



Instructions for Use

HCCBloodTest PCR Kit (M8-01-002)

HCCBloodTest Control Kit (M8-01-003)

Important!

Use only the most current version of the Instructions For Use.

Make sure prior to starting with the test procedure that you have the most current version accessible at www.epigenomics.com.

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



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M8-01-002, M8-01-003



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1. Product and Intended Use

The HCCBloodTest is a real-time polymerase chain reaction (PCR) assay for the assessment of methylated Septin 9 in bisulfite-converted DNA (bisDNA) prepared from DNA extracted from plasma. The HCCBloodTest provides an aid in the detection of hepatocellular carcinoma (HCC) in patients with cirrhosis.

The HCCBloodTest consists of the HCCBloodTest PCR Kit (M8-01-002) and the HCCBloodTest Control Kit (M8-01-003) and shall be used with bisDNA prepared with Epigenomics' Epi BiSKit (M7-01-001).

2. Summary and Explanation

The HCCBloodTest is an in-vitro PCR assay for the qualitative detection of Septin 9 gene methylation (SEPT9) in DNA isolated from 3.5 mL of patient plasma. The SEPT9 gene is a key regulator of cell division and tumor suppressor the hypermethylation of the assessed marker site is associated with liver carcinogenesis. As patients with cirrhosis are at high risk of hepatocellular carcinoma (HCC) HCCBloodTest test offers patients with cirrhosis an option to improve risk prediction and the personalizes management of HCC.

3. Principles of the Procedure

The HCCBloodTest requires bisulfite converted DNA (bisDNA) that was extracted and purified from plasma using Epigenomics' Epi BiSKit (M7-01-001). The HCCBloodTest PCR Kit is a duplex PCR assay that detects methylated Septin 9 as target and ACTB (β -actin) DNA as internal control, to assess adequacy of input DNA. The HCCBloodTest Control Kit provides positive and negative controls for each run starting with the DNA extraction from plasma.

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 bisulfite converted methylated and unmethylated template. HCCBloodTest 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 blocking a primer at its 3' end is added so that preferentially only methylated sequences are amplified. A fluorescent detection probe specific for product from methylated Septin 9 template is used in the reaction in order to exclusively identify methylated sequences amplified during the PCR reaction.

4. Materials Provided

Table 1: Contents of the HCCBloodTest PCR Kit (M8-01-002).

| Reagent | Containers | Volume | Storage Temperature |
|-------------------------|------------|-------------|---------------------|
| HCCBloodTest PCR Mix | 2 tubes | 810 µL each | -25 °C to -15 °C |
| HCCBloodTest Polymerase | 1 tube | 100 µL | -25 °C to -15 °C |

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

Table 2: Contents of the HCCBloodTest Control Kit (M8-01-003)

| Reagent | Containers | Volume | Storage Temperature |
|-------------------------------|------------|--------------|---------------------|
| HCCBloodTest Positive Control | 6 tubes | 3.65 mL each | -25 °C to -15 °C |
| HCCBloodTest Negative Control | 6 tubes | 3.65 mL each | -25 °C to -15 °C |

The concentration of the DNA in the control material is adjusted to an internally established standard based on UV-quantification.

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 (www.epigenomics.com).

HCCBloodTest PCR Kit and HCCBloodTest Control Kit contain no dangerous or harmful chemicals. When used in combination with Epigenomics' Epi BiSKit (M7-01-001) the safety information for the DNA extraction and the bisulfite conversion is applicable, found under Section 9.0 - Test Procedure.

5. Storage and Stability

Reagents provided with the HCCBloodTest PCR Kit and the HCCBloodTest Control Kit are stable until the expiration date when stored and handled according to Instructions for Use. Do not use material past expiration date. Do not mix components from different kit lots.

HCCBloodTest PCR Kit (M8-01-002)

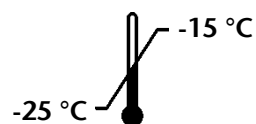


Store HCCBloodTest PCR Mix and HCCBloodTest Polymerase at -25 to -15 °C.

Each HCCBloodTest PCR Mix tube may be thawed and refrozen **one time only**.

