



## Epi proLung<sup>®</sup>

Epi proLung PCR Kit (M6-02-002)

Epi proLung Control Kit (M6-02-003)

### Important!

Use only the current version of the instructions for use.

Make sure prior to starting with the test procedure that you have the most current version available under [www.epigenomics.com](http://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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M6-02-002, M6-02-003



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## 1.0 Product and Intended Use

Epi proLung is a real-time PCR assay for the assessment of methylated DNA biomarkers (SHOX2, PTGER4) in bisulfite-converted DNA (bisDNA) prepared from DNA extracted from plasma. Epi proLung provides an aid in the diagnosis of lung cancer in patients at increased risk for the disease. Lung cancer risk factors include patient life history, and/or presentation with symptoms, and/or radiological findings in the lung.

Epi proLung consists of Epi proLung PCR Kit (M6-02-002) and Epi proLung Control Kit (M6-02-003) and has been validated with bisDNA prepared with Epigenomics Epi BiSKit (M7-01-001).

## 2.0 Summary and Explanation

Epi proLung is a non-automated in-vitro polymerase chain reaction (PCR) assay for the qualitative detection of SHOX2 and PTGER4 gene methylation in DNA isolated from 3,5 mL of patient plasma. SHOX2 and PTGER4 gene methylation can be detected by specific amplification of free-circulating DNA present in the blood stream. Measurement of SHOX2 and PTGER4 methylation in plasma DNA has been demonstrated to allow detection of lung cancer and differentiation of non-malignant diseases<sup>1</sup>.

## 3.0 Principles of the Procedure

Epi proLung requires bisulfite converted DNA (bisDNA) that was extracted and purified from plasma using Epigenomics Epi BiSKit (M7-01-001). The Epi proLung PCR Kit is a triplex PCR assay that detects methylated SHOX2 and PTGER4 DNA as targets and ACTB ( $\beta$ -actin) DNA as internal control, to assess adequacy of input DNA. The Epi proLung 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 using the Epi BiSKit (M7-01-001) 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 complete procedure of DNA extraction, bisulfite conversion and purification using the Epi BiSKit (M7-01-001) is described in sections 9.1 of this document and no additional information is required.

In the subsequent PCR reaction the blockers and probes discriminate between methylated and unmethylated DNA sequences. Epi proLung detects bisDNA sequences containing methylated CpG sites within the SHOX2 and PTGER4 gene and total bisDNA of a region of the ACTB gene. The SHOX2 and PTGER4 portion of the triplex assay consists of primers that are placed in regions lacking CpG dinucleotides. Two individual blockers specific for bisulfite converted unmethylated SHOX2 and PTGER4 sequences within the region are added so that methylated sequences are preferentially amplified. Two methylated SHOX2- and PTGER4-specific fluorescent detection probes are used in the reaction in order to exclusively identify methylated target sequences amplified during the PCR reaction<sup>2</sup>.

#### 4.0 Materials Provided

Epi proLung is comprised of the Epi proLung PCR Kit and the Epi proLung Control Kit.

Table 1: Contents of the Epi proLung PCR Kit (M6-02-002)

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

Table 2: Contents of the Epi proLung Control Kit (M6-02-003)

Reagent	Containers	Volume	Storage Temperature
Epi proLung Positive Control	6 tubes	3,65 mL each	-25 °C to -15 °C
Epi proLung 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 a internally established standard based on UV-quantification.

#### Safety Information

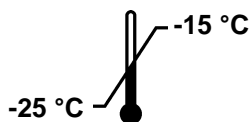
When working with chemicals, always wear a laboratory coat and disposable gloves. Clean contaminated surfaces with water. For more information, please consult the respective material safety data sheets (MSDS) available on our webpage ([www.epigenomics.com](http://www.epigenomics.com)).

Epi proLung PCR Kit and Epi proLung 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.

## 5.0 Storage and Stability

Reagents provided with the Epi proLung PCR Kit and the Epi proLung 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.

### Epi proLung PCR Kit (M6-02-002)

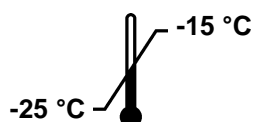


Store Epi proLung PCR Mix and Epi proLung Polymerase at -25 to -15 °C.

Each Epi proLung PCR Mix tube may be thawed and refrozen **one** (1) time.

