October 2023

PreCursor-M+

Instructions For Use

For the assessment of hypermethylation of the genes

FAM19A4 and *hsa-miR124-2*

Version 1

IVD	For in vitro diagnostic use
CE	
	Self-screen B.V., Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands
REF	QHR15100
Σ 72	
Rn	3

Contents

Intended Use

The PreCursor-M+ is a multiplex real-time methylation-specific PCR assay for the detection of promoter hypermethylation of the genes *FAM19A4* and *hsa-mir124-2*. Samples that may be tested with PreCursor-M+ Test include bisulfite-converted DNA isolated from specimens collected in the following ways:

- Cervical specimens collected using a brush/broom-type collection device and placed in PreservCyt® Solution or in SurePath™ Solution (physician collected)
- Cervical specimens collected with the digene® HC2 DNA Collection Device (physician collected)
- Vaginal specimens collected using a brush/broom device (self-collected)

Indications for use:

1. As a follow-up test for women with a positive human papillomavirus (HPV) test, to determine the need for referral to colposcopy or other follow-up procedures.

2. As a follow-up test for women with Pap test results with atypical squamous cells of undetermined significance (ASC-US), to determine the need for referral to colposcopy or other follow-up procedures.

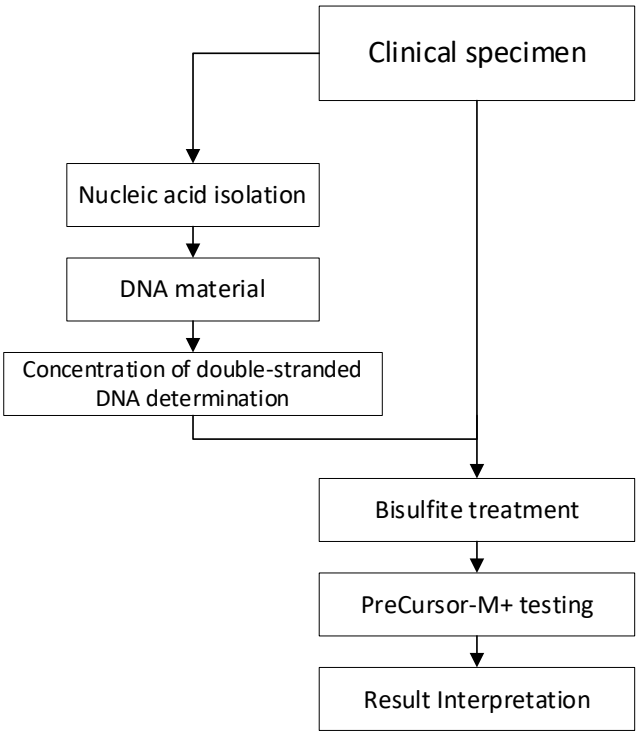
This product is intended to be used by professional users, such as technicians and laboratorians who are trained in in vitro diagnostics procedures, molecular biological techniques, and the Mic qPCR cycler.

Summary and Explanation

DNA methylation is a biochemical process that is important for normal development in higher organisms[1]. It involves the addition of a methyl group to the 5th position of the pyrimidine ring of the cytosine nucleotide. Abnormal patterns of DNA methylation also play a major role in carcinogenesis. In several human cancers and cancer cell lines, including cervical cancer and endometrial cancer, promoter hypermethylation of the genes *FAM19A4* (Family with sequence similarity 19 (chemokine (C-C motif)-like)member A4) and/or hsa-*mir124-2 (homo sapiens micro RNA 124-2)* has been detected [2-6]. Host-cell promoter methylation analysis specifically detects cancers and so-called “advanced” cervical intraepithelial neoplasia (CIN) lesions, which harbor a cancer-like methylation profile and have a high short-term risk of progression to cancer [3, 7, 8, 10, 11, 12, 14]. The PreCursor-M+ assay allows the detection of promoter hypermethylation of the genes *FAM19A4* and *hsa-mir124-2* on bisulfite-converted DNA isolated from cervical or vaginal specimens using human β-actin gene as an internal sample quality control.

