



Epi proColon 2.0 CE

Epi proColon Plasma Quick Kit	(M5-02-001)
Epi proColon Sensitive PCR Kit	(M5-02-002)
Epi proColon Control Kit	(M5-02-003)

Important!

Use only the current version of the IFU.

Make sure prior to starting with the test procedure that you have the most current version available under <http://www.epiprocolon.com/en/e-support-library/instructions-for-use.html>.

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



IFU 0009GB, rev 9, copyright©, 27 April 2018, Epigenomics AG



M5-02-001, M5-02-002, M5-02-003



Epigenomics AG, Geneststr. 5, 10829 Berlin, Germany

Contents

1. Name and Intended Use	3
2. Summary and Explanation	3
3. Principles of the Procedure	3
4. Materials Provided	4
4.1. Contents	4
4.2. Safety Information	4
5. Storage and Stability	5
5.1. Epi proColon Plasma Quick Kit (M5-02-001) & Epi BiSKit (M7-01-001)	5
5.2. Epi proColon Sensitive PCR Kit (M5-02-002)	5
5.3. Epi proColon Control Kit (M5-02-003)	6
6. Materials Required, but not Provided	6
6.1. General Laboratory Equipment	6
6.2. General Laboratory Consumables and Reagents	6
6.3. Required Special Equipment and Consumables	7
6.4. Installation Requirements	7
7. Warnings and Precautions	8
7.1. Laboratory Precautions	8
7.2. Microbiological and Infectious State	8
8. Sample Collection and Handling	8
8.1. Blood Collection and Blood Storage	8
8.2. Plasma Sample Preparation and Plasma Storage	8
9. Test Procedure	9
9.1. Preparation of Working Solutions	9
9.2. DNA Extraction and Bisulfite Conversion from Patient Plasma	9
9.3. PCR Setup	13
10. Analysis with the Applied Biosystems 7500 Fast and 7500 Fast Dx PCR Instruments	14
10.1. Software Requirement	14
10.2. PCR Plate Preparation (Applied Biosystems 7500 Fast / Fast Dx)	14
10.3. Plate Loading (Applied Biosystems 7500 Fast / Fast Dx)	15
10.4. Analysis Settings (Applied Biosystems 7500 Fast / Fast Dx)	18
10.5. Run Validity by the Epi proColon Controls (Applied Biosystems 7500 Fast / Fast Dx)	19
10.6. Interpretation of Results for a Single PCR (Applied Biosystems 7500 Fast / Fast Dx)	19
11. Analysis with Roche LightCycler 480 Instrument I	20
11.1. Plate Preparation (LightCycler 480 Instrument I)	20
11.2. Plate Loading (LightCycler 480 Instrument I)	20
11.3. Analysis Settings (LightCycler 480 Instrument I)	20
11.4. Run Validity by the Epi proColon Controls (LightCycler 480 Instrument I)	21
11.5. Interpretation of Results for a Single PCR (LightCycler 480 Instrument I)	22
12. Analysis with the Roche LightCycler 480 Instrument II	22
12.1. Plate Preparation (LightCycler 480 Instrument II)	22
12.2. Plate Loading (LightCycler 480 Instrument II)	22
12.3. Analysis Settings (LightCycler 480 Instrument II)	23
12.4. Run Validity of the Epi proColon Test Result (LightCycler 480 Instrument II)	24
12.5. Interpretation of Results for a Single PCR (LightCycler 480 Instrument II)	24
13. Interpretation of Results for a Patient Sample	25
14. Quality Control	25
14.1. External Controls	25
14.2. Internal Controls	25
15. Limitations of the Procedure	26
16. Performance Characteristics	26
16.1. Analytical Sensitivity	26
16.2. Reproducibility	26
16.3. Clinical Sensitivity and Specificity	26
16.4. Concordance of the Two Real-Time PCR instruments	27
16.5. Interference	27
17. Meaning of Symbols	28
18. References	29
19. Contact Information	29

1. Name and Intended Use

Epi proColon 2.0 CE is a qualitative assay for the real-time PCR detection of methylated Septin 9 DNA in bisulfite converted DNA from human plasma samples. Presence of methylated Septin 9 is associated with, and may aid in, the detection of colorectal cancer.

Epi proColon 2.0 CE is comprised of the Epi proColon Plasma Quick Kit (M5-02-001), the Epi proColon Sensitive PCR Kit (M5-02-002) and the Epi proColon Control Kit (M5-02-003).

2. Summary and Explanation

Epi proColon 2.0 CE is an *in vitro* polymerase chain reaction (PCR) assay for the qualitative detection of Septin 9 gene methylation in DNA isolated from 3.5 ml of patient plasma. Cytosine residues in the v2 region of the Septin 9 gene become methylated in colorectal cancer (CRC) tissue but not in normal colon mucosa. This aberrant methylation can be detected by specific amplification of DNA present in the blood stream. Detection of CRC DNA in plasma using the Septin 9 methylation biomarker has been demonstrated in multiple case control studies of CRC patients and colonoscopy-verified negative controls¹⁻³. The blood-based Epi proColon 2.0 CE offers patients, who currently refuse screening colonoscopy, a reasonable alternative to non-invasive CRC screening options.

3. Principles of the Procedure

Epi proColon 2.0 CE involves two steps. In the first step DNA is extracted from plasma followed by bisulfite conversion of the purified DNA using the Epi proColon Plasma Quick Kit (M5-02-001) or Epi BiSKit (M7-01-001). In the second step bisulfite converted DNA (bisDNA) is assayed in a duplex PCR using the Epi proColon Sensitive PCR Kit (M5-02-002) which detects both the target methylated Septin 9 DNA and an internal control, ACTB (β -actin) DNA, to assess adequacy of input DNA. Controls provided with the Epi proColon Control Kit (M5-02-003) are required as positive and negative controls for each run.

The extraction of DNA contained in patient plasma is based on the binding of free-circulating DNA to magnetic particles, which are then magnetically separated from the plasma. Remaining impurities are removed from the magnetic particles during the washing steps. In the elution step purified DNA is removed from the magnetic particles by dissolving in elution buffer. The eluate containing DNA is then subjected to a chemical reaction that specifically alters unmethylated cytosine residues within the DNA. Bisulfite treatment is utilized as the method of choice for analyzing DNA methylation. The conversion is based on the nucleophilic addition of a bisulfite ion to a cytosine nucleotide and a subsequent deamination reaction to yield uracil sulfonate, while 5-methylcytosine (methylated cytosine) does not undergo the deamination reaction and remains unchanged.

The blockers and probes used in the subsequent PCR reaction discriminate between methylated and unmethylated sequences. Epi proColon 2.0 CE detects a bisDNA sequence containing methylated CpG sites within the v2 region of the Septin 9 gene and total bisDNA of a region of the ACTB gene. The Septin 9 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. A methylated Septin 9-specific fluorescent detection probe is used in the reaction in order to exclusively identify methylated sequences amplified during the PCR reaction⁴.

The Epi proColon Plasma Quick Kit (M5-02-001) and the Epi BiSKit (M7-01-001) are interchangeable for DNA extraction and bisulfite conversion.

4. Materials Provided

4.1. Contents

Table 1: Contents of the Epi proColon Plasma Quick Kit and Epi BiSKit.

Reagent Epi proColon Plasma Quick Kit	Reagent Epi BiSKit	Containers	Volume	Storage Temperature
Epi proColon Lysis Binding Buffer	Lysis Binding Buffer	1 bottle	125 ml	15° C to 30° C
Epi proColon Wash A Concentrate	Wash A Concentrate	1 bottle	60 ml	15° C to 30° C
Epi proColon Magnetic Beads	Magnetic Beads	1 bottle	4 ml	15° C to 30° C
Epi proColon Wash B Concentrate	Wash B Concentrate	1 bottle	7 ml	15° C to 30° C
Epi proColon Elution Buffer	Elution Buffer	1 tube	6 ml	15° C to 30° C
Epi proColon Bisulfite Solution	Bisulfite Solution	4 tubes	1.9 ml each	15° C to 30° C
Epi proColon Protection Buffer	Protection Buffer	1 tube	1 ml	15° C to 30° C

Note: The color of the (Epi proColon) Protection Buffer can vary from a clear to a brownish color.

Table 2: Contents of the Epi proColon Sensitive PCR Kit.

Reagent	Containers	Volume	Storage Temperature
Epi proColon PCR Mix	2 tubes	810 µl each	-25° C to -15° C
Epi proColon Polymerase	1 tube	85 µl	-25° C to -15° C

Table 3: Contents of the Epi proColon Control Kit.

Reagent	Containers	Volume	Storage Temperature
Epi proColon Negative Control	6 tubes	3.65 ml each	-25° C to -15° C
Epi proColon Positive Control	6 tubes	3.65 ml each	-25° C to -15° C

4.2. Safety Information

When working with chemicals, always wear a laboratory coat and disposable gloves. Clean contaminated surfaces with water. For more information, please consult the respective material safety data sheets (MSDS) available on our webpage (<http://www.epiprocolon.com/en/e-support-library/safety-data-sheets.html>).

(Epi proColon) Lysis Binding Buffer and (Epi proColon) Wash A Concentrate: contain TRITON X-100 and Guanidinium thiocyanate

Hazard statements: H302: Harmful if swallowed. H312: Harmful in contact with skin. H318: Causes serious eye damage. H332: Harmful if inhaled. H412: Harmful to aquatic life with long lasting effects. EUH032: Contact with acids liberates very toxic gas.

Precautionary statements: P261: Avoid breathing aerosols. P273: Avoid release to the environment. P301 + P312: IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell. P302 + P352: IF ON SKIN: Wash with plenty of soap and water. P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.



