair change at each unmethylated cytosine. The blockers and probes used in the subsequent PCR reaction are sensitive to these base-pair changes and will discriminate between methylated and unmethylated sequences.

The Epi *pro*Colon assay detects, in a duplex PCR reaction, a DNA sequence containing methylated CpG sites within the v2 region of the Septin 9 gene (*m*SEPT9) and total DNA of a region of the ACTB gene. The *m*SEPT9 portion of the duplex assay consists of primers that are placed in regions lacking CpG dinucleotides. A blocker specific for bisulfite converted unmethylated sequences within the region is added so that methylated sequences are preferentially amplified. In order to exclusively identify methylated sequences amplified during the PCR reaction, a *m*SEPT9-specific fluorescent probe is used for detection in the reaction[5].

Bisulfite converted DNA from each patient sample is used as the input material for the Epi *pro*Colon real-time PCR Kit (M5-01-002). Two duplex reactions are run for each patient bisulfite DNA sample. Results from both reactions must be evaluated according to the criteria provided in these Instructions for Use in order to determine the *m*SEPT9 result.

Contents

This kit contains reagents required to analyze 24 patient samples or controls (measured in duplicates to yield a total of 48 PCR reactions):

Epi *pro*Colon PCR Mix: 2 tubes each containing 700 µl, ready-to-use, contains Septin 9 primers, blocker and probe; ACTB primers and probe; dNTPS, buffer, $MgCl_2$, water

Epi *pro*Colon Polymerase: 1 tube containing 57 µl ready-to-use Taq Polymerase

Instruments, Supplies and Reagents Required but not Provided

Water, molecular biology grade

Pipette tips with aerosol barrier 0.5-10 µl, 10-100 µl, 100-1000 µl

Disposable gloves

Vortex (VWR International GmbH, catalog no. 444-1372), or equivalent

Thermomixer comfort (catalog no. 5355 000.011) with dry heating block - 2 ml (Eppendorf AG, catalog no. 5362 000.019), or equivalent

Safe-Lock reaction-tubes 2 ml PCR tubes (e.g. Eppendorf AG, catalog no. 0030 120.094), or equivalent

Centrifuge 5810 (Eppendorf AG) or Centrifuge Sigma 4-15C (Qiagen) capable for PCR plates, or equivalent

LightCycler® 480 System with 96 heat block (Roche Molecular Systems) with Software version 1.5.0

LightCycler 480 Multiwell Plate 96 (Roche, catalog no. 04729692001)

LightCycler 480 Sealing Foil (Roche, catalog no. 04729757001)

Safety Information

This Kit does not contain any harmful or dangerous components.

Always wear a laboratory coat and disposable gloves. Clean contaminated surfaces with water.

Storage and Stability

-15°C

Store all reagents of the Epi *pro*Colon real-time PCR kit at –15 to –25°C.

-25°C

Epi *pro*Colon PCR Mix may be frozen and thawn three times.

Test Materials and Preparation for Analysis

Runs or batches shall consist of a minimum of one patient specimen tested together with the Epi *pro*Colon Positive Control and Epi *pro*Colon Negative Control (from Epi *pro*Colon Work Flow Control Kit, M5-01-003), up to a maximum of 22 patient specimens analyzed together with the Epi *pro*Colon Positive Control and Epi *pro*Colon Negative Control. Each sample and control must be measured in duplicate.

The Epi *pro*Colon real-time PCR Kit has been designed and validated for use with sulfonated, bisulfite converted DNA obtained from EDTA plasma samples using the Epi *pro*Colon Plasma DNA Preparation Kit (M5-01-001).

Bisulfite converted DNA can be stored up to 72 hours at –15 to –25 °C.

Experimental Protocol

For accurate results each sulfonated, bisulfite converted DNA sample (patient sample or Epi *pro*Colon Positive/Negative Control) must be tested in duplicate.

Preparation of PCR Master Mix

Thaw sufficient amounts of Epi *pro*Colon PCR Mix for the desired number of patient and control sample determinations according to Table 1. Combine Epi *pro*Colon PCR Mix and Epi *pro*Colon Polymerase in a 2.0 ml reaction tube. For a single determination 27.7 µl Epi *pro*Colon PCR Mix and 1.1 µl Epi *pro*Colon Polymerase are required.