Principle of the procedure

The PreCursor-M+ is a multiplex real-time PCR test that amplifies the methylated promoter regions of the tumor suppressor genes *FAM19A4* and *hsa-mir124-2*, as well as a methylation-unspecific fragment of the reference gene β-actin (*ACTB*). The sample reference is used for normalization and quality control. The master mix is intended for amplification of bisulfite-converted DNA prepared from clinical specimens. Amplification of the genes is detected with fluorescent hydrolysis probes. The multiplex format of the assay enables simultaneous amplification and detection of the three targets within one reaction. The Cq (quantification cycle) value represents the number of PCR cycles necessary for detection of a fluorescent signal above a background signal, which is correlated to the number of target molecules present in the sample. DNA methylation is quantified by ΔΔCq values of the target genes *FAM19A4* and *hsa-mir124-2*. First the ΔCq value is calculated by deducting the Cq value of the sample reference gene β-actin from the Cq value of the sample target gene. For normalization, the ΔCq value of the calibrator is deducted from the ΔCq of the sample target genes resulting in a ΔΔCq value. The calibrator contains a standard concentration of low-copy DNA fragments of the three targets of the assay, FAM19A4, hsa-mir124-2, and β-actin.



Materials Provided

PreCursor-M+ kit content

Number of reactions		72
PreCursor-M+ Master Mix (2 tubes)	Brown color tube	630 µL
PreCursor-M+ Calibrator (2 tubes)	Transparent color tube	25 µL

Materials required but not provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Consumables and reagents for preparation for self-collected samples

- Hologic PreservCyt® Solution (Hologic)

Consumables and reagents for preparation of samples stored in SurePath collection medium

- Buffer AL (QIAGEN, cat.no. 19075)

Consumables and reagents for bisulfite-conversion

Verified bisulfite-conversion kits include:

- EZ DNA Methylation Kit (ZYMO Research, cat.no. D5001 or cat.no. D5002)
- EZ DNA Methylation Lightning (ZYMO Research, cat.no. D5030 or cat.no. D5031)
- Epitect Fast 96 Bisulfite Kit (QIAGEN, cat.no. 59720)
- QIAsymphony Bisulfite Kit (QIAGEN, cat.no. 931106)

Consumables and reagents for the Mic qPCR cycler

- Strip tubes and Caps, 0.1 ml (Bio Molecular Systems, cat.no. MIC-TUBES)
- Purified water (molecular biology grade, distilled or deionized)

Equipment

- Adjustable pipets (1–10 µL; 10–100 µL) and filter tips
- Disposable gloves
- Benchtop centrifuge with a speed >10,000 rpm
- Vortex mixer
- Qubit® (Thermo Fisher Scientific, cat.no. Q33216), NanoDrop® 3300 Fluorospectrometer (Thermo Fisher Scientific, cat.no. ND-3300), or equivalent
- QIAsymphony SP (QIAGEN, cat.no. 9001297) for use with QIAsymphony Bisulfite kit

Equipment for real-time PCR

- Mic qPCR cycler (Bio Molecular Systems)
- QPCR software (Bio Molecular Systems)
- PreCursor-M+ run profile named ‘PreCursor-M+ v.1.0.mictemplate’.

Warnings and Precautions

For in vitro diagnostic use only.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs).

PreCursor-M+ Master Mix

Contains 1,2,4 triazole. Danger! Suspected of damaging fertility or the unborn child. Wear protective gloves/protective clothing/eye protection/face protection. If exposed or concerned: get medical advice. Store locked up. Dispose of contents and container to an approved disposal plant.

General precautions

Use of PCR tests require good laboratory practices, including maintenance of equipment, that are dedicated to molecular biology and is compliant with applicable regulations and relevant standards.

Always pay attention to the following:

- Wear protective disposable powder-free gloves, a laboratory coat, and eye protection when handling specimens.
- Prevent microbial and nuclease (DNase) contamination of the specimen and the kit. DNase may cause degradation of the DNA template.
- Sample preparation and PCR set up should be performed in a dedicated area separated from the area in which PCR amplification will be performed. To minimize the risk for PCR reaction contamination the use of a PCR-cabinet with UV-radiation is strongly recommended.
- The working area needs to be kept clean especially from PCR contaminants. Cleaning can be performed with 0.1 N HCl and/or UV-radiation, or by a user preferred and proven method.
- Include a no template control in your PCR experiments to confirm that reagents are free of contamination.
- Avoid DNA or PCR product carryover contamination, which could result in a false-positive signal.
- Use extreme caution to prevent contamination of the mixes with the synthetic materials that are contained in the PCR reagents.
- Always use DNase-free disposable pipet tips with aerosol barriers.
- Reagents of PreCursor-M+ are optimally diluted. Do not dilute reagents further as this may result in a loss of performance.
- All reagents supplied in the PreCursor-M+ kit are intended to be used solely with the other reagents supplied in the same kit. Do not substitute any reagent from one kit with the same reagent from another PreCursor-M+ kit, even from the same batch, as this may affect performance.
- Refer to the Mic qPCR cycler user manual for additional warnings, precautions, and procedures.
- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Do not use components of the kit that have passed their expiration date, or that have been incorrectly stored.
- Minimize the exposure of components to light; reaction mixes may be altered due to exposure.
- Discard sample and assay waste according to your local safety procedures.