After first use store all reagents at -25 to -15 °C for up to six (6) weeks.

### Epi proLung Control Kit (M6-02-003)



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

## 6.0 Materials Required But Not Provided

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

### Essential Special Equipment and Consumables

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

Special Equipment/Consumables	Vendor
Epi BiSKit	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	Thermo Fisher Scientific
MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Thermo Fisher Scientific
MicroAmp® 96- & 384-Well Optical Adhesive Film	Thermo Fisher Scientific
Magnetic Separator: DynaMag™-15 magnet	Thermo Fisher Scientific
Magnetic Separator: DynaMag™-2 magnet	Thermo Fisher Scientific

### Installation Requirements

The installation, calibration, performance verification and maintenance of the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument or Applied Biosystems 7500 Fast PCR Instrument must 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™, VIC®, TEXAS RED®.

## 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:  $\pm 2$  °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  $\mu$ L and 100 - 1000  $\mu$ L
- Eppendorf Multipette® 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  $\mu$ L, 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

## 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  $\mu$ L and 50 - 1000  $\mu$ L), 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  $-40$  °C to  $+120$  °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

## 7.0 Precautions and Safety

### 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.

### 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.

## 8.0 Specimen Collection and Handling

### Blood Collection and Blood Storage

- Use BD Vacutainer® K2EDTA 10 mL tube, S-Monovette® 9 mL K3E, or S-Monovette® 8,5 mL CPDA 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.

### 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  $1350 \pm 150$  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  $1350 \pm 150$  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.

## 9.0 Test Procedure

Epi proLung contains sufficient reagents to run up to 32 samples including controls. One Epi proLung Positive Control and one Epi proLung 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 Epi BiSKit (M7-01-001)

#### 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 to use 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 Epi proLung Positive and Negative Control

- Thaw one Epi proLung Positive Control and one Epi proLung 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 µL 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.

### 9.1.5 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  $1,000 \pm 100$  rpm and incubate at  $80\text{ }^{\circ}\text{C}$  for  $10 \pm 1$  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  $8\text{ }^{\circ}\text{C}$  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 µL DNA solution):
  - 150 µL Bisulfite Solution
  - 25 µL 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  $45 \pm 5$  min at  $80\text{ }^{\circ}\text{C}$  without shaking.
- Remove microtube from the thermoshaker immediately after  $45 \pm 5$  min.
- Reset thermoshaker temperature to  $23\text{ }^{\circ}\text{C}$ , or set up a second thermoshaker to  $23\text{ }^{\circ}\text{C}$  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 µL Wash A Buffer
  - 20 µL Magnetic Beads (freshly suspended).
- Mix by vortexing for 5 – 10 sec.
- Wait until thermoshaker reaches  $23\text{ }^{\circ}\text{C}$ .
- Place the microtube in the thermoshaker at  $1000 \pm 100$  rpm and incubate for  $45 \pm 5$  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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $10 \pm 1$  min at 23 °C without shaking.

#### 9.1.14 Elution

- Transfer microtube into a non-magnetic rack: Add 60  $\mu$ L Elution Buffer.
- Close the microtube.
- Resuspend the magnetic beads by vortexing for 5 – 10 sec.
- Incubate for  $10 \pm 1$  min at 23 °C in a thermoshaker at  $1000 \pm 100$  rpm.
- Briefly spin down the microtube.
- Place microtube on the DynaMag™-2 magnetic racks for 2 – 6 min.
- Using a 10 - 100  $\mu$ L reference pipette, transfer the complete eluate (~ 60  $\mu$ 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 3.

Table 3: Recommended Layout for a bisDNA Storage Plate.

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

<sup>#</sup>Epi proLung Positive Control, <sup>§</sup>Epi proLung 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, Epi proLung Positive Control, or Epi proLung Negative Control) must be tested in triplicate. Prior to use spin Epi proLung 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 Epi proLung PCR Mix and Epi proLung Polymerase, directly after usage.