DANGER

(Epi proColon) Bisulfite Solution: contains aqueous solution of Ammonium bisulfite (Ammonium hydrogen sulfite)

Hazard statements: H319: Causes serious eye irritation. EUH031: Contact with acids liberates toxic gas.

Precautionary statements: P264: Wash hands thoroughly after handling. P271: Use only outdoors or in well-ventilated areas. P280: Wear protective gloves/protective clothing. P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P312: Call a POISON CENTER or doctor/physician if you feel unwell.



WARNING

(Epi proColon) Protection Buffer: contains 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Tetrahydrofurfuryl alcohol

Hazard statements: H302: Harmful if swallowed. H315: Causes skin irritation. H319: Causes serious eye irritation. H360Df: May damage the unborn child. Suspected of damaging fertility. H335: May cause respiratory irritation

Precautionary statements: P101: If medical advice is needed, have product container or label at hand. P102: Keep out of reach of children. P201: Obtain special instructions before use. P271: Use only outdoors or in a well-ventilated area. P280: Wear protective gloves/protective clothing/eye protection/face protection. P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P308+P313: If exposed or concerned: Get medical advice/attention. P405: Store locked up.



DANGER

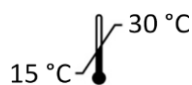
(Epi proColon) Wash B Concentrate, (Epi proColon) Elution Buffer, (Epi proColon) Magnetic Beads, Epi proColon PCR Mix, Epi proColon Polymerase, Epi proColon Positive Control, and Epi proColon Negative Control are not harmful.

5. Storage and Stability

Reagents provided with the Epi proColon Plasma Quick Kit (M5-02-001), Epi BiSKit (M7-01-001), Epi proColon Sensitive PCR Kit (M5-02-002) and Epi proColon Control Kit (M5-02-003) are stable until the expiration date when stored and handled as directed. Do not use material past expiration date. Do not mix components from different kit lots.

5.1. Epi proColon Plasma Quick Kit (M5-02-001) & Epi BiSKit (M7-01-001)

Store all reagents of the Epi proColon Plasma Quick Kit and the Epi BiSKit at 15 to 30°C.



(Epi proColon) Bisulfite Solution is sensitive to oxygen contact. Use only unopened tubes of (Epi proColon) Bisulfite Solution. Discard used tubes!

Store reconstituted (Epi proColon) Wash A Buffer and reconstituted (Epi proColon) Wash B Buffer at 15 to 30°C for up to 6 weeks.

After first use store all reagents at 15 to 30°C for up to 6 weeks.

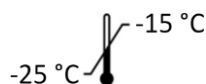
5.2. Epi proColon Sensitive PCR Kit (M5-02-002)

Store Epi proColon PCR Mix and Epi proColon Polymerase at -25 to -15°C.



Each Epi proColon PCR Mix tube may be thawed and refrozen one time. After first use store all reagents at -25 to -15°C for up to 6 weeks.

5.3. Epi proColon Control Kit (M5-02-003)



Store Epi proColon Control Kit at -25 to -15°C.

6. Materials Required, but not Provided

6.1. General Laboratory Equipment

The following general laboratory equipment is required to perform the Epi proColon 2.0 CE test. All laboratory equipment must be installed, calibrated, operated, and maintained according to the manufacturer’s recommendations.

<i>Required Equipment</i>	<i>Suggested Equipment</i>
Tube rack for 15 ml tubes and 2 ml tubes	VWR International, catalog no. 211-0200, or equivalent
Rotator	VWR International, catalog no. 445-2102; Carl Roth GmbH + Co. KG, catalog no. Y549.1, or equivalent
Vortex	VWR International, catalog no 444-1372, or equivalent
Thermoshaker for 2 ml tubes	ThermoMixer® C, Eppendorf, catalog no. 5382 000.015, with dry heating block 2 ml, Eppendorf, catalog no. 5362 000.035, or equivalent (Temperature accuracy of the thermoshaker: ±2°C at 23 - 80°C)
Pipettes with adjustable volumes of the following ranges 10 - 100 µl, 100 - 1000 µl	Eppendorf Reference®, adjustable volume pipette, Eppendorf, catalog no. 4910 000.042 and 4910 000.069, or equivalent
Repeat pipettor capable of repetitively dispensing volumes in an adjustable range	Eppendorf Multipette® M4, Eppendorf, catalog no. 4982 000.012, or HandyStep® electronic, Brand, catalog no. 705000/705001, or equivalent
Bench top centrifuge with rotor for 1.5/2.0 ml tubes	Centrifuge 5418, Eppendorf, catalog no. 5418 000.017 with rotor F-45-30-11, Eppendorf, catalog no. 5490 015.002, or equivalent
Multichannel pipette	Eppendorf Research® plus 8-Channel, 10 - 100 µl, Eppendorf, catalog no. 3122 000.035, or equivalent
Plate centrifuge capable for PCR plates	Centrifuge 5804 R, Eppendorf, catalog no. 5804 000.013, with rotor A-2-DWP, Eppendorf, catalog no. 5804 740.009, or equivalent
100 ml graduated cylinder or 50 ml serological pipette	Carl Roth, catalog no. C177.2, or equivalent, or Fisher Scientific, catalog no. 11889181, or equivalent

6.2. General Laboratory Consumables and Reagents

<i>Consumables and Reagents</i>	<i>Suggested Consumables and Reagents</i>
Ethanol absolute (for molecular biology, ≥99.5 %)	Merck KGaA, catalog no. 1.08543.0250, or equivalent
15 ml polypropylene centrifuge tubes with conical bottom, PP/sterile	Sarstedt, catalog no. 62.554.502, or equivalent
2.0 ml microtubes with round bottom and with attached PP cap with lid seal mechanism	Sarstedt SafeSeal, catalog no. 72.695.400 or Eppendorf Safe-Lock™, catalog no. 0030 120.094, or equivalent
Pipette tips with aerosol barrier	ep Dualfilter T.I.P.S.®, Eppendorf: <ul style="list-style-type: none"> ▪ 2 - 100 µl, catalog no. 0030 077.547, or equivalent ▪ 50 - 1000 µl, catalog no. 0030 077.571, or equivalent
Tips for repetitive pipettes for volumes of 0.5 ml, 1 ml, 10 ml, 25 ml	Combitips advanced®, Eppendorf: <ul style="list-style-type: none"> ▪ 0.5 ml, catalog no. 0030 089.421, or equivalent ▪ 1 ml, catalog no. 0030 089.430, or equivalent ▪ 10 ml, catalog no. 0030 089.464, or equivalent ▪ 25 ml, catalog no. 0030 089.472, or equivalent
Disposable transfer pipettes, non-sterile bulk packaged, length 15 cm (about 6 inches), stem diameter 5 mm, capacity about 5 ml	VWR Disposable Transfer Pipettes with Reference Lines Non-sterile, catalog no. 612-4520, or equivalent
Disposable transfer pipettes, non-sterile bulk packaged, length 22.5 cm (about 9 inches), stem diameter 5 mm, capacity about 5 ml	Disposable transfer pipette, graduated, Carl Roth, catalog no. EA61.1, or equivalent

<i>Consumables and Reagents</i>	<i>Suggested Consumables and Reagents</i>
96 well plates for DNA storage	MicroAmp® Fast 96-Well Reaction Plate, 0.1 ml , Applied Biosystems (Life Technologies Co.), catalog no. 4346906, or equivalent
Adhesive film or foil for DNA storage plate	VWR Thermalseal PCR Sealing Film, catalog no. 391-1254 or Eppendorf Storage Foil (self-adhesive), catalog no. 0030 127.889, or equivalent
Applicator to form a tight seal between a microplate and an adhesive film	MicroAmp® Adhesive Film Applicator, Applied Biosystems, catalog no. 4333183
Re-closable plastic bags, 10 x 15 cm for disposing of used PCR plates.	Carl Roth, catalog no. P279.2, or equivalent
Cryovial, 5 ml, self standing	VWR, catalog no. 479-1218, or equivalent
Blood collection tubes	<ul style="list-style-type: none"> ▪ BD Vacutainer® K₂EDTA Blood Collection Tubes (Becton Dickinson, e.g. catalog no. 367525) or ▪ S-Monovette® 9 ml K3E (Sarstedt catalog no. 02.1066.001) or ▪ S-Monovette® 8.5 ml CPDA (Sarstedt catalog no. 01.1610.001)

6.3. Required Special Equipment and Consumables

The following special equipment and consumables are required to perform the Epi proColon 2.0 CE test and cannot be replaced by other equipment.