Reagent storage and handling

PreCursor-M+ is shipped on dry ice. If any component of the PreCursor-M+ is not frozen upon arrival, the outer packaging has been opened during transit please contact the local distributor.
Upon receipt store immediately at –30 to –15°C in a constant-temperature freezer and protected from light. It is advised to store the kit in a laboratory that is free of PCR amplicons.
When stored under the specified storage conditions, PreCursor-M+ is stable until the expiration date stated on the box label. Once opened, reagents can be stored in their original packaging at –30 to –15°C. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 3 freeze–thaw cycles. Before use, thaw the reagents completely. Mix by gently vortexing and centrifuge the tubes before opening.

Sample handling

Cervical specimens

The PreCursor-M+ is for use with bisulfite-converted genomic DNA samples obtained from cervical specimens. Validated collection media for cervical specimens (scrapes) are PreservCyt® collection medium, SurePath™ collection medium and Digene Specimen Transport Medium (STM). Optimal storage temperature of the clinical samples is 2–8°C upon arrival at the lab. Under these storage conditions, samples in PreservCyt or SurePath™ collection medium are stable for 3 months prior to DNA extraction.

Self-collected vaginal brush specimens

The PreCursor-M+ is for use with bisulfite-converted genomic DNA samples extracted from self-collected vaginal brush specimens. Self-collected vaginal brush specimens can be collected and shipped dry and upon arrival in the laboratory stored in PreservCyt collection medium with a recommended storage volume of 2-3 mL. Samples in PreservCyt collection medium may be stored at 2–8°C or room temperature for no more than 3 months.

Genomic DNA samples

Once genomic DNA is extracted, DNA samples can be stored and shipped at –30°C to –15°C for up to 12 months.

Sample preparation for samples stored in PreservCyt

The PreCursor-M+ has been validated for use with bisulfite-converted genomic DNA derived from cervical specimens. Bisulfite-conversion of genomic DNA can be performed:

i) with prior sample DNA extraction and DNA quantity control
ii) directly on the cervical specimen using the EpiTect Fast 96 Bisulfite Kit (QIAGEN, cat.no. 59720) or
iii) directly on the cervical specimen using the QIAsymphony Bisulfite Kit (QIAGEN, cat.no. 931106)

Our recommendations are outlined below.

- i) Bisulfite-conversion with prior DNA extraction and DNA quantity control

This protocol requires DNA Extraction, DNA concentration measurement, followed by aliquoting of optimal eluate volume before starting with the bisulfite-conversion protocol, and has been verified for the EZ DNA Methylation™ Kit (cat.no. D5001) and the EZ DNA Methylation Lightning Kit (cat.no. D5030) from ZYMO Research. We recommend the following workflow and methods:

- *DNA extraction*
  Standard DNA extraction kits (e.g., column-based and magnetic bead-based kits) are compatible with the PreCursor-M+ (e.g. QIAamp DNA mini kit). Use 5-10%* (i.e. 1 to 2 mL from 20 mL) of the cervical sample for DNA extraction.
  IMPORTANT: PreservCyt medium can interfere with the DNA extraction process. To overcome this the PreservCyt sample can be 1) diluted (1:1) with lysis buffer that is compatible with the DNA extraction kit prior to starting DNA extraction, or 2) pellet the sample by centrifugation and remove the PreservCyt by pipetting. The pellet is resuspended in appropriate volume of PBS or lysis buffer that is compatible with the DNA extraction kit prior to starting DNA extraction. Equivalent media should be processed similarly.
- *DNA concentration measurement*
  Prior to bisulfite-conversion of DNA, measure DNA concentration. Suitable systems for measuring the DNA concentrations are Qubit® Fluorometer, NanoDrop 3300 Fluorospectrometer (both from Thermo Fisher Scientific) or equivalents.
- *Aliquoting DNA eluate*
  Recommended DNA input for bisulfite-conversion is 200 ng that is to be eluted in 10 µL elution buffer. DNA concentration input for bisulfite-conversion can range from 100 ng to 2 µg for valid test results. If DNA concentration is too low for bisulfite-conversion, repeat the DNA extraction with a higher input volume of the clinical sample or elute DNA in a smaller elution volume.
- *Bisulfite-conversion with EZ DNA Methylation Kit and EZ DNA Methylation Lightning Kit* is performed according to the manufacturer's recommendations.
  Note: According to EZ DNA Methylation Kit, maximum amount of sample DNA should not exceed 2 µg to obtain a sufficiently high conversion-efficiency (>98%).