Note: Prior to use pulse spin Epi *pro*Colon Polymerase to remove drops from the lid.

Table 1: Preparation of PCR Master Mix (MM)

Component	Volume for 8 PCRs (4 determinations)	Volume for 12 PCRs (6 determinations)	Volume for 24 PCRs (12 determinations)	Volume for 48 PCRs (24 determinations)
Epi *pro*Colon PCR Mix	221.2 µl	331.7 µl	663.5 µl	1327.0 µl
Epi *pro*Colon Polymerase	8.8 µl	13.2 µl	26.5 µl	53.0 µl

LightCycler® 480 Plate Preparation

Note: Strictly follow the specified setup order to avoid cross-contamination.

1. Transfer 25 µl PCR Master Mix (MM) into each PCR well.

2. Add 15 µl of bisulfite converted DNA to respective wells of the PCR plate.
3. Seal the plate with an optical sealing foil.
4. Briefly spin the plate down with a plate centrifuge.

Note: The filled PCR plate can be stored in a refrigerator at 2 to 8 °C for up to 1h.

LightCycler® 480 Plate Loading

1. Place the loaded PCR plate into the frame (the short plate edge with the beveled corner points to the front of the instrument).
2. Start the PCR on the LightCycler® 480 using the cycling program below.

Table 2: Recommended Cycling Program

Program Parameter		Denaturation	Cycling			Cooling
	Analysis Mode	None	Quantification mode			None
	Cycles	1	50			1
	Segment	1	1	2	3	1
	Target [°C]	94	93	62	57	40
	Hold [hh:mm:ss]	00:30:00	00:00:10	00:00:05	00:00:30	00:00:30
LC 480*	Ramp Rate [°C/s]	4.4	2.2	2.2	2.2	2.2
	Acquisition Mode	None	None	None	Single	None

* LightCycler® 480 System: select the Dual Color Hydrolysis Probe/UPL Probe as detection format, activate the filter combination 483 – 533 nm and 523 – 568 nm. For LightCycler® 480 II activate the filter combination 465 – 510 nm and 533 – 580 nm.

LightCycler® PCR Result Analysis and Experiment/Analysis File Transfer

Important: Select *Color Compensation* "ON" and load the respective "Epi *pro*Colon Color Compensation" File for the Epi *pro*Colon real-time PCR assay.

1. Click "Analysis" on the LightCycler® 480 Basic Software Module bar opening the "Analysis Overview" Window.

Select "Abs Quant/Fit Points" for all samples.

Activate "Epi *pro*Colon Color Compensation".

Activate the "Filter Comb 483-533" (465 – 510 for LightCycler® 480 II).

Set "First Cycle" to "1" and "Last Cycle" to "50".

Set the background (click blue background button) to "2-25" by setting "Min Offset" to "1" and "Max Offset" to "24" in the "Cycle Range" window.

Set the Noise Band to "Noise Band (fluorescent)" in the "Noise Band" window.

Set the threshold to 2.0 in the "Analysis" window.

Note: If the background is greater than 2.0 adjust the threshold such that it is slightly above the background to ensure correct readout.

Click "Calculate".

^{m}SEPT9 Crossing Point ("CP") for each sample is calculated automatically and displayed in the Sample Table.

Export the CP values by clicking with the right mouse button on the Sample Table. Choose "Export". Save the file with a unique and meaningful file name.

Activate the "Filter Comb 523-568" (533-580 for LightCycler® 480 II).

Set "First Cycle" to "1" and "Last Cycle" to "50".

Set the background (click blue background button) to "2-25" by setting Min Offset to 1 and Max Offset to 24 in the "Cycle Range" window.

Set the Noise Band (fluorescent) to 2.0 in the "Noise Band" window.

Set the Threshold to 2.0 in the "Analysis" window.

Click "Calculate".

ACTB CP values for each sample are calculated automatically and displayed in the Sample Table.

Export the CP values by clicking with the right mouse button on the Sample Table. Choose "Export". Save the file with a unique and meaningful file name.

Quality Control

External Controls

The Epi *pro*Colon Work Flow Control Kit (M5-01-003) contains Epi *pro*Colon Positive and Negative Controls. These controls must be included with each assay run to monitor the successful execution of the work flow to ensure validity of test results. The Epi *pro*Colon Positive / Negative Control values must be within the validity limits (Table 3) specified below. If a control is out of its specified range, the associated test results are invalid, must not be reported and the patient must be retested.