When performing the Epi proColon 2.0 CE test on the **Applied Biosystems 7500 Fast or 7500 Fast Dx PCR instruments**:

- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with Sequence Detection Software v1.4 21 CFR Part 11 Module for Windows XP or with Sequence Detection Software v1.4.1 21 CFR Part 11 Module for Windows 7 (Life Technologies Co., catalog no. 4406984 or 4406985) or Applied Biosystems 7500 Fast Real-Time PCR Instrument (Life Technologies Co., catalog no. 4351106 or 4351107) with Sequence Detection Software v1.4 21 CFR Part 11 Module for Windows XP or with Sequence Detection Software v1.4.1 21 CFR Part 11 Module for Windows 7 (Life Technologies Co., catalog no. 4377355)
- MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 ml (Applied Biosystems (Life Technologies Co.), 20 plates, catalog no. 4346906; 200 plates, catalog no. 4366932)
MicroAmp® 96- & 384-Well Optical Adhesive Film (Applied Biosystems (Life Technologies Co.), 25 sheets, catalog no. 4360954 or 100 sheets, catalog no. 4311971)

When performing the Epi proColon 2.0 CE test on the **Roche LightCycler 480 instruments I and II**:

- LightCycler 480 Instrument I with 96-well heat block (Roche, catalog no. 05015278001) and with Software version 1.5.x or
LightCycler 480 Instrument II with 96-well heat block (Roche, catalog no. 05015278001) and with Software version 1.5.x
- LightCycler 480 Multiwell Plate 96 (white plate) with sealing foils (Roche, catalog no. 04729692001)
- LightCycler 480 Sealing Foil (Roche, catalog no. 04729757001)

Additional equipment required, compatible with all Real-Time PCR instrument configurations:

- Magnetic Separator: DynaMag™-15 magnet (Invitrogen, catalog no. 123.01D)
- Magnetic Separator: DynaMag™-2 magnet (Invitrogen, catalog no. 123.21D)

6.4. Installation Requirements

The installation, calibration, performance verification and maintenance of the Applied Biosystems 7500 Fast Dx PCR Instrument, Applied Biosystems 7500 Fast PCR Instrument or the Roche LightCycler 480 Instrument I or II needs to be performed according to the manufacturer’s instructions.

Note: Monthly background calibration as described in the manufacturer’s maintenance procedure is mandatory for the Applied Biosystems 7500 Fast Dx PCR Instrument and Applied Biosystems 7500 Fast PCR Instrument.

Semi-annual maintenance tasks are required for the Applied Biosystems 7500 Fast Dx and 7500 Fast PCR Instrument according to manufacturer's instructions, including calibration of the pure dyes FAM™, JOE™, TAMRA™.

7. Warnings and Precautions

7.1. Laboratory Precautions

Compliance with good laboratory practices is essential to minimize the risk of cross-contamination between samples during and after the DNA extraction, bisulfite conversion, and purification procedure.

Prevent the introduction of nucleases into samples during the extraction procedure. We recommend using only single-use pipettes and pipette tips to prevent cross-contamination of patient samples. This procedure is for professional laboratory use only and assumes familiarity with DNA extraction methods and real-time PCR assays.

To prevent contamination by amplicons generated from previous PCR we recommend a strict separation of pre-PCR activities (e.g. plasma DNA extraction and purification, PCR setup) and post-PCR activities (e.g. real-time PCR). Further, we recommend that used PCR plates are disposed in a way that no PCR product can be released. E. g. used PCR plates should be placed in a re-closable plastic bag immediately after removal from the PCR instrument; the bag closed and disposed of in dedicated waste container. Never store a used PCR plate outside of the PCR instrument. Never open a used PCR plate.

7.2. Microbiological and Infectious State

The product does not contain any infectious substances or agents which cause disease in humans or animals.

Human blood and plasma samples analyzed with this assay should be handled as potentially infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories, Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work, or with other appropriate biosafety practices.

8. Sample Collection and Handling

8.1. Blood Collection and Blood Storage

Blood collection and blood storage can be performed according to the following condition(s):

- Use BD Vacutainer® K₂EDTA tube or S-Monovette® 9 ml K3E for drawing blood. Follow manufacturer's recommendations for performing blood draw. Blood should be processed immediately. At maximum the blood may be stored at 2 to 8°C for up to 24 hours prior to plasma preparation. Do not freeze blood samples.
- Alternatively S-Monovette® 8.5 ml CPDA tube can be used for drawing blood. Follow manufacturer's recommendations for performing blood draw. Blood should be processed immediately. At maximum the blood may be stored in S-Monovette® 8.5 ml CPDA tube at 15 to 25°C for 48 hours. Do not freeze blood samples.

8.2. Plasma Sample Preparation and Plasma Storage

- Disable the brake function in the centrifuge to prevent disruption of the cell layer.
- Centrifuge the blood in blood collection tube for 12 min at 1350 ± 150 rcf. For conversion of revolutions per minute (rpm) to rcf, refer to the centrifuge user manual.
- Remove blood collection tube from centrifuge.
- Use a fresh 15 cm disposable transfer pipette to transfer plasma from the collection tube to a 15 ml polypropylene centrifuge tube with conical bottom.
- Centrifuge plasma in the 15 ml centrifuge tube for 12 min at 1350 ± 150 rcf.
- Using a fresh extra-long (22.5 cm) disposable transfer pipette or serological pipette, transfer 3.5 ml plasma into a labeled cryovial or centrifuge tube.
- Plasma samples may be stored at -15 to -25°C for up to 4 weeks.
- When using Vacutainer® K₂EDTA tube, plasma sample may be stored at 2 to 8°C for up to 18 hours.

Note: Take care not to disturb or transfer the buffy coat (white blood cells) layered above the red blood cells in the blood collection tube after the first centrifugation, or sedimented at the bottom of the conical centrifuge tube after the second centrifugation.

9. Test Procedure

A repeat pipettor is recommended for repetitive dispensing of the following reagents:

- (Epi proColon) Lysis Binding Buffer
- (Epi proColon) Magnetic Bead Suspension
- Ethanol in DNA Binding Step 9.2.3.
- (Epi proColon) Wash A Buffer
- (Epi proColon) Wash B Buffer
- (Epi proColon) Elution Buffer
- (Epi proColon) Bisulfite Solution
- (Epi proColon) Protection Buffer and the PCR Master Mix.

Further we strongly recommend to use a ferris wheel rotator not a rocking platform, and to pipette extracted and bisulfite treated DNA with reference pipettes.

9.1. Preparation of Working Solutions

9.1.1. Preparation of (Epi proColon) Wash A Buffer

- Add 60.0 ml of Absolute Ethanol (for molecular biology, $\geq 99.5\%$) to the (Epi proColon) Wash A Concentrate using a sterile graduated cylinder or a serological pipette.
- Close lid, mix thoroughly by inverting the bottle five times, avoiding formation of foam. Label the bottle with date of dilution and mark the “Ethanol added” checkbox.
- Store reconstituted (Epi proColon) Wash A Buffer at 15 to 30°C for up to 6 weeks.

9.1.2. Preparation of (Epi proColon) Wash B Buffer

- Add 40.0 ml of Absolute Ethanol (for molecular biology, $\geq 99.5\%$) to the (Epi proColon) Wash B Concentrate using a sterile graduated cylinder or a serological pipette.
- Close lid, mix thoroughly by inverting the bottle five times. Label the bottle with date of dilution and mark the “Ethanol added” checkbox.
- Store reconstituted (Epi proColon) Wash B Buffer at 15 to 30°C for up to 6 weeks.

9.2. DNA Extraction and Bisulfite Conversion from Patient Plasma

Epi proColon 2.0 CE contains sufficient reagents to run up to 32 samples including controls. One Epi proColon Positive Control and one Epi proColon Negative Control must be included in each independent test run. There are 4 single-use tubes of (Epi proColon) Bisulfite Solution that will enable a maximum of 4 independent test runs (e.g., 4 runs of 8 samples each).

Note: Brief centrifugation of microtubes (stated as ‘Briefly spin down the tubes’) is required in several steps of this instruction to remove drops from the lid and/or to collect remaining liquid. It is recommended to centrifuge for 10 - 20 sec at $1,000 \pm 150$ rcf using a Bench-Top centrifuge. Avoid stronger centrifugation to prevent the compacting of magnetic bead pellets in specific steps.

Note: Vortexing of tubes and containers is required in several steps of this instruction to ensure homogeneous mixing of liquid. It is recommended to use a vortexer adjusted to medium speed for 5 to 10 sec.

9.2.1. Thawing of Plasma and Epi proColon Positive and Negative Control

- Thaw one Epi proColon Positive Control and one Epi proColon Negative Control for about 30 min at 15 to 30°C.
- If frozen plasma sample is used, thaw sample for about 30 min at 15 to 30°C.
- Start lysis within 60 min after thawing.

9.2.2. Lysis

Note: Prior to use, briefly shake the (Epi proColon) Lysis Binding Buffer and visually check for precipitates. If precipitates are present heat the (Epi proColon) Lysis Binding Buffer in a water bath at 37°C for 60 min and shake gently until the precipitate is completely dissolved. Equilibrate (Epi proColon) Lysis Binding Buffer to room temperature before use.

- Add the following to a labeled 15 ml centrifuge tube:
 - 3.5 ml plasma sample, or Epi proColon Positive Control, or Epi proColon Negative Control
 - 3.5 ml (Epi proColon) Lysis Binding Buffer
- Cap the tube and mix by vortexing for 5-10 sec.
- Incubate tube on the bench top at 15 to 30°C for 10 ± 1 min.

9.2.3. DNA Binding

Note: A homogeneous suspension of the (Epi proColon) Magnetic Beads is essential for proper test performance. Deviations from the specified amount of beads may lead to false results. To ensure the correct magnetic bead concentration, the bottle should be mixed thoroughly just before pipetting. There should be no visible sediment at the bottom of the bottle. Mix to ensure a homogeneous suspension between the pipetting steps.

- Add to the 15 ml centrifuge tube in the following order:
 - 90 µl (Epi proColon) Magnetic Beads (freshly suspended)
 - 2.5 ml of Absolute Ethanol (for molecular biology, ≥99.5 %)
- Cap the tube and mix by inverting the tube 5 - 6 times.
- Place 15 ml tube into a rotator.
- Rotate at room temperature for 45 ± 5 min at medium speed (approx. 10 - 20 rpm); adjust rotator angle to 35 - 45 degrees.

9.2.4. DNA Washing

Note: Before starting the wash procedure set the thermoshaker to 80°C for later use in the elution and bisulfite conversion steps.

- Place the 15 ml tube into the DynaMag™-15 magnetic racks for 5 - 10 min.