**Note:** For a single PCR 16 µL Epi proLung PCR Mix and 0,95 µL Epi proLung 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 Epi proLung PCR Mix tubes depending on the desired number of patient and control sample determinations (see Table 4).
- Vortex the Epi proLung PCR Mix tube(s) for 5 – 10 sec, briefly spin down the tube(s).
- Transfer the corresponding volumes of Epi proLung PCR Mix and Epi proLung Polymerase as indicated in Table 4 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 4: 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	22,8 µL	45,6 µL	68,4 µL	91,2 µL

Table 5: 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 <sup>\$</sup>	NC <sup>\$</sup>	NC <sup>\$</sup>	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						

<sup>#</sup>Epi proLung Positive Control, <sup>\$</sup>Epi proLung Negative Control

## 10.0 Analysis

### 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 9.2.13 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:** 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 Epi proLung template file)
  - Run Mode: Standard 7500.
- Click "Next".
- Click "New Detector...".
- Create a new detector using following properties:
  - Name: SHOX2
  - Description: Epi proLung
  - Reporter dye: FAM
  - Quencher dye: (none)
  - Color: Red.
- Create a new detector using following properties:
  - Name: PTGER4

- Description: Epi proLung
- Reporter dye: VIC
- Quencher dye: (none)
- Color: Blue.
- Click "Create Another" and define following properties:
  - Name: ACTB
  - Description: Epi proLung
  - Reporter dye: TEXAS RED
  - Quencher dye: (none)
  - Color: Green.
- Click "ok".
- Select all three 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 detectors "SHOX2", "PTGER4" and "ACTB".
- Check the Passive Reference setting to be "(none)" (see Figure 2).
- Click "Close".
- Go to tab "Instrument" to program the cycling conditions as described in Table 6
- 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 6.
- Change the "Ramp Rate" according to Table 6.
- Set "Data Collection" for "Stage 2, Step 2 (55,5 @ 0:35)".
- Confirm the Thermal Cycler Protocol settings according to Table 6 (see Figure 1).
- 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 6: 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)				