After first use store all reagents at -25 to -15 °C for up to six weeks.

HCCBloodTest Control Kit (M8-01-003)



Store HCCBloodTest Control Kit at -25 to -15 °C.

6. Materials required, but not provided

The tables below provide information on general laboratory equipment that is required to perform HCCBloodTest along with suggested vendors. All laboratory equipment should be installed, calibrated, operated, and maintained according to the manufacturer’s recommendations.

6.1. Essential Special Equipment and Consumables

The following special equipment and consumables are essential to perform HCCBloodTest and cannot be replaced by other equipment.

| Special Equipment/Consumables | Vendor |
|--|--------------------------|
| Epi BiSKit (M7-01-001) | Epigenomics AG |
| Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument or Applied Biosystems® 7500 Fast Real-Time PCR Instrument Both instruments require Sequence Detection Software 7500 Fast System SDS v1.4 21 CFR Part 11 Module for Windows XP or v1.4.1 21 CFR Part 11 Module for Windows 7 with MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL MicroAmp® 96- & 384-Well Optical Adhesive Film | Thermo Fisher Scientific |
| 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 with LightCycler® 480 Multiwell Plate 96 (white plate) with sealing foils LightCycler® 480 Sealing Foil | Roche |
| Magnetic Separator: DynaMag®-15 magnet | Thermo Fisher Scientific |
| Magnetic Separator: DynaMag®-2 magnet | Thermo Fisher Scientific |

Installation Requirements

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 Applied Biosystems® 7500 Fast PCR Instrument according to manufacturer’s instructions, including calibration of the pure dyes FAM, JOE, TAMRA to manufacturer’s instructions.

6.2. General Laboratory Equipment

- Four-way microtube and test tube racks for 15 mL tubes and 2 mL tubes from VWR International, or equivalent
- Rotators, SB2 and SB3 from VWR International, or equivalent
- Vortex mixer from VWR International, or equivalent
- Eppendorf ThermoMixer® C with Eppendorf SmartBlock™ 2.0 mL (Temperature accuracy of the thermoshaker: ±2 °C at 23 - 80 °C), or equivalent thermoshaker for 2 mL tubes
- Eppendorf Reference® 2, adjustable volume pipette, or equivalent pipettes with adjustable volumes of the following ranges 10 - 100 µL and 100 - 1000 µL

- Eppendorf Multipipette® M4, or HandyStep® electronic, Brand, or equivalent repeat pipettor capable of repetitively dispensing volumes in an adjustable range
- Eppendorf Centrifuge 5418, not cooled, with fixed-angle rotor F-45-30-11, or equivalent bench top centrifuge with rotor for 1.5/2.0 mL tubes
- Eppendorf Research® plus 8-Channel, variable, 10 - 100 µL, or equivalent multichannel pipette
- Eppendorf Centrifuge 5804 R, not cooled, with rotor A-2-DWP, or equivalent plate centrifuge capable for PCR plates
- 100 mL graduated cylinder from e.g. Carl Roth or 50 mL serological pipette from e.g. Fisher Scientific