Note: If incomplete bead capturing is observed after this step, incubate the affected tube at 56°C for up to 10 min (e.g. water bath) and place the tube back into the DynaMag™-15 magnetic racks for 5 - 10 min.

- Pour off the supernatant carefully, taking care not to remove magnetic beads.
 - Add 1.5 ml (Epi proColon) Wash A Buffer
- Resuspend magnetic beads completely by vortexing for 5 - 10 sec.
- Using an extra-long (22.5 cm) disposable transfer pipette, transfer magnetic beads suspension into a labeled 2.0 ml microtube.
- Place disposable transfer pipette back into 15 ml tube to collect remaining magnetic beads and transfer them into the 2.0 ml microtube.
- Place the microtube into the DynaMag™-2 magnetic racks for 2 - 6 min.
- Using a 15 cm disposable transfer pipette, remove as much buffer as possible while microtube is still in the DynaMag™-2 magnetic rack, taking care not to remove magnetic beads.
- Briefly spin down the microtube.
- Place the 2.0 ml microtube into the DynaMag™-2 magnetic rack for 2 - 6 min.
- Using a 10 - 100 µl reference pipette, remove as much residual buffer as possible while microtube is still in magnetic rack.

9.2.5. Elution

- Transfer the microtube into a non-magnetic rack.

- Vortex (Epi proColon) Elution Buffer for 5 - 10 sec.
- Add 100 µl (Epi proColon) Elution Buffer to each microtube.
- Close the microtube.
- Resuspend the magnetic beads by vortexing for 5 - 10 sec.
- Place microtube into a thermoshaker set to $1,000 \pm 100$ rpm and incubate at 80°C for 10 ± 1 min.
- Briefly spin down the microtube.
- Place microtube into the DynaMag™-2 magnetic rack for 2 - 6 min.
- Transfer the complete eluate, while microtube is still in the magnetic rack, (~100 µl DNA solution) into fresh 2.0 ml microtube.
- Discard the 2 ml microtube containing the magnetic beads.

9.2.6. Storage of Extracted DNA

Note: If extracted DNA is not used immediately, store material at 2 to 8°C for up to 24 hours. **Do not freeze** the extracted DNA.

9.2.7. Bisulfite Conversion

Note: (Epi proColon) Bisulfite Solution is sensitive to oxygen contact. Use only unopened tubes of (Epi proColon) Bisulfite Solution. Do not store but discard any left-over solution.

- Add the following reagents to the 2.0 ml microtubes containing the eluate (~100 µl DNA solution):
 - 150 µl (Epi proColon) Bisulfite Solution
 - 25 µl (Epi proColon) Protection Buffer

Note: The color of the (Epi proColon) Protection Buffer can vary from a clear to a brownish color.

- Cap the microtube and mix the bisulfite reaction by vortexing for 5 - 10 sec.
- Briefly spin down the microtube.
- Place microtube into thermoshaker and incubate for 45 ± 5 min at 80°C **without** shaking.
- Remove microtube from the thermoshaker immediately after 45 ± 5 min.
- Reset thermoshaker temperature to 23°C , or set up a second thermoshaker to 23°C for later use.

9.2.8. Binding Step

Note: A homogeneous suspension of beads in the (Epi proColon) Magnetic Beads suspension is essential for proper performance. Deviations from the specified amount of beads may lead to false results. To ensure correct magnetic bead concentration, the bottle should be mixed thoroughly just before pipetting. There should be no visible sediment at the bottom of the bottle. Mix to ensure a homogeneous suspension between the pipetting steps.

- Briefly spin down the 2.0 ml microtube containing the bisulfite reaction.
- Add the following components to the microtube:
 - 1000 µl (Epi proColon) Wash A Buffer
 - 20 µl (Epi proColon) Magnetic Beads (freshly suspended).
- Mix by vortexing for 5 - 10 sec.
- Wait until thermoshaker reaches 23°C .
- Place the microtube in the thermoshaker at 1000 ± 100 rpm and incubate for 45 ± 5 min.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 - 6 min.
- Using a fresh 15 cm disposable transfer pipette, remove as much liquid as possible while tube is still in the magnetic rack, taking care not to remove magnetic beads.

9.2.9. **First Wash**

- Remove the sample rack from the magnet for washing and vortexing:
 - Add 800 µl (Epi proColon) Wash A Buffer.
- Resuspend by vortexing for 5 - 10 sec.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 - 6 min.
- Using a fresh 15 cm disposable transfer pipette, remove as much liquid as possible while tube is still in the magnetic rack, taking care not to remove magnetic beads.

9.2.10. **Second Wash**

- Remove the sample rack from the magnet for washing and vortexing:
 - Add 800 µl (Epi proColon) Wash B Buffer.
- Resuspend by vortexing for 5 - 10 sec.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 - 6 min.
- Using a fresh 15 cm disposable transfer pipette, remove as much liquid as possible while tube is still in the magnetic rack, taking care not to remove magnetic beads.

9.2.11. **Third Wash**

- Remove the sample rack from the DynaMag™-2 magnet for washing and vortexing:
 - Add 400 µl (Epi proColon) Wash B Buffer.
- Resuspend by vortexing for 5 - 10 sec.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 - 6 min.
- Using a fresh 15 cm disposable transfer pipette, remove as much liquid as possible while tube is still in the magnetic rack, taking care not to remove magnetic beads.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 - 6 min.
- Using a 10 - 100 µl reference pipette, remove as much remaining liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads.

Note: This step is very important. If the remaining droplets are not removed from the magnetic beads, the remaining (Epi proColon) Wash B Buffer may cause performance issues in the final PCR.

9.2.12. **Drying**

Note: Do not increase drying time or temperature as over-drying might reduce bisDNA recovery.

- Open microtube lid.
- Place open microtube into thermoshaker.
- Allow the pellet to dry for 10 ± 1 min at 23°C without shaking.

9.2.13. **Elution**

- Transfer microtube into a non-magnetic rack:
 - Add 60 µl (Epi proColon) Elution Buffer.
- Close the microtube.
- Resuspend the magnetic beads by vortexing for 5 - 10 sec.
- Incubate for 10 ± 1 min at 23°C in a thermoshaker at 1000 ± 100 rpm.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 - 6 min.

- Using a 10 - 100 µl reference pipette, transfer the complete eluate (~ 60 µl DNA solution) into a 96-well plate and seal the plate with adhesive film using an adhesive film applicator.
- Set up the bisDNA storage plate according to the recommended plate layout in Table 4.

Table 4: Recommended Layout for a bisDNA Storage Plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC [#]	S7										
B	NC [§]	S8										
C	S1	S9										
D	S2	S10										
E	S3	S11										
F	S4	S12										
G	S5	S13										
H	S6	S14										

[#]Epi proColon Positive Control, [§]Epi proColon Negative Control

9.2.14. **Storage of Bisulfite Converted DNA**

If extracted bisulfite converted DNA (bisDNA) is not used immediately, store material at 2 to 8°C for up to 24 hours or at -25 to -15°C for up to 72 hours.

9.3. **PCR Setup**

Note: Each bisDNA (patient sample, Epi proColon Positive Control, or Epi proColon Negative Control) must be tested in triplicate.

Note: Prior to use spin Epi proColon Polymerase for 10 - 20 sec at 1,000 ± 150 rcf using a Bench-Top centrifuge to remove drops from the lid.

9.3.1. **Preparation of PCR Master Mix**

- Thaw 1 or 2 Epi proColon PCR Mix tubes depending on the desired number of patient and control sample determinations (see Table 5).
- Vortex the Epi proColon PCR Mix tube(s) for 5 - 10 sec, briefly spin down the tube(s).
- Transfer the corresponding volumes of Epi proColon PCR Mix and Epi proColon Polymerase as indicated in Table 5 into a 2.0 ml microtube.
- Mix the PCR Master Mix by vortexing for 5 - 10 sec.
- Briefly spin the PCR Master Mix to remove drops from the lid.

Note: Use PCR Master Mix immediately. Do not store. Refreeze unused Epi proColon PCR Mix and Epi proColon Polymerase, directly after usage.

Note: For a single PCR 16 µl Epi proColon PCR Mix and 0.8 µl Epi proColon Polymerase are required. Indicated volumes already contain pipetting margin. There is no need to prepare an additional reaction for Master Mix preparation.

Table 5: Preparation of PCR Master Mix with pipetting margin.

Component	Volume for 8 Determinations (24 PCRs)	Volume for 16 Determinations (48 PCRs)	Volume for 24 Determinations (72 PCRs)	Volume for 32 Determinations (96 PCRs)
Epi proColon PCR Mix	384 µl	768 µl	1152 µl	1536 µl
Epi proColon Polymerase	19.2 µl	38.4 µl	57.6 µl	76.8 µl

Table 6: Recommended PCR Plate Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC [#]	PC [#]	PC [#]	S7	S7	S7						
B	NC [§]	NC [§]	NC [§]	S8	S8	S8						
C	S1	S1	S1	S9	S9	S9						
D	S2	S2	S2	S10	S10	S10						
E	S3	S3	S3	S11	S11	S11						
F	S4	S4	S4	S12	S12	S12						
G	S5	S5	S5	S13	S13	S13						
H	S6	S6	S6	S14	S14	S14						

[#]Epi proColon Positive Control, [§]Epi proColon Negative Control

Note: If the PCR will be performed with the Applied Biosystems 7500 Fast PCR Instrument or 7500 Fast Dx PCR Instruments follow the instructions in section 10 and section 13.

If the PCR will be performed with the Roche LightCycler 480 Instrument I follow the instructions in section 11 and section 13.