- ii) Bisulfite-conversion directly on cervical specimen with the Epitect Fast 96 Bisulfite Kit

Bisulfite-conversion directly performed on the cervical specimen collected in PreservCyt® Solution has been verified for the EpiTect Fast 96 Bisulfite Kit (QIAGEN). For the Epitect Fast 96 Bisulfite Kit we refer to the Handbook section for high-concentration DNA samples (1 ng – 2 µg) according to the manufacturer's recommendation, except for the following items:

- Step 1 of the protocol. Take 2.5% to 10%* of the cervical specimen in PreservCyt collection medium (i.e. 500 µLL to 2 mL* from 20 ml) and pellet by centrifugation for 10 min at minimal 3390 x g. Discard the supernatant leaving the cell pellet at a maximum 20 µL of residual PreservCyt collection medium. For the bisulfite-conversion reaction, use this cell pellet sample and continue with step 2 of the manufacturer's protocol.
- Buffer BL: Do not add carrier RNA.
- Elution volume of the bisulfite-conversion DNA is 50 µL of Buffer EB for each sample.

*) for samples with low cellularity this can be increased to 10% (i.e. 2 mL from 20 mL)

- iii) Bisulfite-conversion directly on cervical specimen with the QIAsymphony Bisulfite Kit

Bisulfite-conversion directly performed on the cervical specimen collected in PreservCyt Solution has been verified for the QIAsymphony Bisulfite kit from QIAGEN. We refer to the handbook of the QIAsymphony Bisulfite kit and testing is performed according to the manufacturer's recommendation.

- Take 2.5% to 10%* of the cervical specimen in PreservCyt collection medium (i.e. 500 µLto 2 mL* from 20 ml) and pellet by centrifugation for 10 minutes at minimal 3390 x g. Discard the supernatant leaving the cell pellet and at a maximum 20 µL of residual PreservCyt collection medium.
- For the bisulfite-conversion reaction, follow the "QIAsymphony SP protocol sheet – Bisulfite 140_HC_V" starting at step 2, instead of DNA use the cell pellet.
- Once the bisulfite conversion reaction is completed start an SP run on the QIAsymphony following the steps described in the protocol "Bisulfite conversion of unmethylated Cytosines in different sample types" from the QIAsymphony Bisulfite Kit handbook. Elution is done in 40 µL.

Sample Preparation for samples stored in SurePath™

Samples stored in SurePath need to be pretreated before DNA extraction. The PreCursor-M+ assay has been validated for use with bisulfite-converted genomic DNA derived from cervical specimens collected in SurePath according to the procedure outlined below.

- Take 2.5% to 10% of the cervical sample (250 µL to 1 mL from 10 mL)
- Centrifuge for 10 minutes at 4000g, remove the supernatant but leave 100 µL SurePath
- Add 100 µL buffer AL and resuspend the pellet
- Mix well and incubate for 20 minutes at 90°C
- Cool down for 5 min at room temperature.
- Perform DNA extraction using the QIAamp DNA mini kit (or equivalent) according manufacturers instruction, elute DNA in 50 µL.
- Measure DNA concentration, suitable systems are Qubit® Fluorometer, NanoDrop 3300 Fluorospectrometer (both from Thermo Fisher Scientific) or equivalents
- 200 ng DNA is recommended for bisulfite conversion, if 200 ng is not available use the maximum input volume of DNA extract. Perform the conversion with the EZ DNA methylation kit or EZ DNA Methylation-Lightning kit (Zymo) according manufacturers instruction.
- Use 2.5 µL eluate for the PreCursor-M+.

General recommendations for bisulfite-conversion

The bisulfite-conversion reaction should be performed in a designated area separate from where the PreCursor-M+ Master Mix is stored and dispensed, to avoid contaminating the reagents.

The input in the PreCursor-M+ reaction is 2.5 µL of bisulfite-converted DNA.

If the internal sample quality control is negative (i.e., β-actin Cq values are >26.4), the specimen bisulfite-converted DNA preparation resulted in material of insufficient quantity and/or quality and is scored

invalid. Perform the recommended steps to reach an β-actin Cq that is within the valid range for the following:

- *Bisulfite-conversion with prior DNA extraction and DNA quantity control:*

  Repeat bisulfite-conversion reaction with a higher input of sample DNA and/or repeat DNA isolation with a higher input of cervical specimen

- *Bisulfite-conversion directly on cervical specimen:*

  Repeat bisulfite-conversion reaction with 10%[1] of the cervical specimen in PreservCyt collection medium (i.e. 2 ml from 20 ml).