6.3. General Laboratory Consumables and Reagents

- Ethanol absolute (for molecular biology, $\geq 99.5\%$) from Merck KGaA, or equivalent
- Tube 15 mL, 120 x 17 mm, conical base, PP, with print, assembled red cap, sterile and pyrogen-free, from Sarstedt, or equivalent 15 mL tubes
- SafeSeal reaction tube, 2 mL, PP, with attached lid with retaining cams, with moulded graduation and frosted writing space, certified PCR Performance Tested (free of DNase/RNase, DNA and PCR inhibitors) from Sarstedt or Eppendorf Safe-Lock Tubes, 2.0 mL, Eppendorf Quality™, or equivalent tubes
- ep Dualfilter T.I.P.S.®, from Eppendorf (2 - 100 µL and 50 - 1000 µL), or equivalent pipette tips with aerosol barrier
- Combitips advanced®, from Eppendorf (0.5 mL, 1 mL, 10 mL, and 25 mL), or equivalent tips for repetitive pipettes for volumes of 0.5 mL, 1 mL, 10 mL, 25 mL
- Disposable transfer pipettes, Standard Line (low affinity surface, non-sterile bulk packaged, length 14 cm, capacity about 3.0 mL from VWR International, or equivalent disposable transfer pipettes
- Disposable pasteur pipettes, graduated (transparent LDPE, non-sterile bulk packaged, length 21.7 cm, stem diameter 5 mm, capacity about 5 mL) from Carl Roth or equivalent disposable transfer pipettes
- MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL from Thermo Fisher Scientific, or equivalent 96-Well Reaction Plates
- Adhesive PCR film seals, PP, non-sterile, for temperatures from $-40\text{ }^{\circ}\text{C}$ to $+120\text{ }^{\circ}\text{C}$, DNase, RNase and nuclease-free from VWR or Eppendorf Storage Film (self-adhesive) or equivalent adhesive film or foil for DNA storage plate
- MicroAmp™ Adhesive Film Applicator from Thermo Fisher Scientific or equivalent applicator to form a tight seal between a microplate and an adhesive film
- Re-closable plastic bags, 10 x 15 cm for disposing of used PCR plates
- Only for storage of plasma required: Cryogenic vials, PP, free-standing, 5.0 mL With external thread, lip and silicone washer seal, certified RNase-, DNase-, pyrogen- and DNA-free, from VWR or equivalent
- Blood collection tubes:
 - BD Vacutainer® K2EDTA 10 mL Blood Collection Tubes, Becton Dickinson, Cat. No. 367525 or
 - S-Monovette® 9 mL K3E, Sarstedt, Cat. No. 02.1066.001 or
 - S-Monovette® 8.5 mL CPDA, Sarstedt, Cat. No. 01.1610.001

7. Precautions and Safety

7.1. Laboratory Precautions

Compliance with good laboratory practices is recommended 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 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

- Use 10 mL 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°C to 25°C for 48 hours. Do not freeze blood samples.

8.2. Plasma Sample Preparation and Plasma Storage

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.

- 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 -25 to -15 °C for up to 4 weeks.
- When using Vacutainer® K2EDTA 10 mL tubes, plasma samples may be stored at 2 to 8 °C for up to 18 hours.

9. Test Procedure

HCCBloodTest contains sufficient reagents to run up to 32 samples including controls. One HCCBloodTest Positive Control and one HCCBloodTest Negative Control must be included in each independent test run.

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.

9.1. DNA Extraction and Bisulfite Conversion using Epigenomics' Epi BiSKit (M7-01-001)

Table 3: Contents of the Epi BiSKit (M7-01-001):

| Reagent | Containers | Volume | Storage Temperature |
|----------------------|------------|-------------|---------------------|
| Lysis Binding Buffer | 1 bottle | 125 mL | 15 °C to 30 °C |
| Wash A Concentrate | 1 bottle | 60 mL | 15 °C to 30 °C |
| Magnetic Beads | 1 bottle | 4 mL | 15 °C to 30 °C |
| Wash B Concentrate | 1 bottle | 7 mL | 15 °C to 30 °C |
| Elution Buffer | 1 tube | 6 mL | 15 °C to 30 °C |
| Bisulfite Solution | 4 tubes | 1.9 mL each | 15 °C to 30 °C |
| Protection Buffer | 1 tube | 1 mL | 15 °C to 30 °C |

Safety information for the Epi BiSKit (M7-01-001):

Lysis Binding Buffer and 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

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

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

Wash B Concentrate, Elution Buffer and Magnetic Beads of the Epi BiSKit (M7-01-001) are not harmful.

Storage information for the Epi BiSKit (M7-01-001):

- Store all reagents at 15 to 30 °C.
- Bisulfite Solution is sensitive to oxygen contact. Use only unopened tubes of Bisulfite Solution. Discard used tubes! There are four (4) single-use tubes of Bisulfite Solution that will enable a maximum of four (4) independent test runs (e.g., 4 runs of 8 samples each).
- Store reconstituted Wash A buffer and reconstituted 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.