If the PCR will be performed with the Roche LightCycler 480 Instrument II follow the instructions in section 12 and section 13.

10. Analysis with the Applied Biosystems 7500 Fast and 7500 Fast Dx PCR Instruments

10.1. Software Requirement

This product has been validated using the SDS v1.4 software with 21 CFR Part 11 Module.

10.2. PCR Plate Preparation (Applied Biosystems 7500 Fast / Fast Dx)

- Set up the PCR plate. The plate layout as shown in Table 6 is recommended.
- Transfer 15 µl PCR Master Mix into the selected wells of the MicroAmp® Fast Optical 96-Well Reaction Plate.
- Briefly centrifuge the bisDNA storage plate created in section 9.2.13 if required for 1 min at 1000 ± 100 rcf using the plate centrifuge.
- Add 15 µl of bisDNA solution to respective wells of the PCR plate.
- Seal the plate with MicroAmp® Optical Adhesive Film.
- Briefly spin down the plate with a plate centrifuge for 1 min at 1000 ± 100 rcf.

Note: The filled PCR plate can be stored in a refrigerator at 2 to 8 °C for up to 4 hours.

10.3. Plate Loading (Applied Biosystems 7500 Fast / Fast Dx)

Note: The PCR Master Mix does not contain ROX or any other reference dye. Accordingly, the passive reference setting must be set to “none”.

Note: It is recommended to save a template file (*.sdt) with the defined cycling and analysis settings.

- Start the Software.
- Load the specified experiment template file or create a new plate document.
- Click “Create New Document”.
- Define the following plate document:
 - Assay: Standard Curve (Absolute Quantification)
 - Container: 96-Well Clear
 - Template: Blank Document (or select respective Epi proColon 2.0 CE Template file)
 - Run Mode: Standard 7500.
- Click “Next”.
- Click “New Detector”.
- Create a new detector using following properties:
 - Name: Septin9
 - Description: Epi proColon 2.0 CE
 - Reporter dye: FAM
 - Quencher dye: (none)
 - Color: Red.
- Click “Create Another” and define following properties:
 - Name: ACTB
 - Description: Epi proColon 2.0 CE
 - Reporter dye: JOE
 - Quencher dye: (none)
 - Color: Green.
- Click “ok”.
- Select both detectors and click “Add >>” to assign the detectors to the plate document.
- Select “(none)” in the drop down menu of “Passive Reference”.
- Click “Done”.
- Go to the tab “Setup” and “Plate”.
- Select all 96 wells of the plate.
- Go to the menu point “View” and open the “Well Inspector”.
- Select detector “Septin9” and “ACTB”.
- Check the Passive Reference setting to be “(none)” (see Figure 1).
- Click “Close”.
- Go to tab “Instrument” to program the cycling conditions as described in Table 7
- Change the following settings:
 - Sample Volume: 30 µl,
 - Run Mode: Standard 7500,
 - Data Collection: Stage 2, Step 2.
- Create a “Thermal Profile” with 3 stages.
- Create a “Stage 2” having 3 steps, and a “Stage 1” and “Stage 3” having 1 step.
- Enter repetitions, target temperature, and hold time according to Table 7.

- Change the “Ramp Rate” according to Table 7.
- Set “Data Collection” for “Stage 2, Step 2 (55.5 @ 0:35)”.
- Confirm the Thermal Cycler Protocol settings according to Table 7 (see Figure 2).
- Save the run plate document under an appropriate file name. Open the tray.
- Place the PCR plate into the frame (position A1 goes to the upper left corner), ensure that the plate fits accurately in the frame. Close the tray.
- Start the run by pressing the “Start” button.

Table 7: Thermal Cycler Program for Applied Biosystems 7500 Fast / Fast Dx.

Program Parameter	Denaturation	Cycling			Holding
Stage	”Stage 1”	”Stage 2”			”Stage 3”
Repetitions	1	45			1
Step	1	1	2	3	1
Target [°C]	94	62	55.5	93	40
Hold [mm:ss]	20:00	00:05	00:35	00:30	00:05
Auto Increment		0	0	0	
Ramp Rate [%]	40	80	80	40	80
Data Collection	Stage 2, Step 2 (55.5 @ 0:35)				

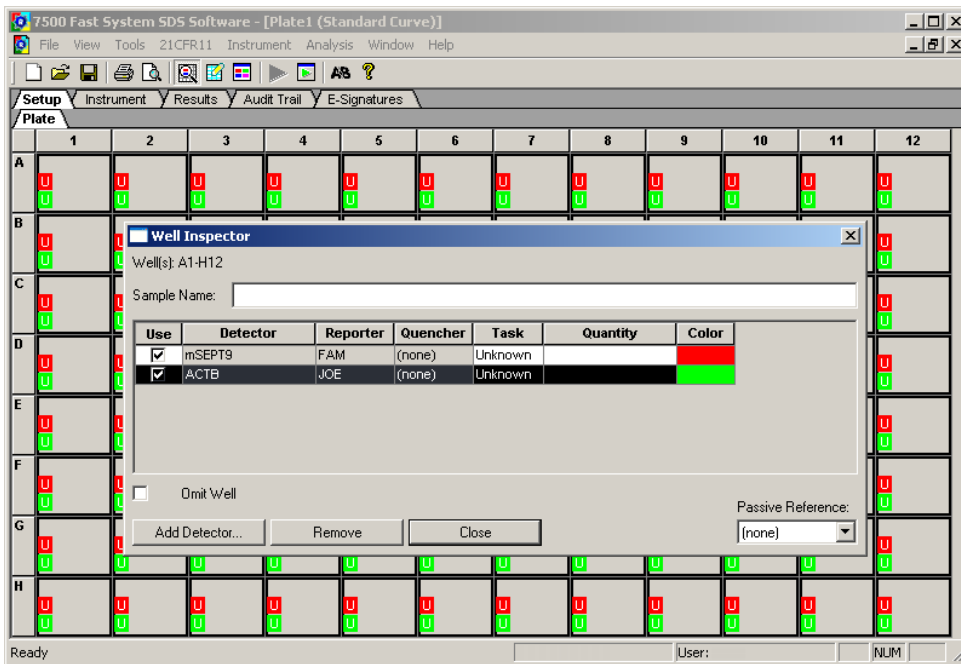


Figure 1: Screenshot after confirming settings in the 'Well Inspector' window.

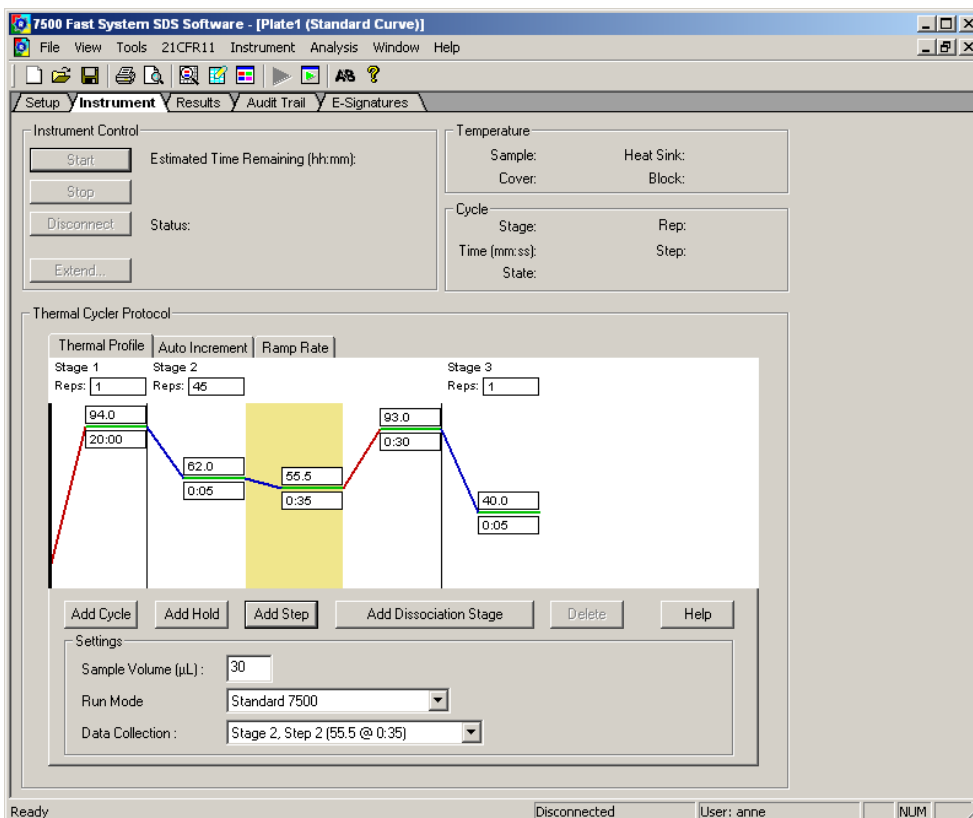


Figure 2: Screenshot after setting cycler program and detection mode.

10.4. Analysis Settings (Applied Biosystems 7500 Fast / Fast Dx)

Note: Analyze PCR runs with software version SDS v1.4 or software version SDS v1.4.1 only.