If laboratory quality control procedures require more frequent use of controls to verify test results, follow those procedures.

Internal Controls

The internal control allows the detection of ACTB (ß-actin) DNA, assessing the adequacy of DNA obtained from each patient specimen. This control monitors the specimen quality, specimen preparation and adequate DNA concentration of the specimen.

A positive curve of the ^{m}SEPT9 PCR must coincide with a CP value of the ACTB PCR $\geq$ 24.3 in order to be valid. Lower CP values of the ACTB PCR have been observed when plasma samples have been prepared improperly, potentially leading to false positive results.

"No curve" of the ^{m}SEPT9 PCR must coincide with a CP value of the ACTB PCR ≤ 34.4 in order to be valid. Very high CP values of the ACTB PCR are associated with very low DNA content or PCR inhibition.

Interpretation of Results

Validity of Epi *pro*Colon Assay Batches or Runs

Any assay run or batch (one or more patient sample(s) processed together with Epi *pro*Colon Positive and Negative Controls) is considered valid when criteria set forth in Table 3 are met for BOTH PCR replicates per Control:

Table 3: Validity Limits for Epi proColon Positive /Negative Controls

Result of Control	Determination	^{m}SEPT9 Result	ACTB Result
Positive Control valid	PCR 1	+ Curve	CP < 31.1
	PCR 2	+ Curve	CP < 31.1
Negative Control valid	PCR 1	- Curve	CP < 35.2
	PCR 2	- Curve	CP < 35.2

Interpretation of Results for a Single PCR

The interpretation of a single PCR is performed according to the following Table:

Single PCR Result	^{m}SEPT9	ACTB
Valid positive ^{m}SEPT9 PCR	+ Curve	CP ≥ 24.3
Valid negative ^{m}SEPT9 PCR	- Curve	CP ≤ 34.4
Invalid ^{m}SEPT9 PCR	- Curve	CP > 34.4
Invalid ^{m}SEPT9 PCR	+ Curve	CP < 24.3

Interpretation of Results for a Patient Sample

The test result for a single patient sample is interpreted according to the following Table:

Sample	Positive Control Negative Control	Interpretation

PCR 1	Valid positive ^{m}SEPT9 PCR	Valid	Sample ^{m}SEPT9 positive
PCR 2	Valid positive ^{m}SEPT9 PCR		
PCR 1	Any result	Valid	Sample ^{m}SEPT9 positive
PCR 2	Valid positive ^{m}SEPT9 PCR		
PCR 1	Valid positive ^{m}SEPT9 PCR	Valid	Sample ^{m}SEPT9 positive
PCR 2	Any result		
PCR 1	Valid negative ^{m}SEPT9 PCR	Valid	Sample ^{m}SEPT9 negative
PCR 2	Valid negative ^{m}SEPT9 PCR		
PCR 1	Valid negative ^{m}SEPT9 PCR	Valid	Sample invalid
PCR 2	Invalid ^{m}SEPT9 PCR		
PCR 1	Invalid ^{m}SEPT9 PCR	Valid	Sample invalid
PCR 2	Valid negative ^{m}SEPT9 PCR		
PCR 1	Invalid ^{m}SEPT9 PCR	Valid	Sample invalid
PCR 2	Invalid ^{m}SEPT9 PCR		

Important! Only ^{m}SEPT9 PCR measurements from valid test runs or batches shall be evaluated.

Limitations of the Procedure

This product has been validated for use with bisulfite-converted DNA derived from human EDTA plasma using the Epi *pro*Colon Plasma DNA Preparation Kit (M5-01-001).

The product shall be used by personnel experienced in molecular biological techniques, specifically real-time PCR. General recommendations on the organization and procedures of the laboratory shall be obeyed in order to prevent DNA contamination.

Only single-use pipettes and pipette tips are recommended to prevent cross-contamination of patient samples.