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

- Lysis Binding Buffer
- Magnetic Bead Suspension
- Ethanol in DNA Binding Step 9.1.4
- Wash A buffer
- Wash B buffer
- Elution Buffer
- Bisulfite Solution
- Protection Buffer and the PCR Master Mix.

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

9.1.1. Preparation of Working Solutions

Preparation of Wash A Buffer

- Add 60.0 mL of Absolute Ethanol (for molecular biology, $\geq 99.5\%$) to the 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 Wash A buffer at 15 to 30 °C for up to 6 weeks.

Preparation of Wash B Buffer

- Add 40.0 mL of Absolute Ethanol (for molecular biology, $\geq 99.5\%$) to the 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 Wash B Buffer at 15 to 30 °C for up to 6 weeks.

9.1.2. Thawing of Plasma and HCCBloodTest Positive and Negative Control

- Thaw one HCCBloodTest Positive Control and one HCCBloodTest 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.

9.1.3. Lysis

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

- Add the following to a labeled 15 mL centrifuge tube:
 - 3.5 mL plasma sample, or Positive Control, or Negative Control
 - 3.5 mL 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.

9.1.4. DNA Binding

Note: A homogeneous suspension of the 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 μ L Magnetic Beads (freshly suspended)
 - 2.5 mL of Absolute Ethanol (for molecular biology, $\geq 99.5\%$)
- 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.

9.1.5. 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 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.

9.1.6. Elution

- Transfer the microtube into a non-magnetic rack.
- Vortex Elution Buffer for 5 - 10 sec.
- Add 100 µL 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 ± 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.

9.1.7. Storage of Extracted DNA

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.

9.1.8. Bisulfite Conversion

Note: Bisulfite Solution is sensitive to oxygen contact. Use only unopened tubes of 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 μ L DNA solution):
 - 150 μ L Bisulfite Solution
 - 25 μ L Protection Buffer

Note: The color of the 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.

9.1.9. Binding Step

Note: A homogeneous suspension of beads in the 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 μ L Wash A Buffer
 - 20 μ L 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.

9.1.10. First Wash

- Remove the sample rack from the magnet for washing and vortexing: Add 800 μ L 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.

9.1.11. Second Wash

- Remove the sample rack from the magnet for washing and vortexing: Add 800 μ L 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.

9.1.12. Third Wash

- Remove the sample rack from the DynaMag®-2 magnet for washing and vortexing: Add 400 μ L 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 μ L reference pipette, remove as much remaining liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads.

9.1.13. 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.

9.1.14. Elution

- Transfer microtube into a non-magnetic rack: Add 60 μ L 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 μ L reference pipette, transfer the complete eluate (~ 60 μ L 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 | | | | | | | | | | |

[#]HCCBloodTest Positive Control, [§]HCCBloodTest Negative Control

9.1.15. 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.

9.2. PCR Setup

Each bisDNA (patient sample, HCCBloodTest Positive Control, or HCCBloodTest Negative Control) must be tested in triplicate. Prior to use spin HCCBloodTest Polymerase for 10 - 20 sec at 1,000 ± 150 rcf using a Bench-Top centrifuge to remove drops from the lid.

9.2.1. Preparation of PCR Master Mix

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

Note: For a single PCR 16 µL HCCBloodTest PCR Mix and 0.95 µL HCCBloodTest Polymerase are required. Indicated volumes already contain pipetting margin. There is no need to prepare an additional reaction for Master Mix preparation.

- Thaw 1 or 2 HCCBloodTest PCR Mix tubes depending on the desired number of patient and control sample determinations (see Table 5).
- Vortex the HCCBloodTest PCR Mix tube(s) for 5 - 10 sec, briefly spin down the tube(s).
- Transfer the corresponding volumes of HCCBloodTest PCR Mix and HCCBloodTest 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.

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) |
|------------|--|---|---|---|
| PCR Mix | 384 µL | 768 µL | 1152 µL | 1536 µL |
| 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 | | | | | | |

[#]HCCBloodTest Positive Control, [§]HCCBloodTest Negative Control

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 Sequence Detection Software 7500 Fast System SDS v1.4 21 CFR Part 11 Module (Windows XP) and SDS v1.4.1 21 CFR Part 11 Module (Windows 7).