- **Note:** Incomplete runs or runs where an error message occurs must not be analyzed. The run document must contain fluorescence data for 45 cycles. After completion of the PCR cycling program click “ok”.
- Select the tab “Results”, then select the tab “Amplification Plot”.
- Set “Analysis Setting” for Septin 9 detector to be the following:
 - Data: “Delta Rn vs Cycle”
 - Detector: “Septin9”
 - Line color: “Detector Color”
 - Manual Ct, Threshold: “50000” (appears as “5.0e+004”)
 - Manual baseline, Start (cycle): “10”
 - Manual baseline, End (cycle): “22”
- Set “Analysis Setting” for ACTB detector to be the following:
 - Data: “Delta Rn vs Cycle”
 - Detector: “ACTB”
 - Line color: “Detector Color”
 - Manual Ct, Threshold: “25000” (appears as “2.5e+004”)
 - Manual baseline, Start (cycle): “10”
 - Manual baseline, End (cycle): “22”
- Click “Analyze”.
- Click “Save”.
- Septin 9 Ct values and ACTB Ct values are calculated automatically.
- Select the wells to be analyzed.
- Amplification curves are displayed in the tab “Amplification Plot”.
- Ct values are displayed in the tab “Report”.

Note: Each amplification curve should be visually inspected. Amplification curves crossing the threshold due to inconsistent data points (noise peaks) or linear curve shape should be evaluated as negative. Examples are provided in Figure 3.

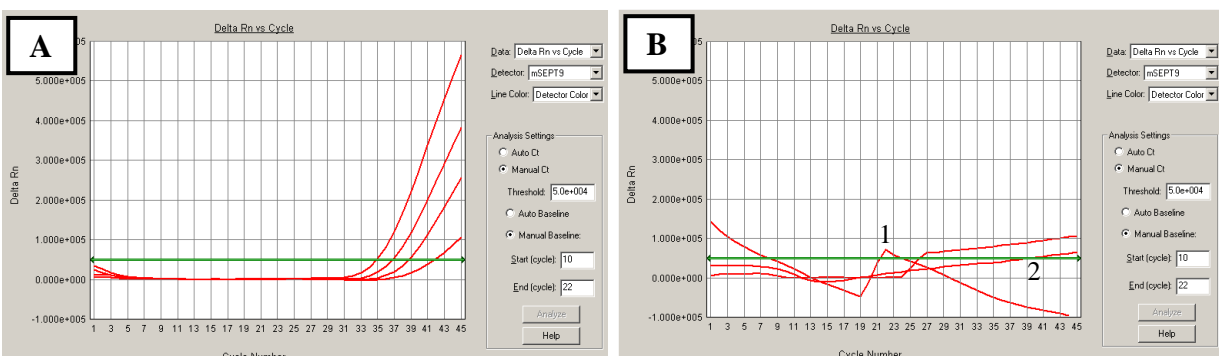


Figure 3: Screenshots of Septin 9 amplification curves using the Applied Biosystems 7500 Fast.

A: Examples of valid positive curves. B: Examples of negative curves due to inconsistent data points (1) or linear curve shape (2).

10.5. Run Validity by the Epi proColon Controls (Applied Biosystems 7500 Fast / Fast Dx)

Any run of one or more patient sample(s) processed together with Epi proColon Positive Control and Epi proColon Negative Control is considered valid, when criteria set forth in Table 8 are met for **ALL THREE (3)** PCR replicates per control.

If either the Epi proColon Positive Control or Epi proColon Negative Control, or both is/are invalid, the data for patient samples processed together with the controls cannot be interpreted. Testing must be repeated for all patient samples included in this test run.

Table 8: Validity limits of Epi proColon Controls analyzed with the Applied Biosystems 7500 Fast / 7500 Fast Dx.

Result of Control	Determination	Septin 9 Result	ACTB Result
Positive Control valid	PCR1	Ct* ≤ 41.1	Ct* ≤ 29.8
	PCR2	Ct* ≤ 41.1	Ct* ≤ 29.8
	PCR3	Ct* ≤ 41.1	Ct* ≤ 29.8
Negative Control valid	PCR1	no Ct* provided ("Undetermined")	Ct* ≤ 37.2
	PCR2		Ct* ≤ 37.2
	PCR3		Ct* ≤ 37.2

*Cycle threshold

10.6. Interpretation of Results for a Single PCR (Applied Biosystems 7500 Fast / Fast Dx)

The interpretation of a single PCR is performed according to Table 9. Provided that the result of the internal control assay ACTB indicates sufficient input of bisDNA into the single PCR (ACTB cycle threshold specified in Table 9), the result of the Septin 9 PCR defines the result for this single PCR (see section 13). An ACTB value above the cycle threshold specified in Table 9 determines the single PCR result to "Invalid PCR".

Table 9: Interpretation of Results for Single PCR (Applied Biosystems 7500 Fast / Fast Dx).

Single PCR Result	Septin 9 Result	ACTB Result
Septin 9 Positive	Ct* < 45	Ct* ≤ 32.1
Septin 9 Negative	no Ct* provided ("Undetermined")	Ct* ≤ 32.1
Invalid	any result	Ct* > 32.1 Or "Undetermined"

*Cycle threshold

11. Analysis with Roche LightCycler 480 Instrument I

11.1. Plate Preparation (LightCycler 480 Instrument I)

- Set up the PCR plate. The plate layout as shown in Table 6 is recommended.
- Transfer 15 µl PCR Master Mix into the selected wells of LightCycler 480 Multiwell Plate 96 Reaction Plate.
- Briefly centrifuge the bisDNA storage plate created in section 9.2.13 for 1 min at 1000 ± 100 rcf using the plate centrifuge.
- Add 15 µl of bisDNA solution to respective wells of the PCR plate.
- Seal the PCR plate with LightCycler 480 Sealing Foil.
- Briefly spin down the PCR plate with a plate centrifuge for 1 min at 1000 ± 100 rcf.

Note: The filled PCR plate can be stored at 2 to 8°C for up to 4 h.

11.2. Plate Loading (LightCycler 480 Instrument I)

Note: It is recommended to save a template file (*.ixc) with the defined cycling and analysis settings.

- Start the software version 1.5.x.
- Create a new experiment, click “New Experiment”
- Load the specified experiment template file or define the following experiment according to the PCR program parameters detailed in Table 10 below.
- Open the tray.
- Place the PCR plate into the frame (position A1 goes to the upper left corner), ensure that the plate fits accurately in the frame. Close the tray.
- Click “Start Run” to start.

Table 10: Standard PCR Program for LightCycler 480 Instrument I.

Program Parameter	Denaturation	Cycling			Cooling
Analysis Mode	None	Quantification mode			None
Cycles	1	50			1
Segment	1	1	2	3	1
Target [°C]	94	62	56	93	40
Hold [hh:mm:ss]	00:20:00	00:00:05	00:00:35	00:00:30	00:00:30
Ramp Rate [°C/s]*	1.0	1.3	1.3	1.3	1.3
Acquisition Mode	None	None	Single	None	None

* LightCycler 480 Instrument I: select the “Dual Color Hydrolysis Probe/UPL Probe” as detection format; activate the filter combination 483 - 533 nm and 523 - 568 nm and set the “Reaction Volume” to “30”.

11.3. Analysis Settings (LightCycler 480 Instrument I)

Note: Users of the LightCycler 480 Instrument I need to select *Color Compensation* “ON” and load the respective “Epi proColon Color Compensation” file for the filter combinations “Filter Comb 483 - 533” and “Filter Comb 523 - 568”.

Note: Perform data analysis only on used wells by generating a respective sample sub set as described in the manual of the LightCycler 480 Instrument I.

- 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 proColon Color Compensation”.
- Activate the “Filter Comb 483 - 533”.

- Set “First Cycle” to “1” and “Last Cycle” to “50”.
- Set the background (click blue background button) to “5 - 22” by setting “Min Offset” to “4” and “Max Offset” to “21” in the “Cycle Range” window.
- Set the noise band to ”Noise Band (Fluoresc)” and set the noise band manually to “1.6” in the “Noise Band” window.
- After selecting “Threshold (Manual)” in the “Analysis” window set the threshold to “1.6”.
- Set the number of Fit Points to “2” in the “Analysis” window.
- Click “Calculate”.

Septin 9 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”.
- Set “First Cycle” to “1” and “Last Cycle” to “50”.
- Set the background (click blue background button) to “5 - 22” by setting Min Offset to “4” and Max Offset to “21” in the “Cycle Range” window.
- Set the noise band to ”Noise Band (Fluoresc)” and set the noise band manually to “0.8” in the “Noise Band” window.
- After selecting “Threshold (Manual)” in the “Analysis” window set the threshold to “0.8”.
- Set the number of Fit Points to “2” in the “Analysis” window.
- Click “Calculate”.

ACTB CP values 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.

Note: Adjust the “Noise Band” and “Threshold” such that it is slightly above the background and that it crosses the amplification curves at the beginning of the exponential increase. Amplification curves without a significant exponential increase should be evaluated as negative.

11.4. Run Validity by the Epi proColon Controls (LightCycler 480 Instrument I)

Any run (patient samples processed together with Epi proColon Positive Control and Epi proColon Negative Control) is considered valid if criteria set forth in Table 11 are met for **ALL THREE (3)** PCR replicates per control.

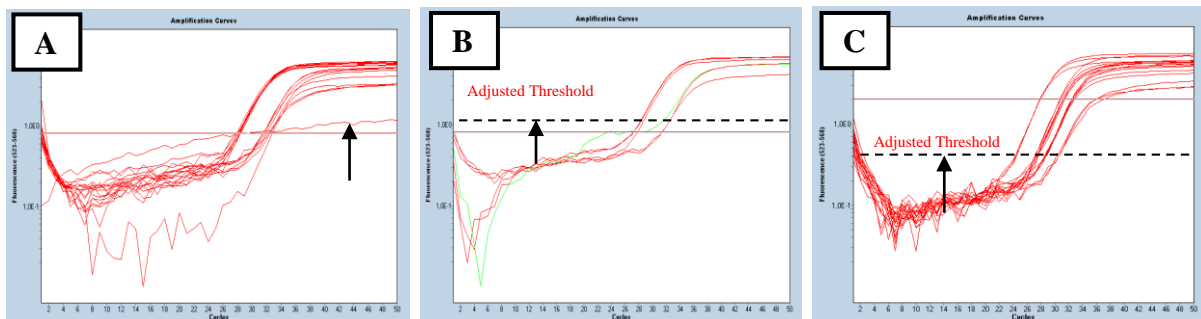


Figure 4: Screenshots of Septin 9 amplification curves using LightCycler 480. A: Amplification curve without a significant exponential increase should be evaluated as negative. B: Threshold should be adjusted that it is slightly above the background. C: Threshold should be adjusted that it crosses the amplification curves at the beginning of the exponential increase.