Bisulfite-converted DNA can be stored up to 24 hours at 2–8°C and up to 3 months –20°C or lower. Repeat freeze-thawing of the bisulfite-converted DNA should be avoided at all times. The number of freeze-thaw cycles should not exceed three, to maintain sufficient quality.

1 Sample volume for direct bisulfite-conversion can be increased when success rate is unsatisfactory due to sampling variability, for example as a result of inadequate sampling.

Procedure: PreCursor-M+ PCR in the Mic qPCR cycler

PreCursor-M+ Reaction Setup for Mic qPCR cycler

Before you start:

- To minimize the risk for PCR contamination, it is strongly recommended that a PCR-cabinet with UV-irradiation capability is used.
- Dispensing of the PreCursor-M+ Master Mix must be performed in an area separate from the area where the DNA bisulfite-conversion reaction is performed.
- Clean the bench area, pipets, and tube rack prior to use with a DNA-degrading solution to prevent template or nuclease contamination

In a designated DNA- and amplicon-free area:

1. Thaw the PreCursor-M+ master mix and calibrator completely, and protect the PreCursor-M+ master mix from light whenever possible
   NOTE: *Do not exceed 30 minutes for the thawing step, to avoid any material degradation.*
2. Briefly vortex and spin down prior to pipetting.
3. Dispense 17.5 µL of ready-to-use PreCursor-M+ master mix into the appropriate tubes of the tube-strips (at maximum 48 tubes per run). Reaction setup can be done at room temperature.
   NOTE: *Each tube is preloaded with silicone oil to prevent evaporation and condensation. Make sure you pipet tip does not touch the silicone oil.*
4. Return the vial with remaining PreCursor-M+ master mix to the freezer to avoid any material degradation.
5. Transfer tube strips to designated amplicon-free area.

In a designated amplicon-free area:

6. Add 2.5 µL of water for the No Template Control (NTC) reaction to tube position 2.
7. Add 2.5 µL of PreCursor-M+ Calibrator to tube position 1.
8. Add 2.5 µL of bisulfite-converted DNA to the appropriate tubes containing the PreCursor-M+ master mix.
9. Once a set of 4 tubes has been filled, use the Mic capping tool to close the tubes.
   NOTE: *The PCR tubes can be stored up to 30 minutes between pipetting samples into the PCR tubes and start of the experiment in the machine at 2-8°C in the dark.*
10. Return the PreCursor-M+ Calibrator to the freezer to avoid any material degradation.
11. Transfer tube strips to designated PCR amplification area.

In a designated PCR amplification area:

12. Place tube strips into 48-well rotor according to the assigned positions, unused positions should be filled with dummy tube strips holding 20 µL of water with caps as counterweight.
13. Attach locking ring.

Mic qPCR machine set up

14. Start the micPCR Software.
15. Click New, Run from Template
16. Select template: PreCursor-M+ v.1.0 and click *Ok.*
17. Select Samples and enter the sample identification number(s) into this list. The Calibrator is always on position 1 and the NTC on position 2. The list represents the expected layout of the rotor. Sample identification number(s) can also be added after the run is completed.
18. Click on the Mic instrument icon (right top).
19. Click *Start Run.*
20. Enter the run (experiment) name and click *Save*.
21. Click Start. The PreCursor-M+ run should start.

Acquiring Cq values

21. After the run is successfully completed click OK.
22. Click on save.
23. If the sample ID's haven't been added yet, fill in the sample identification number(s).
24. Go in the left pane to Analysis and click on 'Cycling +'. Select target FAM19A4.
25. Go in the left pane to Analysis and click on 'Cycling +'. Select target hsa-miR124-2.
26. Go in the left pane to Analysis and click on 'Cycling +'. Select target β-Actin.
    The fluorescent signals in the 3 detection channels (Green, Yellow and Orange) are automatically analysed according to their channel specific settings.
27. Check the fluorescent data in each detection channel for each reaction for typical logarithmic amplification curves (i.e., logarithmic increase of fluorescence). If the amplification curve(s) is/are not satisfactory, repeat the sample with the PreCursor-M+ or contact support.
28. To export go to the left pane and click on 'Reports +'. A preview of the report will be displayed.
29. Click on the icon 'Export' (top of right pane) and select export format 'XLSX' and click OK.

Interpretation of results

Analysis of the data is divided in the following steps and must be performed in this specified order.

1. Validation criteria PreCursor-M+ controls

30. The PCR run is valid when the controls comply with below criteria for all targets

	β-actin	FAM19A4	hsa-miR124-2
NTC	>40 (no Cq value)	>40 (no Cq value)	>40 (no Cq value)
Calibrator	<30	<30	<30

In case the above criteria for the controls are met, the run can be considered valid and the patient samples can be analyzed and interpreted. If the Cq value criteria for one of the controls are not met, the associated test results are invalid and the run should be repeated.