10.2. PCR Plate Preparation

- 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 9.2.14 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: Immediately start the PCR plate.

10.3. Plate Loading

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 HCCBloodTest template file)
 - Run Mode: Standard 7500.
- Click “Next”.
- Click “New Detector”.
- Create a new detector using following properties:
 - Name: Septin9
 - Description: HCCBloodTest
 - Reporter dye: FAM
 - Quencher dye: (none)
 - Color: Red.
- Click “Create Another” and define following properties:
 - Name: ACTB
 - Description: HCCBloodTest
 - 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) | | | | |

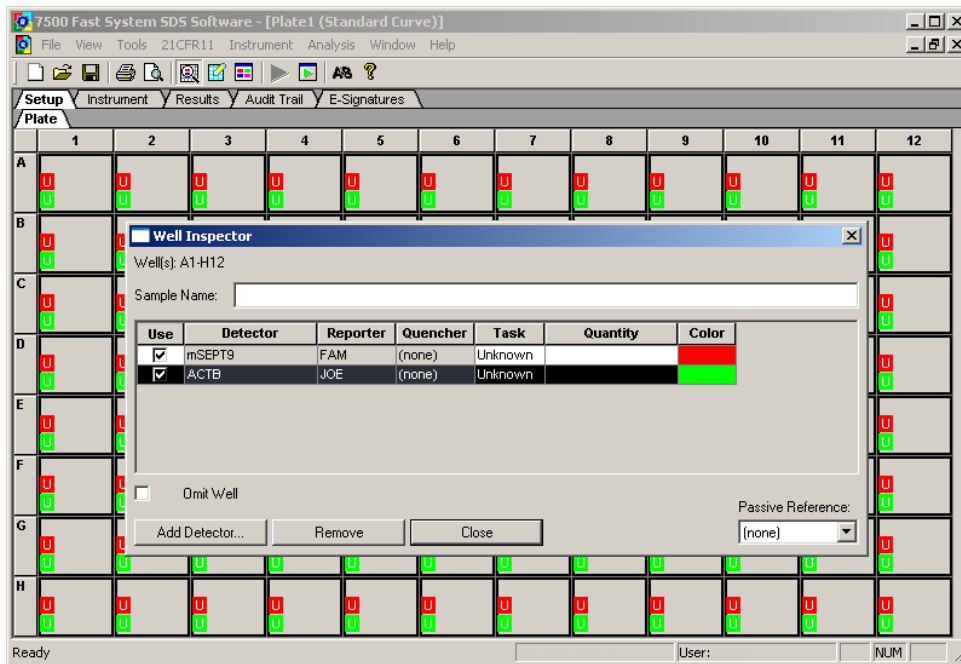


Figure 1: Screenshot after confirming settings in the 'Well Inspector' window.