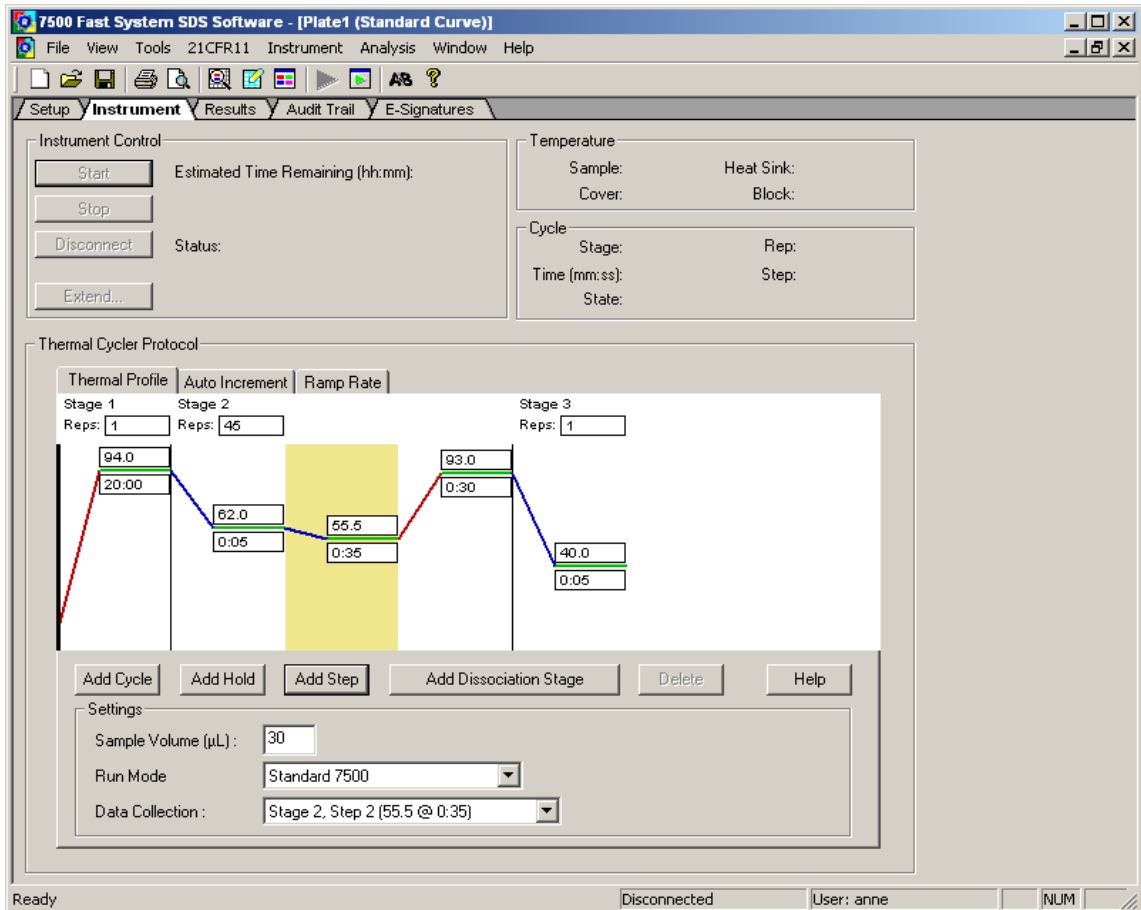


Figure 1: Screenshot after setting cycler program and detection mode.

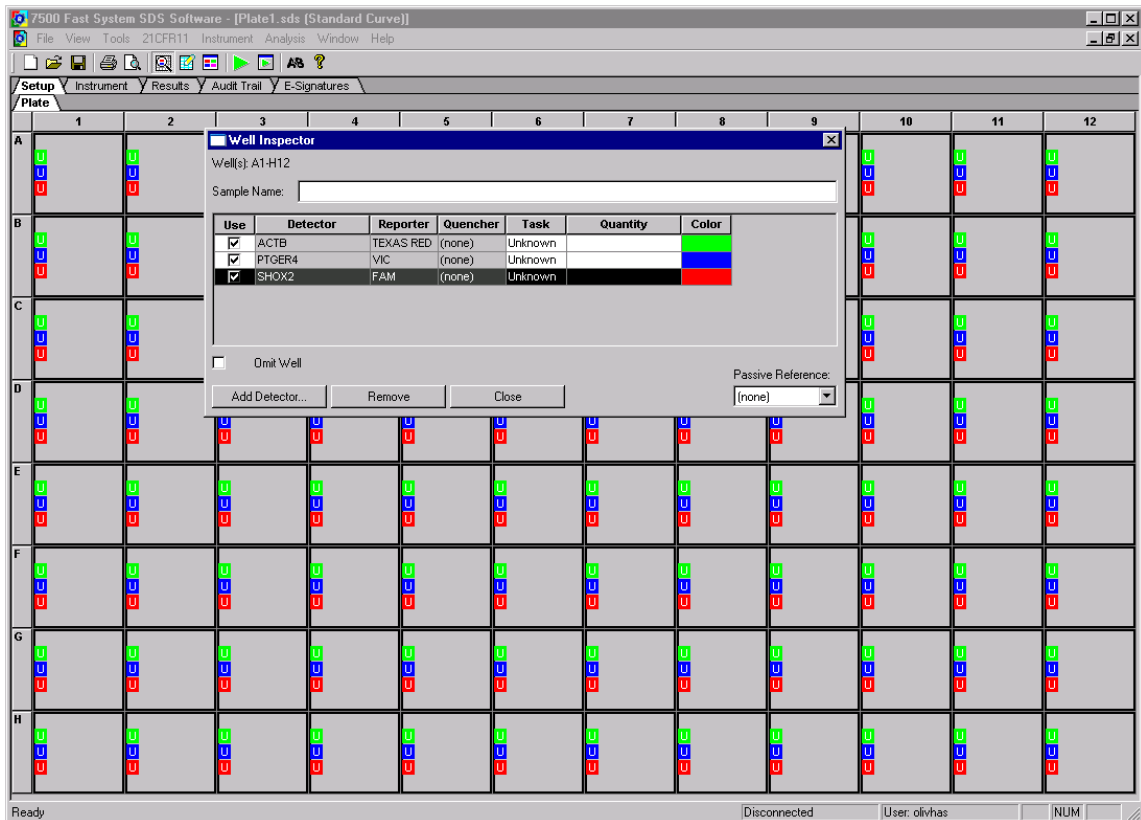


Figure 2: Screenshot after confirming settings in the 'Well Inspector' window.