2. Validation criterion of each individual sample

31. The Cq value of β-Actin of a sample should be ≤ 26.4 for a valid result.
    When the above criterion is met, the sample results can be used for analysis. If the Cq value of the target 'β-actin' does not match this requirement, the sample is invalid and should not be used for ΔΔCq calculations.

3. Calculating ΔΔCq values

The result of a sample is expressed in ΔΔCq for each target (i.e. FAM19A4 and hsa-miR124-2), being a measure for hyper methylation.

32. Use the below formula for each sample to calculate the ΔΔCq values of the target genes FAM19A4 and hsa-miR124-2.

$$\Delta\Delta Cq_{\text{target gene}} = (Cq_{\text{target gene sample}} - Cq_{\textit{β-actin} \text{ sample}}) - (Cq_{\text{target gene calibrator}} - Cq_{\textit{β-actin} \text{ calibrator}})$$

For targets with no Cq values the target is negative for hypermethylation.

4. Scoring of ΔΔCq values

32. If a ΔΔCq value for FAM19A4 or hsa-miR124-2 is below the cut-off values listed in the tables below, the target is scored "Hypermethylation positive".
    ***NOTE**: Partial or low levels of methylation are a natural occurring phenomenon that are, unlike hypermethylation levels, not directly related to the development of cancer.*

33. A sample is 'Methylation-positive" when at least one of the targets is scored "Methylation-positive".

Cut-off values for physician-collected cervical specimens

	Cq value β-Actin	ΔΔCq	Sample result
1	≤26.4	FAM19A4: ≤9.66 and/or hsa-miR124-2: ≤6.0	Methylation-positive
2	≤26.4	FAM19A4: >9.66 and hsa-miR124-2: >6.0	Methylation-negative
3	>26.4	Any	Invalid

Cut-off values for self-collected vaginal specimens and its interpretation

	Cq value β-Actin	ΔΔCq	Sample interpretation
1	≤26.4	FAM19A4: ≤11.59 and/or hsa-miR124-2: ≤7.58	Methylation-positive
2	≤26.4	FAM19A4: >11.59 and hsa-miR124-2: >7.58	Methylation-negative
3	>26.4	Any	Invalid

- Samples with low DNA quality/quantity (i.e., ACTB C_q values just within acceptance criterion; C_q values from 25 to 26.4) could be scored false-negative. Repeat testing of the PCR in single is advised. A negative result for the repeat test means that the sample is hypermethylation negative, a positive result means that the sample is hypermethylation positive.
- Samples with high cellularity that are processed with a direct conversion protocol have the risk of overloading the PCR reaction with DNA, which can lead to a false negative result for the FAM19A4 target. Therefore, for samples with 1) a negative hypermethylation result, 2) an ACTB C_q value ≤23 and 3) a FAM19A4 $\Delta\Delta C_q$ value between 10.36 and 11.36, it is advised to repeat the PCR with 5 times less input of converted DNA by diluting the sample in water.

Troubleshooting

This troubleshooting guide may be helpful in solving any problems that may arise. If the problem persists or in case of questions or other information contact your local distributor or Self-screen at info@self-screen.nl.

Comments and suggestions	
Sample DNA concentration too low for bisulfite conversion	
Check DNA extract	Repeat DNA extraction with more concentrated clinical sample
Sample is scored invalid: the amplification of β-actin is too low or absent	
a) Pipetting error or omitted reagents	Check pipetting scheme and the reaction setup. Repeat the PCR run.
b) Check the DNA concentrate	Increase DNA input for bisulfite-conversion to a maximum. Bisulfite-conversion reaction has optimal performance for DNA input ranging from 100 ng–2 µg
c) For the protocol "Bisulfite-conversion directly on cervical specimen", check the clinical specimen for cellularity	Repeat the bisulfite-conversion reaction with 10% of the cervical specimen in PreservCyt collection medium (i.e. 2 ml from 20 ml).
d) Check the bisulfite-converted eluate	Repeat bisulfite conversion, when necessary a higher DNA input can be used.
Sample is scored invalid: the targets *FAM19A4* and/or *hsa-mir124-2* are invalid	
Insufficient mixing	Mix sample and reaction mix by pipetting (approximately 10 times per tube). Repeat sample.
Positive control is scored invalid: the amplification is too low or absent for one or more of the targets	
a) Pipetting error or omitted reagents	Check pipetting scheme and the reaction setup. Repeat the PCR run.
b) Partial degradation	Store kit contents at –30 to –15°C. Avoid repeated freezing and thawing to a maximum of three cycles.
c) PCR reagents partially degraded	Store kit contents at –30 to –15°C and keep the reaction mixes protected from light. Avoid repeated freezing and thawing.
d) Strip tube inversion	Check the pipetting scheme and the reaction setup.
e) Expiry date	Check the expiry date of the used kit.