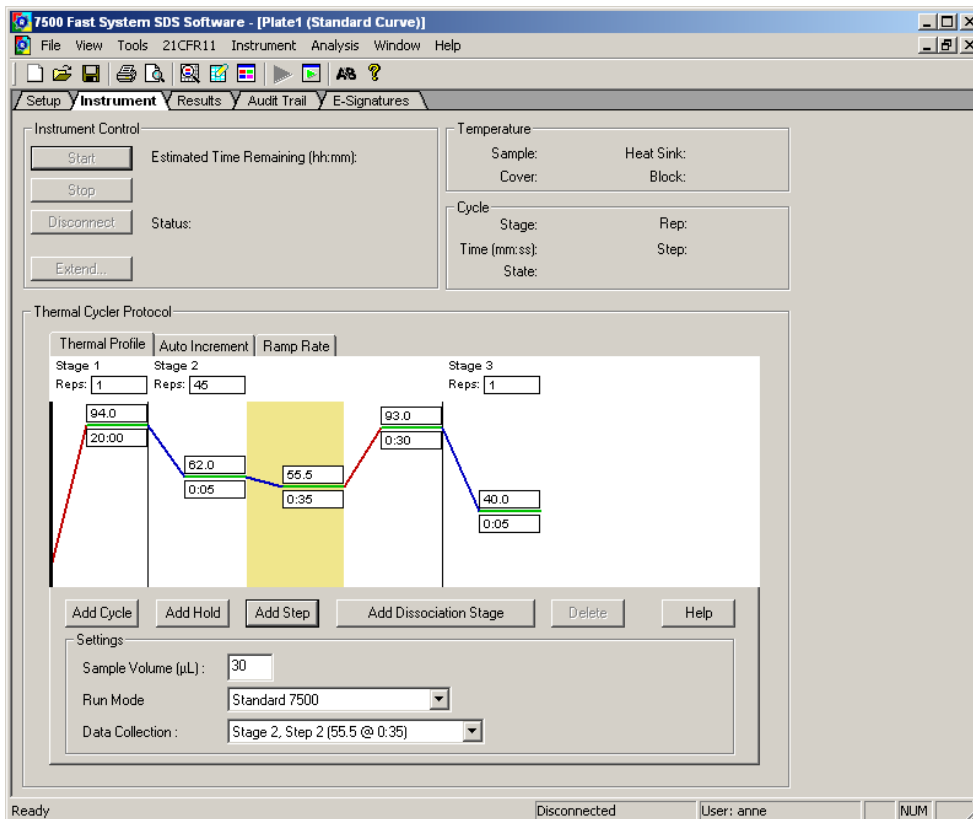


Figure 2: Screenshot after setting cycler program and detection mode.

10.4. Analysis Settings

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 1.

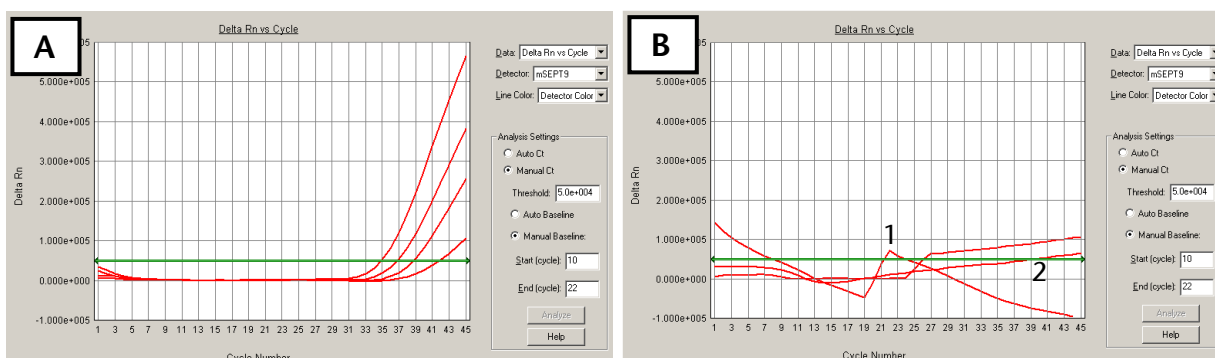


Figure 1: Screenshots of Septin 9 amplification curves using the Applied Biosystems® 7500 Fast.

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 HCCBloodTest Controls

Any run of one or more patient sample(s) processed together with HCCBloodTest Positive Control and HCCBloodTest 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 HCCBloodTest Positive Control or HCCBloodTest 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 HCCBloodTest 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

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

- 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 9.2.14 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

Note: It is recommended to save a template file (*.ixo) 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

Note: Users of the LightCycler® 480 Instrument I need to select *Color Compensation* “ON” and load the respective “HCCBloodTest 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 “HCCBloodTest 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.