## 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" to be the following:
- Data: "Delta Rn vs Cycle"
  - Detector: "All"
  - 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".
- Ct values for SHOX2, PTGER4 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

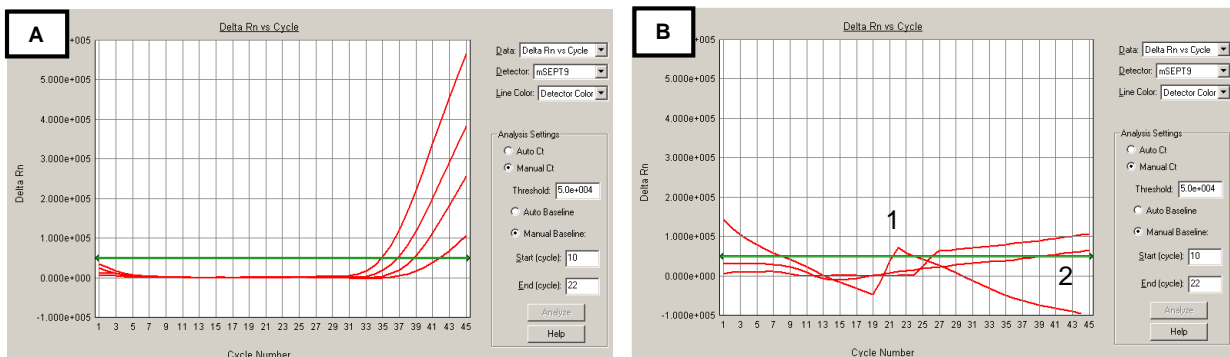


Figure 3: Screenshots of amplification curves using the Applied Biosystems 7500 Fast. A: Examples of valid positive curves. B: Examples of negative curves due to inconsistent data points (1) or linear curve shape (2).

## 10.5 Run Validity by the Epi proLung Controls

Any run of one or more patient sample(s) processed together with Epi proLung Positive Control and Epi proLung Negative Control is considered valid, when criteria set forth in Table 7 are met for **ALL THREE (3)** PCR replicates per control.

If either the Epi proLung Positive Control or Epi proLung 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 7: Validity limits of Epi proLung Controls.

Result of Control	Determination	SHOX2 Result	PTGER4 Result	ACTB Result
Positive Control valid	PCR1	Ct* ≤ 39,7	Ct* ≤ 42,3	Ct* ≤ 32,0
	PCR2	Ct* ≤ 39,7	Ct* ≤ 42,3	Ct* ≤ 32,0
	PCR3	Ct* ≤ 39,7	Ct* ≤ 42,3	Ct* ≤ 32,0
Negative Control valid	PCR1	no Ct* provided ("Undetermined")	no Ct* provided ("Undetermined")	Ct* ≤ 35,2
	PCR2			Ct* ≤ 35,2
	PCR3			Ct* ≤ 35,2

\*Cycle threshold

## 11.0 Interpretation of Results, the EPLT -Score

### 11.1 Patient Sample Validity with internal control assay ACTB

An individual patient sample provides a valid measurement if the Ct-value of the internal control assay ACTB is less or equal to 33,0 for each of the PCR triplicates (see Table 8). A sample showing no Ct or a value higher than 33,0 is considered as invalid.

Table 8: Validity of Patient Sample

	Determination	ACTB Result
Patient sample valid	PCR1	Ct* ≤ 33,0
	PCR2	Ct* ≤ 33,0
	PCR3	Ct* ≤ 33,0
Patient sample invalid	Any PCR	Ct* > 33,0 or no Ct* provided

\*Cycle threshold

### 11.2 EPLT-Score calculation

**Note:** Only if the measurement is determined valid (see section 11.1) an EPLT-Score can be calculated. If the measurement is determined invalid an EPLT-Score must not be calculated.

The EPLT-Score is calculated in a two-step procedure:

#### 1. Ct-value Aggregation:

- Determine the minimal Ct-value from SHOX2 triplicate PCR → minCt.SHOX2
- Determine the minimal Ct-value from PTGER4 triplicate PCR → minCt.PTGER4

**Note:** Ct-value "45,0" is assigned to a PCR providing no Ct value ("undetermined" in SDS software)

#### 2. Calculation of EPLT-Score:

- $EPLT-Score = 13,46 - 0,14 * \min Ct.SHOX2 - 0,23 * \min Ct.PTGER4.$

The following table provides an example for the calculation of the EPLT score for one patient sample:

Table 9: Example for EPLT-Score calculation

Patient Sample Results		
1. Ct-value aggregation <sup>1</sup> :	SHOX2 PCR1: 30,01 SHOX2 PCR2: 29,85 SHOX2 PCR3: 30,05 minCt.SHOX2 = <b>29,85</b>	PTGER4 PCR1: 31,20 PTGER4 PCR2: 31,25 PTGER4 PCR3: 31,24 minCt.PTGER4 = <b>31,20</b>
2. Calculation of EPLT-Score:	$EPLT-Score = 13,46 - (0,14 * \min Ct.SHOX2) - (0,23 * \min Ct.PTGER4)$ $= 13,46 - (0,14 * 29,85) - (0,23 * 31,20)$ $= 13,46 - 4,18 - 7,18$ $= 2,10$ EPLT-Score = <b>2,10</b>	

<sup>1</sup>Ct value "45,0" is assigned to a PCR providing no Ct value ("undetermined" in SDS software)

## 12.0 Quality Control

### External Controls

Epi proLung contains Epi proLung Positive and Negative Controls (M6-02-003). 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 proLung Positive and Negative Control values must be within the validity limits (see Table 7). 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.

### Internal Controls

The internal control allows the detection of bisulfite converted ACTB ( $\beta$ -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 (see section 11.1) invalidate the patient sample; as such high values are associated with very low bisDNA content or PCR inhibition.

## 13.0 Limitations of the Procedure

- For in vitro diagnostic use only.
- This product has been validated for the combination of Epi BiSKit (M7-01-001), Epi proLung PCR Kit (M6-02-002) and Epi proLung Control Kit (M6-02-003) 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 10 mL, S-Monovette® 9 mL K3E and S-Monovette® 8,5 mL CPDA tubes. Use of other sample types and other blood collection tubes have not been validated.
- Use of this product is limited to personnel experienced and trained in PCR assays.

## 14.0 Performance Characteristics

### Clinical Sensitivity and Specificity

Clinical performance of Epi proLung was assessed in a validation study comprising data from 360 clinical specimens from consented patients enrolled at sites in the US and Europe. Of these patients 152 subjects were diagnosed with lung cancer (pathologically confirmed), 208 subjects were not diagnosed with lung cancer (LC), either after a screening computed tomography scan (CT-scan) was negative or after the follow-up of a radiological finding (pulmonary nodule ) did not lead to a LC diagnosis.

In Figure 4 the clinical performance of Epi proLung in terms of discriminating lung cancer patients from clinical controls is shown. The receiver operating characteristics (ROC) curve displays all sensitivity / specificity value pairs over the range of possible thresholds on the EPLT score scale. The area under the ROC curve (AUC) equals 0,82.

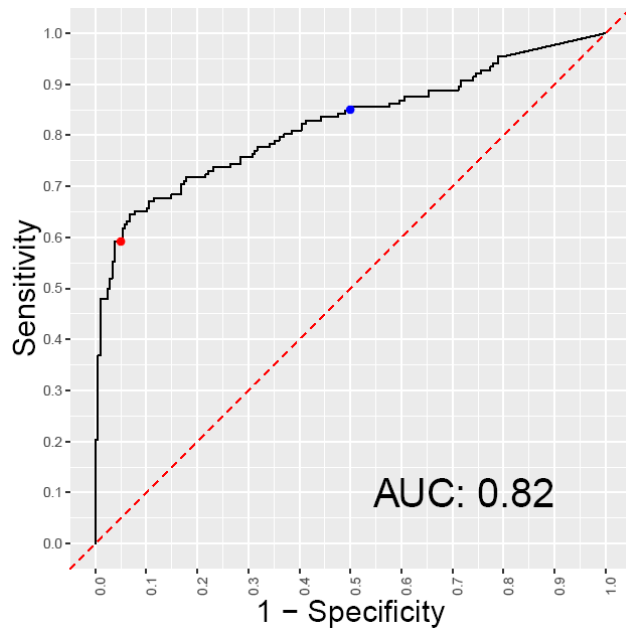


Figure 4: Clinical performance Epi proLung: Receiver operating characteristics (ROC) curve for discrimination of lung cancer patients against clinical controls. Dots indicate sensitivity values at specificity values 50% (blue) and 95% (red), respectively.

Table 10: Threshold, sensitivity and specificity for Epi proLung.