Comments and suggestions	
f) Time-delay between pipetting samples and start of the run	PCR reactions mixes can be stored at 2–8°C for 30 minutes in the dark between dispensing samples into the PCR reactions and starting the run in the machine.
No template control (NTC) is invalid	
a) Pipetting error	Check pipetting scheme and the reaction setup. Repeat the PCR run.
b) Cross-contamination	Replace all critical reagents. Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carryover contamination.
c) Reagent contamination	Replace all critical reagents. Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carryover contamination.
d) Strip tube inversion	Check the pipetting scheme and the reaction setup.
e) Time-delay between pipetting samples and start of the run	PCR reactions mixes can be stored at 2–8°C for 30 minutes in the dark between dispensing samples into the PCR reactions and starting the run in the machine.
f) Probe degradation	Keep reaction mixes protected from light. Check for false positive on the fluorescence curve.
Absent or low signals in sample, but the control run ok	
a) Inhibitory effects	Always check there are no remains of buffer on the filter after centrifugation during bisulfite-conversion. Repeat bisulfite-conversion.
b) Pipetting error	Check pipetting scheme and the reaction setup. Repeat the PCR run.

Limitations

PreCursor-M+ reagents may exclusively be used for in vitro diagnostics.

Use of PCR tests requires good laboratory practices, including maintenance of equipment, that are dedicated to molecular biology and is compliant with applicable regulations and relevant standards.

The PreCursor-M+ is to be used by laboratory professionals trained in the use of the Mic qPCR cycler.

The product is to be used by personnel specially instructed and trained in the techniques of real-time PCR and in the *in vitro* diagnostic procedures only. Any diagnostic results that are generated must be interpreted in conjunction with other clinical or laboratory findings.

Strict compliance with the user manual (handbook) is required for optimal PCR results.

Do not use components beyond their expiration date.

Samples with low DNA quality/quantity (i.e., β-Actin Cq values just within acceptance criterion; Cq values from 25 to 26.4) could be scored false-negative. Repeat testing in single is advised. A negative result for the repeat test means that the sample is methylation-negative, a positive result means that the sample is methylation-positive.

All reagents supplied in the PreCursor-M+ are intended to be used solely with the other reagents supplied in the same kit. This may otherwise affect performance.

The PreCursor-M+ is validated for HPV-positive women.

The PreCursor-M+ is validated for cervical specimens collected and stored in PreservCyt, SurePath or STM collection medium and for self-collected vaginal brush specimens collected dry that have been stored in PreservCyt upon arrival in the laboratory.

Any off-label use of this product and/or modification of the components will void Self-screen B.V.'s liability.

It is the user's responsibility to validate system performance for any procedures used in their laboratory that are not covered by the Self-screen performance studies.

Performance Characteristics

Limit of Detection (LOD)*

The analytical sensitivity of the PreCursor-M+ was determined as the 95% limit of detection (95% LOD) by using a serial dilution series of gBlock (i.e. synthetic double-stranded genomic DNA blocks) dilution series containing all three amplicon sequences (i.e., β-actin, *FAM19A4*, and *hsa-mir124-2*; range 750,000 to 0.25 copies per PCR). The 95% LOD for the targets was assessed as the lowest plasmid dilution giving at least 35 out of 36 positive results (C_q<40). In total, 12 experiments were performed by four different operators (1 run per operator per day) using three different Lots and three different RGQ systems. Each experiment included triplicate testing of 11 plasmid dilutions. The 95% LOD was for all the three different targets 7.5 copies per PCR.

Linearity*

Linearity of the PreCursor-M+ was determined with the data from the 12 experiments that were performed to assess the 95% LOD. The two targets, *FAM19A4* and *hsa-mir124-2*, and the reference β-actin have linear amplification from 750,000 up to 7.5 copies per PCR.

Precision*

Precision of the PreCursor-M+ was determined as the intra-assay variability (variability of multiple results of samples with the same concentration within one experiment) and total variance of the assay (variability of multiple results of the assay generated by different operators, on different instruments, with different batches, in different laboratories). Testing was performed on bisulfite-converted DNA obtained from a high-risk HPV-positive cervical specimen that tested hypermethylation positive with signals for both *FAM19A4* and *hsa-mir124-2* corresponding to approximately 3 times the LOD concentration. Testing was performed in duplicate in 8 runs by four different operators (one run per operator per day) using two different Lots and three different Rotor-Gene Q MDx 5-plex instruments in two different laboratories, giving rise to 16 data points per sample. Coefficient of variation (CV) was determined for the Cq and ΔΔCq values (Table 3).