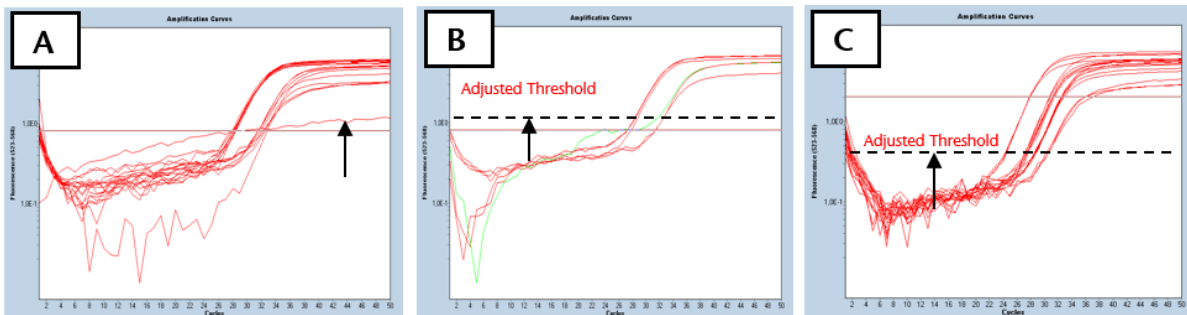


Figure 2: Screenshots of Septin 9 amplification curves using LightCycler® 480. A: Amplification curve without a significant exponential increase should be evaluated as negative. B: Threshold should be adjusted that it is slightly above the background. C: Threshold should be adjusted that it crosses the amplification curves at the beginning of the exponential increase.

11.4. Run Validity by the HCCBloodTest Controls

Any run (patient samples processed together with HCCBloodTest Positive Control and HCCBloodTest negative Control) is considered valid if criteria set forth in Table 11 are met for **ALL THREE (3)** PCR replicates per control. If either the HCCBloodTest Positive Control or HCCBloodTest 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 HCCBloodTest 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

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

- 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 9.2.14 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

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

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.

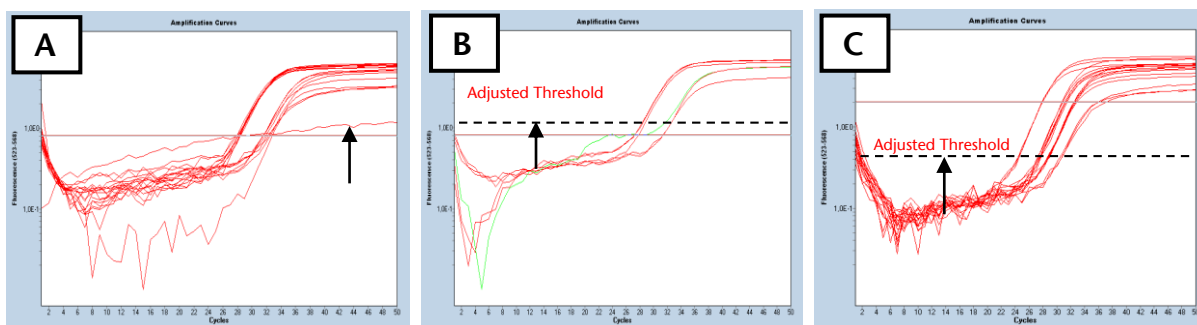


Figure 3: Screenshots of Septin 9 amplification curves using LightCycler® 480. A: Amplification curve without a significant exponential increase should be evaluated as negative. B: Threshold should be adjusted that it is slightly above the background. C: Threshold should be adjusted that it crosses the amplification curves at the beginning of the exponential increase.

12.4. Run Validity of the HCCBloodTest Result

Any run (patient samples processed together with HCCBloodTest Positive Control and HCCBloodTest 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 HCCBloodTest Positive Control or HCCBloodTest 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 HCCBloodTest 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

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 HCCBloodTest 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

HCCBloodTest contains HCCBloodTest Positive and Negative Controls (HCCBloodTest Control Kit M8-01-003). These controls must be included with each run to monitor the successful execution of the work flow to ensure validity of test results. The HCCBloodTest Positive and Negative Control values must be within the validity limits. 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. Ct values of the ACTB PCR outside of the specified range invalidate the patient sample; 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 must be used in the combination of Epi BiSKit (M7-01-001). The parts must not be replaced by other DNA extraction methods.
- 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 HCC 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 HCC. Any test result should be interpreted in the context of all clinical and diagnostic information available in the comprehensive evaluation of the patient.
- Positive test results have been observed in clinically diagnosed patients with the following diseases: chronic gastritis, esophagitis, non-rheumatoid arthritis, colon cancer, lung cancer, breast cancer and prostate cancer.
- Positive test results have been observed in pregnant women.