Threshold	Sensitivity	Specificity
-0,43	59 %	95 %
-1,85	85 %	50 %

Table 10 contains threshold values together with respective sensitivity and specificity values. Figure 5 displays the observed likelihood to be diagnosed with lung cancer in this study as a function of the EPLT score together with corresponding sensitivity and specificity values given a specific threshold on the EPLT score scale.

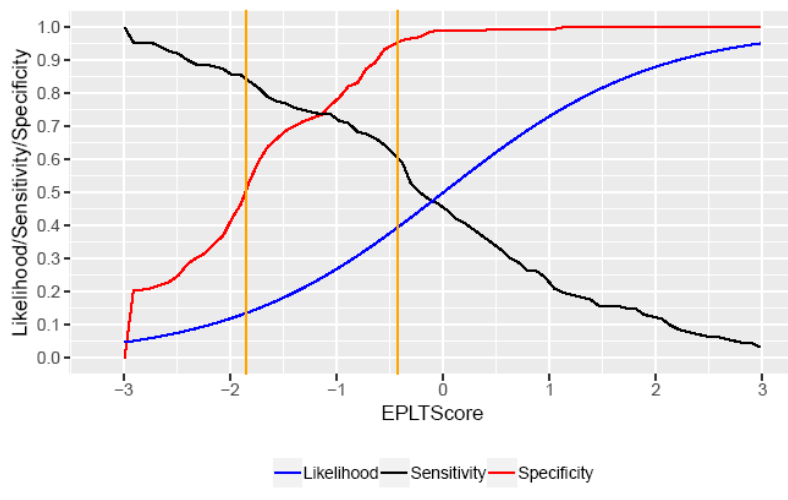


Figure 5: Functional Relationship between the EPLT score and the observed likelihood to be diagnosed with lung cancer in this study (blue); sensitivity (black) and specificity (red) values as a function of choice of threshold on the EPLT score scale for test output dichotomization. The vertical lines (orange) indicate the thresholds at specificity values 50% and 95%, respectively (see Table 10).

## Analytical Sensitivity

The analytical sensitivity was evaluated by four operators in a total of 16 independent runs comprising two independent preparations of six technical Limit of Detection (LoD) samples with methylated DNA (SHOX2 and PTGER4 positive) concentrations of 0, 2, 4, 8, 16 and 32 pg/mL in a background of 10 ng/mL unmethylated DNA. Altogether 192 LoD samples were processed using two different lots Epi proLung PCR Kit. 190 determinations on the Applied Biosystems 7500 Fast Dx with SDS v1.4 were valid. Of the 159 LoD samples with spiked methylated DNA 98 samples were positive for SHOX2 and 117 samples were positive for PTGER4. All 31 samples without spiked methylated DNA were negative for both targets. The estimated LoD95 was determined by a logistic regression model to be 36 pg/mL (CI 95%: 19 pg/mL – 67 pg/mL) for SHOX2 and 40 pg/mL (CI 95%: 18 pg/mL – 87 pg/mL) for PTGER4.

## Reproducibility and Precision

Reproducibility and Precision of the procedure were tested by processing different human plasma pools spiked with plasma from patients diagnosed with lung cancer. Six plasma pools with different concentration levels of methylated SHOX2 and PTGER4 DNA were tested.

80 replicates of each of the six plasma pools were processed in a total of 40 independent runs by four operators on three Applied Biosystems 7500 Fast Dx Real-Time PCR Instruments using three different Kit lots Epi proLung PCR Kit (M6-02-002). 16 out of the 40 runs were performed at an external testing site. 474 out of the 480 determinations were valid. For each valid sample the minCT value was determined from the CT results of the triplicate PCR. Subsequently, for each of the six sample types the mean of these minCT values was calculated. The mean of the minCT values covered a range from CT 32,8 to 37,8 for SHOX2 and for PTGER4 from CT 34,1 to 39,3. Additionally the overall standard deviation (SD) and coefficient of variation (CV) were calculated for each plasma pool. For SHOX2 the ranges of SD and CV were 0,7 – 2,4 CT and 2,0 – 6,5%, respectively. The corresponding ranges for PTGER4 were 0,5 – 2,2 CT and 1,5 – 5,5%.