If either the Epi proColon Positive Control or Epi proColon Negative Control (or both) is invalid, the data for patient samples processed together with the controls cannot be interpreted. Testing must be repeated for all patient samples included in this run.

Table 11: Validity Limits of Epi proColon Controls (LightCycler 480 Instrument I).

Result of Control	Determination	Septin 9 Result	ACTB Result
Positive Control valid	PCR1	CP* ≤ 40.6	CP* ≤ 29.5
	PCR2	CP* ≤ 40.6	CP* ≤ 29.5
	PCR3	CP* ≤ 40.6	CP* ≤ 29.5
Negative Control valid	PCR1	No CP* provided	CP* ≤ 36.5
	PCR2		CP* ≤ 36.5
	PCR3		CP* ≤ 36.5

*Crossing Point

11.5. Interpretation of Results for a Single PCR (LightCycler 480 Instrument I)

If the result of the internal control ACTB indicates sufficient input of DNA into the single PCR (ACTB cycle threshold specified in Table 12), the result of the Septin 9 PCR defines the result for this single PCR (see section 13). An ACTB value above the crossing point specified in Table 12 determines the single PCR result to be “Invalid”.

Table 12: Interpretation of Results for Single PCR (LightCycler 480 Instrument I).

Single PCR Result	Septin 9 Result	ACTB Result
Septin 9 Positive	CP* < 50	CP* ≤ 33.1
Septin 9 Negative	no CP* provided	CP* ≤ 33.1
Invalid	any result	CP* > 33.1

*Crossing Point

12. Analysis with the Roche LightCycler 480 Instrument II

12.1. Plate Preparation (LightCycler 480 Instrument II)

- Set up the PCR plate. The plate layout as shown in Table 6 is recommended.
- Transfer 15 µl PCR Master Mix into the selected wells of a LightCycler 480 Multiwell Plate 96 Reaction Plate.
- Briefly centrifuge the bisDNA storage plate created in section 9.2.13 for 1 min at 1000 ± 100 rcf using the plate centrifuge.
- Add 15 µl of bisDNA solution to respective wells of the PCR plate.
- Seal the PCR plate with LightCycler 480 Sealing Foil.
- Briefly spin down the PCR plate with a plate centrifuge for 1 min at 1000 ± 100 rcf.

Note: The filled PCR plate can be stored in a refrigerator at 2 to 8°C for up to 4 h.

12.2. Plate Loading (LightCycler 480 Instrument II)

Note: It is recommended to save a template file (*.ixc) with the defined cycling and analysis settings.

- Start the software version 1.5.x.
- Create a new experiment, click “New Experiment”
- Load the specified experiment template file or define the following experiment according to the PCR program parameters detailed in Table 13 below.
- Open the tray.

- Place the PCR plate into the frame (position A1 goes to the upper left corner), ensure that the plate fits accurately in the frame. Close the tray.
- Click “Start Run” to start.

Table 13: Standard PCR Program for LightCycler 480 Instrument II.

Program Parameter	Denaturation	Cycling			Cooling
Analysis Mode	None	Quantification mode			None
Cycles	1	50			1
Segment	1	1	2	3	1
Target [°C]	94	62	56	93	40
Hold [hh:mm:ss]	00:20:00	00:00:05	00:00:35	00:00:30	00:00:30
Ramp Rate [°C/s]*	1.0	1.3	1.3	1.3	1.3
Acquisition Mode	None	None	Single	None	None

* LightCycler 480 Instrument II: select the “Dual Color Hydrolysis Probe/UPL Probe” as detection format; activate the filter combination 465 - 510 nm and 533 - 580 nm and set the “Reaction Volume” to “30”.

12.3. Analysis Settings (LightCycler 480 Instrument II)

Note: Perform data analysis only on used wells by generating a respective sample sub set as described in the manual of the LightCycler 480 Instrument II.

- Click “Analysis” on the LightCycler 480 Basic Software Module bar opening the “Analysis Overview” window.
 - Select “Abs Quant/Fit Points” for all samples.
 - Activate the “Filter Comb 465 - 510”.
 - Set “First Cycle” to “1” and “Last Cycle” to “50”.
 - Set the background (click blue background button) to “5 - 22” by setting “Min Offset” to “4” and “Max Offset” to “21” in the “Cycle Range” window.
 - Set the noise band to ”Noise Band (Fluoresc)” and set the noise band manually to “2.0” in the “Noise Band” window.
 - After selecting “Threshold (Manual)” in the “Analysis” window set the threshold to “2.0”.
 - Set the number of Fit Points to “2” in the “Analysis” window.
 - Click “Calculate”.
- Septin 9 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 533 - 580”.
 - Set “First Cycle” to “1” and “Last Cycle” to “50”.
 - Set the background (click blue background button) to “5 - 22” by setting Min Offset to “4” and Max Offset to “21” in the “Cycle Range” window.
 - Set the noise band to ”Noise Band (Fluoresc)” and set the noise band manually to “2.0” in the “Noise Band” window.
 - After selecting “Threshold (Manual)” in the “Analysis” window set the threshold to “2.0”.
 - Set the number of Fit Points to “2” 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.

Note: Adjust the “Noise Band” and “Threshold” such that it is slightly above the background and that it crosses the amplification curves at the beginning of the exponential increase. Amplification curves without a significant exponential increase should be evaluated as negative.

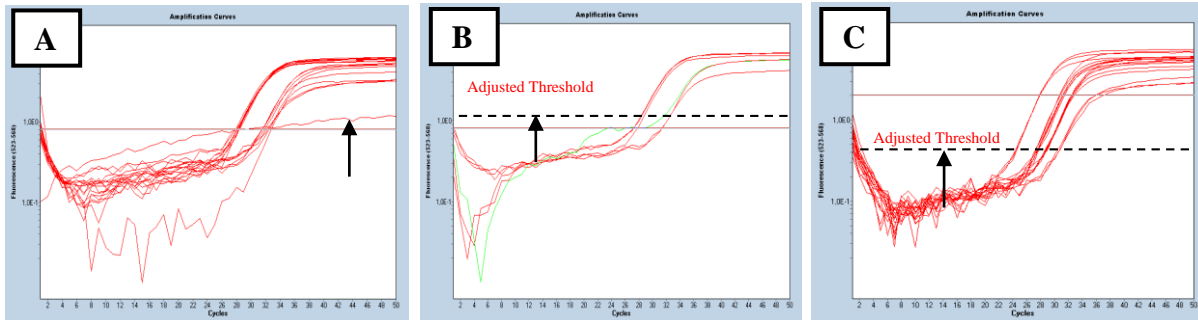


Figure 5: Screenshots of Septin 9 amplification curves using LightCycler 480. A: Amplification curve without a significant exponential increase should be evaluated as negative. B: Threshold should be adjusted that it is slightly above the background. C: Threshold should be adjusted that it crosses the amplification curves at the beginning of the exponential increase.

12.4. Run Validity of the Epi proColon Test Result (LightCycler 480 Instrument II)

Any run (patient samples processed together with Epi proColon Positive Control and Epi proColon Negative Control) is considered valid, if criteria set forth in Table 14 are met for **ALL THREE (3)** PCR replicates per control.

If either the Epi proColon Positive Control or Epi proColon Negative Control (or both) is invalid, the data for patient samples processed together with the controls cannot be interpreted. Testing must be repeated for all patient samples included in this run.

Table 14: Validity Limits of Epi proColon Controls (LightCycler 480 Instrument II).

Result of Control	Determination	Septin 9 Result	ACTB Result
Positive Control valid	PCR1	CP* ≤ 40.5	CP* ≤ 30.3
	PCR2	CP* ≤ 40.5	CP* ≤ 30.3
	PCR3	CP* ≤ 40.5	CP* ≤ 30.3
Negative Control valid	PCR1	No CP* provided	CP* ≤ 37.1
	PCR2		CP* ≤ 37.1
	PCR3		CP* ≤ 37.1

*Crossing Point

12.5. Interpretation of Results for a Single PCR (LightCycler 480 Instrument II)

If the result of the internal control ACTB indicates sufficient input of DNA into the single PCR (ACTB cycle threshold specified in Table 15), the result of the Septin 9 PCR defines the result for this single PCR (see section 13). An ACTB value above the crossing point specified in Table 15 determines the single PCR result to “Invalid PCR”.

Table 15: Interpretation of Results for Single PCR (LightCycler 480 Instrument II).

Single PCR Result	Septin 9 Result	ACTB Result
Septin 9 Positive	CP* < 50	CP* ≤ 33.7
Septin 9 Negative	no CP* provided	CP* ≤ 33.7
Invalid	any result	CP* > 33.7

* Crossing Point

13. Interpretation of Results for a Patient Sample

The test result for a patient sample is interpreted according to Table 16. The test result for a patient sample is “POSITIVE”, if at least two of three PCR replicates are Septin 9 Positive. The test result for a patient sample is “NEGATIVE”, if at least two of three PCR replicates are Septin 9 Negative. The test result is “INVALID” in all other cases.