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 HCCBloodTest.

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 cancer plasma that was expected to test positive for Septin 9. Three pools comprised non-cancer plasma that was expected to test negative for Septin 9. All pools were processed in 6 replicates by three operators using different kit lots of the reagents for DNA Extraction and Bisulfite Conversion as well as the reagents for conduct of the PCR. 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 the HCCBloodTest was determined from two studies that included patients with cirrhosis with and without HCC from France (initial study) and from Germany (replication study), which were designed to evaluate the diagnostic accuracy of the test to diagnose HCC among patients with cirrhosis.¹

For the initial study patients were recruited with cirrhosis with or without HCC with a high degree of ascertainment. The HCC study population included: 1) patients with cirrhosis enrolled in an HCC screening program and for whom a diagnosis of HCC was made and ascertained by an abdominal

contrast-enhanced computed tomography (CT) scan, and 2) patients with cirrhosis who were directly referred for HCC. HCC-free patients with cirrhosis were recruited if the screening abdominal ultrasound showed no evidence of HCC at the time of blood sampling for the mSEPT9 test and on the next visit six months later. The total enrollment of 186 patients included 51 patients with cirrhosis and HCC and 135 patients with cirrhosis without HCC.

The replication study was a matched case-control study. HCC-free patients with cirrhosis were recruited at the same facility and were matched by age and gender. All patients with a diagnosis of HCC were discussed in multidisciplinary consultation meeting (gastroenterologists, radiologists, and surgeons). HCC-free patients with cirrhosis were recruited if the screening abdominal ultrasound showed no evidence of HCC at the time of blood sampling for the mSEPT9 test and on the next visit six months later. The total of 103 patients included 47 patients with cirrhosis and HCC and 56 patients with cirrhosis without HCC.

Of the 51 HCC patients in the initial study 48 specimens were determined to be Septin 9 positive resulting in an estimated clinical sensitivity of 94 % (CI 95 %: 84 % - 99 %). Of the 135 subjects without HCC, 114 specimens were reported to be Septin 9 negative resulting in an estimated clinical specificity of 84 % (CI 95 %: 77 % - 90 %).

In the replication study, 40 out of 47 HCC were Septin 9 positive for sensitivity estimate of 85 % (CI 95 %: 72 % - 94 %). Of the 56 subjects without HCC, 51 were reported to be Septin 9 negative resulting in an estimated clinical specificity of 91 % (CI 95 %: 80 % - 97 %).

A meta analysis combining the results of both studies resulted in a final estimates for clinical sensitivity of 91 % (CI 95 %: 82 % - 99 %) and a clinical specificity of 87 % (CI 95 %: 81 % - 94 %).

The table below summarizes the results on clinical sensitivity and specificity together with reported negative (NPV) and positive predicted values (PPV).

Table 17. Summary of clinical performance data of HCCBloodTest

| | Sensitivity | NPV | Specificity | PPV |
|--------------------------------|-------------|--------|-------------|--------|
| France (initial study) | 94 % | 97.4 % | 84 % | 69.6 % |
| Germany (replication study) | 85 % | 87.9 % | 91 % | 88.9 % |
| Meta (both) | 91 % | 93.3 % | 87 % | 79.2 % |

16.4. Concordance of the Two Real-Time PCR instruments

The concordance on the Applied Biosystems® 7500 Fast Dx System and the LightCycler® 480 Instrument I has been determined in a side-by-side measurement with a set of 100 plasma samples from subjects with and without malignancy. 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



Reorder Number



In Vitro Diagnostic Medical Device



Lot Number



Use-by



Control, negative



Control, positive



Manufacturer



Temperature limit



Sufficient for <n> tests



Do not Re-use



CE mark



UDI Barcode

18. References

- 1 Oussalah A., Rischer S., Bensenane M., et.al., 2018. Plasma mSEPT9: A Novel Circulating Cell-free DNA-Based Epigenetic Biomarker to diagnose Hepatocellular Carcinoma. EBioMedicine 30, 138-147, 2018

19. Contact Information

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