Analysis of variance components was performed for Day-to-Day (comprising run, PCR lot and instrument variability), Operator-to-Operator, Site-to-Site and within run variance (repeatability). Within run variance contributed the most to the variance. The Operator-to-Operator component was the smallest. All minCT, SD and CV values are listed in Table 11.

Table 11: Results for Precision and Reproducibility of Epi proLung represented by total Standard Deviation (SD), total Coefficient of Variation (CV) and Variance Components in terms of Standard Deviations (SD).

Target	Sample	Mean minCt [Ct]	Total SD [Ct]	Total CV [%]	Day-to-Day* SD [Ct]	Operator-to-Operator SD [Ct]	Site-to-Site SD [Ct]	Repeatability (Within Run) SD [Ct]
SHOX2	Plasma Pool 1	33,4	0,7	2,0	0,2	0,1	0,5	0,4
	Plasma Pool 2	35,4	0,9	2,5	0,4	0,2	0,4	0,7
	Plasma Pool 3	37,8	2,4	6,5	1,1	0,0	0,0	2,2
	Plasma Pool 4	32,8	0,7	2,1	0,1	0,2	0,5	0,4
	Plasma Pool 5	34,8	0,8	2,2	0,3	0,2	0,3	0,6
	Plasma Pool 6	37,1	2,2	5,9	0,0	0,8	0,0	2,0
PTGER4	Plasma Pool 1	34,1	0,5	1,5	0,4	0,0	0,2	0,3
	Plasma Pool 2	35,9	0,6	1,8	0,5	0,0	0,1	0,3
	Plasma Pool 3	37,5	0,9	2,4	0,4	0,0	0,3	0,7
	Plasma Pool 4	35,3	0,6	1,6	0,4	0,0	0,3	0,3
	Plasma Pool 5	37,0	0,8	2,3	0,4	0,0	0,4	0,7
	Plasma Pool 6	39,3	2,2	5,5	0,8	0,1	0,6	2,0

\* Day comprises run, PCR lot and instrument variability

## Cross-Reactivity

To test for sequence cross-reactivity, BLAST alignment searches and electronic PCR analyses were performed against the human genome with the Epi proLung PCR assay (blockers, primers and probes). This analysis showed that the test is specific for amplification of the bisDNA sequence of methylated SHOX2 and PTGER4, respectively, and therefore showed no cross-reactivity within the human genome.

Fifty-seven (57) samples from patients with non-malignant diseases of the lung (COPD, pneumonia, lung emphysema, interstitial lung disease) were evaluated to determine cross-reactivity. The Epi proLung test discriminates malignant from non-malignant disease with AUC = 0,73.

#### Interference Substances

A study was conducted to verify that the presence of interfering substances potentially found in plasma samples have no effect on the test results. Ten (10) common substances present in human plasma were selected and tested at the highest concentration that would occur in a clinical setting. Albumin (40 mg/mL), Bilirubin (0,2 mg/mL), Cholesterol (5 mg/mL), CPDA (20% v/v), D-(+) Glucose (10 mg/mL), Hemoglobin (10 mg/mL), K2EDTA (20 mg/mL), Red Blood Cells (0,4 % v/v), Triglycerides (12 mg/mL), and Uric Acid (0,235 mg/mL) were tested in 6 plasma specimens each (3 analyte negative, 3 analyte positive). None of the substances interfere with the test when added to the specimen at the listed concentration level.

## 15.0 Meaning of Symbols

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Consult Instructions for Use

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**IVD**

In-vitro Diagnostica

---

**REF**

Reorder Number

---

**LOT**

Lot Number

---



Use-by

---



Manufacturer

---



Temperature Limit

---



Sufficient for <n> tests

---



Do not Re-use

---

**CONTROL-**

Control, negative

---

**CONTROL+**

Control, positive

---



CE mark

---



UDI Barcode

---

## 16.0 References

- 1 Weiss, G et al. Validation of the SHOX2/PTGER4 DNA Methylation Marker Panel for Plasma-Based Discrimination between Patients with Malignant and Nonmalignant Lung Disease. *Journal of Thoracic Oncology*, Volume 12, Issue 1 (2017)
- 2 Eads, C.A. et al. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Research*, Volume 28, Issue 8, e32 (2000)

## 17.0 Contact Information

Epi proLung is manufactured by:

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Geneststraße 5  
10829 Berlin, Germany

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