Table 16: Interpretation of Epi proColon 2.0 CE Test Results

Test Result	Positive Control Negative Control	Single PCR Results
POSITIVE	Valid	At least two Positive Septin 9 PCR
NEGATIVE	Valid	At least two Negative Septin 9 PCR
INVALID	Valid	All other cases
INVALID	Invalid	n/a

14. Quality Control

14.1. External Controls

Epi proColon 2.0 CE contains Epi proColon Positive and Negative Controls (M5-02-003). These controls must be included with each run to monitor the successful execution of the work flow to ensure validity of test results. The Epi proColon Positive and Negative Control values must be within the validity limits (see Table 8, or Table 11, or Table 14). If a control is out of its specified range, the associated test results are invalid, must not be reported and testing must be repeated.

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

14.2. Internal Controls

The internal control allows the detection of bisulfite converted ACTB (β-actin) DNA. This co-amplified internal control monitors the sample quality, sample preparation and adequate DNA concentration of the sample.

The result of the Septin 9 PCR is linked to the Ct or CP value of the ACTB PCR (see Table 9, or Table 12 or Table 15). Ct or CP values of the ACTB PCR outside of the specified range invalidate the single PCR result; as such high values are associated with very low bisDNA content or PCR inhibition.

15. Limitations of the Procedure

- For *in vitro* diagnostic use only.
- This product has been validated for the combination of Epi proColon Plasma Quick Kit (M5-02-001) or Epi BiSKit (M7-01-001), Epi proColon Sensitive PCR Kit (M5-02-002), and Epi proColon Control Kit (M5-02-003) only. The parts must not be replaced by other DNA extraction methods or PCR kits.
- This product has been validated for use with plasma derived from blood collected with BD Vacutainer® K₂EDTA, S-Monovette® 9 ml Potassium-EDTA and S-Monovette® 8.5 ml CPDA tubes. Use of other sample types and other blood collection tubes have not been validated.
- Storage of blood in S-Monovette® 8.5 ml CPDA tubes at temperatures exceeding 25 °C may lead to false positive test results.
- Use of this product is limited to personnel experienced and trained in PCR assays.
- Detection of colorectal cancer is dependent on the amount of circulating tumor DNA in the specimen and may be affected by sample collection methods and sample storage, patient factors (i.e. age, other diseases) and tumor stage.
- Results are not confirmative evidence of the presence or absence of colorectal cancer. Any positive Epi proColon 2.0 CE test result should be confirmed by colonoscopy or sigmoidoscopy.
- Positive test results have been observed in clinically diagnosed patients with the following diseases: chronic gastritis, esophagitis, non rheumatoid arthritis, lung cancer, breast cancer and prostate cancer.
- Positive test results have been observed in pregnant women.
- The Epi proColon 2.0 CE test result must be evaluated in conjunction with other clinical parameters.

16. Performance Characteristics

16.1. Analytical Sensitivity

The analytical sensitivity was evaluated by 3 operators each performing 4 independent runs with a set of 2 x 7 technical Limit of Detection (LoD) samples with HeLa DNA (Septin 9 positive) concentrations of 0, 6, 12, 18, 25, 35 and 50 pg/ml resulting in 168 Septin 9 determinations. All determinations on the Applied Biosystems 7500 Fast Dx with SDS v1.4 were valid. 133 of 144 LoD samples with spiked HeLa DNA were positive and 24 of 24 samples without spiked HeLa DNA were negative. The estimated LoD₉₅ was determined by a logistic regression model to be 14 pg/ml (CI 95%: 9 pg/ml - 19 pg/ml) for Epi proColon 2.0 CE.

A similar investigational setup was used to determine the analytical sensitivity using the LightCycler 480 Instrument I. The estimated LoD₉₅ was determined to be equivalent.

16.2. Reproducibility

The reproducibility of the procedure was tested by processing plasma aliquots from nine pools generated from human EDTA plasma. Six of the plasma pools comprised plasma from patients diagnosed with colorectal carcinoma. Three pools comprised plasma from patients with no apparent disease as controls. All pools were processed in 6 replicates by three operators using different kit lots of the Epi proColon Plasma Quick Kit (M5-02-001), as well as the Epi proColon Sensitive PCR Kit (M5-02-002). In 54 of the 54 Septin 9 determinations the expected result (cancer pool Septin 9 positive; non-cancer pool Septin 9 negative) was consistently generated.

16.3. Clinical Sensitivity and Specificity

The clinical performance of Epi proColon 2.0 CE was determined with 149 clinical samples with no evidence of disease selected from a prospectively collected average risk screening population and with 197 clinical specimens in a case-control design comprised of samples from histologically confirmed colorectal carcinoma patients of all stages and samples from colonoscopy-verified negative individuals with no evidence of disease.

Bisulfite converted DNA was prepared from plasma which was derived from blood processed within four hours of blood draw with Vacutainer® K₂EDTA Tubes (Becton Dickinson). Valid Septin 9 measurements were obtained for 346 out of 346 specimens (100 %).

In the prospectively collected cohort 1 out of 149 samples provided a positive test result. The estimated false positive rate in this group of patients is 1 % (CI 95 %: 0 % - 4 %). These numbers translate into a clinical specificity of 99 % (CI 95 %: 96 % - 100 %).

Within the 99 subjects from the case-control cohort with no evidence of disease (controls), 96 specimens were determined to be Septin 9 negative resulting in an estimated clinical specificity of 97 % (CI 95 %: 91 % - 99 %), see Table 17.

Of the 98 subjects diagnosed with colorectal carcinoma 79 specimens were determined to be Septin 9 positive resulting in an estimated clinical sensitivity of 81 % (CI 95 %: 72 % - 87 %). In early, localized colorectal cancer the Epi proColon 2.0 CE test was positive in 43 (18 stage I, 25 stage II) of the 56 patients (77 %).

The table below summarizes the data obtained.

Table 17. Summary of clinical performance data of Epi proColon 2.0 CE

	Controls (screening cohort)	Controls (case-control)	All colorectal carcinoma	Localized colorectal carcinoma
Valid results	149	99	98	56
Septin 9 positive	1	3	79	43
Septin 9 negative	148	96	19	13
Positivity	1%	3%	81%	77%

16.4. Concordance of the Two Real-Time PCR instruments

The concordance of Epi proColon 2.0 CE on the Applied Biosystems 7500 Fast Dx System and the LightCycler 480 Instrument I has been determined in a side-by-side measurement with 48 plasma samples from patients with histologically confirmed colorectal carcinoma of all stages and 52 samples from subjects without apparent colon disease as verified by colonoscopy. In 93 % (93/100) of cases, both measurement systems generated concordant Septin 9 results.

16.5. Interference

No interference was observed within experimental controls and nonreactive or reactive samples tested with elevated levels of unmethylated genomic DNA (100 ng/ml), Bilirubin (0.20 mg/ml), Hemoglobin (1 mg/ml), Triglycerides (12 mg/ml), Protein (Albumin from human serum) (40 mg/ml), Red Blood Cells (0.4% v/v), K₂EDTA (20 mg/ml), Cholesterol (5 mg/ml), Uric acid (0.235 mg/ml), and Glucose (10 mg/ml).

17. Meaning of Symbols



Consult Instructions for Use



Catalogue Number



In Vitro Diagnostic Medical Device



Batch code



Use-by date



Negative Control



Positive Control



Manufacturer



Temperature limit



Number of Tests



Do not Re-use



UDI Barcode

Restricted to USA only:

R x O n l y Caution: Federal Law restricts this device to sale by or on the order of a licensed practitioner.

18. References

- 1 deVos, T et al. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clinical Chemistry* 55:7, 1337-46 (2009)
- 2 Lofton-Day, C. et al. DNA methylation biomarkers for blood-based colorectal cancer screening. *Clinical Chemistry* 54:2, 414-423 (2008)
- 3 Model, F. et al. Identification and validation of colorectal neoplasia-specific methylation markers for accurate classification of disease. *Mol Cancer Res* 5, 153-163 (2007)
- 4 Eads, C.A. et al. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Research*, Volume 28, Issue 8, e32 (2000)

19. Contact Information

Epi proColon 2.0 CE is manufactured by:

Epigenomics AG
Geneststr. 5
10829 Berlin, Germany

For further information and support send an e-mail to or call:

support@products.epigenomics.com

Phone: +49 30 24345 222

Fax: +49 30 24345 555

Notice to Purchaser

Epi proColon (Epi proColon®) and Epi BiSKit™ are registered trademarks of Epigenomics AG. All other trademarks, brands, and names contained herein are the property of their respective owners. The LightCycler® 480 System is a registered trademark of F. Hoffmann-La Roche Ltd.

The Applied Biosystems®, MicroAmp® are registered trademarks of Life Technologies Corporation; DynaMag™ is a trademark of Life Technologies Corporation.

FAM™, JOE™ and TAMRA™ are registered trademarks of Applied Biosystems Corporation.

BD Vacutainer® is a registered trademark of Becton Dickinson Inc./Corporation.

S-Monovette® is a registered trademark of Sarstedt AG & Co.

ThermoMixer®, Eppendorf Research®, Reference®, Multipette®, ep Dualfilter T.I.P.S.®, Combitips advanced® are registered trademarks of Eppendorf AG; Safe Lock™ is a trademark of Eppendorf AG.

HandyStep® is a registered trademark of Brand GmbH + Co KG.

The purchase of this product grants the purchaser rights under certain Roche patents to use it solely for providing human in vitro diagnostic services. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.