Positive test results have been observed in a portion of patients with the following diseases: bladder cancer, lung cancer, inflammatory bowel disease, Systemic Lupus Erythematodes, chronic heart disease, respiratory disease, rheumatoid arthritis, non-rheumatoid arthritis, pyelonephritis, cholecystitis.[3]

Performance Characteristics

Analytical Performance

To determine the Limit of Detection, a panel of three technical samples was tested. Two different concentrations (8 pg/ml and 16 pg/ml) of lyophilyzed DNA detectable with the ^{m}SEPT9 PCR were spiked into a matrix containing genomic DNA at a concentration of 10 ng/ml. A blank sample consisting of matrix with genomic DNA only, and the two technical samples were processed with 24 replicates each, by three technicians. Bisulfite converted DNA was prepared using reagents from different lots of the Epi *pro*Colon Plasma DNA Preparation kit and converted DNA was assayed with Epi *pro*Colon real-time PCR kits from different lots adopting a schedule from NCCLS EP 17-A[7]. All 24 replicates of the blank sample were determined ^{m}SEPT9 negative, 23 replicates of the technical samples containg 8 pg/ml and 16 pg/ml DNA spikes were determined ^{m}SEPT9 positive. The Table below summarizes the data obtained.

	0pg/ml lyophilyzed DNA	8pg/ml lyophilyzed DNA	16pg/ml lyophilyzed DNA
Valid replicates	24	23	24
^{m}SEPT9 positive	0	23	23
^{m}SEPT9 negative	24	0	1

Reproducibility

The reproducibility of the procedure was tested by processing plasma aliquots from four pools generated from human EDTA plasma. Two of the plasma pools comprised plasma from patients diagnosed with invasive colorectal adenocarcinoma. Two pools comprised plasma from patients with no apparent disease as controls. Both cancer pools were processed in 9 replicates, both non-cancer pools were processed in 18 replicates. Processing of replicates was performed by three operators using different lots of the Epi *pro*Colon Plasma DNA Preparation kit (M5-01-001) as well as the Epi *pro*Colon real-time PCR kit. In 51 of the 54 ^{m}SEPT9 determinations the expected result (cancer pool ^{m}SEPT9 positive; non-cancer pool ^{m}SEPT9 negative) was consistently generated.

Clinical Performance

The clinical performance of the Epi *pro*Colon real-time PCR kit was tested in a case – control design comprised of specimens from histologically confirmed invasive colorectal adeno-carcinoma patients of all stages and specimens from colonoscopy-verifed negative individuals with no apparent disease. Bisulfite converted DNA was prepared using the Epi *pro*Colon Plasma DNA Preparation kit from 261 plasma aliquots from individual subjects and converted DNA was assayed with the Epi *pro*Colon real-time PCR kit. Based on internal and

external control values for each run, valid ^{m}SEPT9 measurements were determined for 257 out of 261 specimens (98.5 %).

Within the 154 subjects with no evidence of disease (controls), 135 specimens were determined ^{m}SEPT9 negative resulting in an estimated clinical specificity of 88 % (95 % confidence interval [82 %; 92 %]).

Of the 103 subjects diagnosed with invasive colorectal adenocarcinoma (cases), 69 specimens were determined ^{m}SEPT9 positive resulting in an estimated clinical sensitivity of 67 % (95 % confidence interval [57 %; 76 %]). ^{m}SEPT9 was detected positive in 44 of the 66 patients with cancer in early, still localized disease state (44 stage I, 22 stage II). The Table below summarizes the data obtained.

	Controls	All adenocarcinoma	Localized adenocarcinoma	Advanced adenocarcinoma
Valid results	154	103	66	37
^{m}SEPT9 positive	19	69	44	25
^{m}SEPT9 negative	135	34	22	12

Interference

No interference was observed within experimental controls and nonreactive or reactive specimens tested with elevated levels of bilirubin (20 mg/dL), triglycerides (1200 mg/dL), protein (12 g/dL), red blood cells (0.4% v/v), or hemoglobin (1 g/dL).

References

1. De Vos, T. et al. Circulating methylated SEPT9 DNA in Plasma is a Biomarker for Colorectal Cancer, Clinical Chemistry 55:7, 1337-46 (2009)

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Cottrell, S. et al. A real-time PCR assay for DNA-methylation using methylation-specific blockers. Nucleic Acids Research, Vol. 32, No. 1 e10 (2004)

Roche LightCycler® Real-time PCR System Brochure, Roche-Applied-Science

NCCLS EP 17-A, Protocols for Determination of Limits of Detection and Limits of Quantitation; approved guideline.

Contact Support

The Epi *pro*Colon Plasma real-time PCR Kit is manufactured by:

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