Table 3. CV% of Cq and ΔΔCq values in a methylation-positive cervical sample

	Sample type	Inter-assay variability	Total variance of the assay
Cq value	Internal sample quality control (i.e., β-=actin)	0.3%	1.32%
	FAM19A4	1.02%	1.52%
	hsa-mir124-2	1.16%	1.64%
ΔΔCq value	*FAM19A4*	3.70%	5.97%
	hsa-mir124-2	4.21%	5.75%

The overall statistical spread in Cq values of a sample with the mentioned concentration is 1.32% for the internal sample quality control (β-actin), 1.52% for *FAM19A4*, and 1.64% for *hsa-mir124-2*. The overall statistical spread in ΔΔCq values of a sample with the mentioned concentration is 5.97% for *FAM19A4* and 5.75% for *hsa-mir124-2*.

*) determined on Rotor-Gene® Q MDx 5plex instrument

Interfering substances*

Inhibitory substances selected for their potential effect on PCR were the desulfonation and wash buffer of the bisulfite-conversion kit. Substances potentially present in the original sample were not tested given the fact that sample DNA is purified twice with silica beads, i.e., DNA extraction from original sample and DNA clean-up after bisulfite-conversion. Traces of the desulfonation and wash buffer showed interference in the PCR, which was detected by an invalid test result for the internal sample quality control.

Clinical Performance*

HPV-positive physician-collected cervical specimens[2]

The clinical performance of the FAM19A4/miR124-2 methylation test for cervical intraepithelial neoplasia grade 3 (CIN 3) and cervical cancer (i.e., CIN 3+), was assessed in a EU-multicenter, retrospective study [13]. The FAM19A4/miR124-2 methylation test (e.g. PreCursor-M+) was evaluated in 2384 HPV-positive cervical screening samples of women (age 29-76 years) derived from four EU countries (Scotland, Slovenia, Denmark, The Netherlands) [13]. Specimens were collected in PreservCyt or SurePath media. The study population comprised 2012 women with no evidence of disease within 2 years of follow-up (abbreviated as ≤CIN1), 124 with CIN2, 228 with CIN3 and 20 with cancer. DNA was extracted from the cervical samples, and 250 ng DNA was used for input in the bisulfite-conversion reaction (EZ DNA Methylation Kit, ZYMO Research). Of the 250 ng modified DNA, 20% was used in the PCR (corresponding to 50 ng original target DNA/PCR). The overall FAM19A4/miR124-2 methylation test positivity rates stratified by clinical endpoint are given below (Table 4).

Table 4. Overall FAM19A4/miR124-2 methylation test positivity rates

Clinical endpoint	Fraction	Positivity rate (95% CI)
≤CIN 1	437/2012	21.7% (20.0– 23.6)
CIN 2	58/124	46.8% (38.1– 56.6)
CIN 3	176/228	77.2% (71.3– 82.2)
Cervical cancer	19/20	95.0% (70.7–99.3)

Overall specificity of FAM19A4/miR124-2 methylation test was 78.3% (n = 2013; 95%CI: 76-80). The negative predictive value of hrHPV positive, methylation-negative outcomes were 99.9% for cervical cancer (N = 1694; 95%CI: 99.6-99.99), 96.9% for ≥CIN3 (95%CI: 96-98), and 93.0% for ≥CIN2 (95%CI: 92-94). Overall sensitivity for CIN3 using FAM19A4/miR124-2 methylation test was 77.2% (n = 228; 95%CI: 71-82).

Additionally, the sensitivity for cervical cancer was evaluated in a multicenter worldwide retrospective study on 519 invasive cancers with different histotypes from over 25 countries (11). DNA was extracted from the cervical samples, and 250 ng DNA was used for input in the bisulfite-conversion reaction (EZ DNA Methylation Kit, ZYMO Research). Of the 250 ng modified DNA, 20% was used in the PCR (corresponding to 50 ng original target DNA/PCR). 510 out of the 519 cancers tested positive with the QIAsure Methylation

*) determined on Rotor-Gene® Q MDx 5plex instrument

test, yielding a positivity rate of 98.3% (95% CI: 96.7–99.2), the positivity rates stratified per histotype are given below in table 5.

Table 5.

Clinical endpoint	Fraction	Positivity rate (95% CI)
Squamous cell carcinoma	313/318	98.4% (96.4–99.5)
Adenocarcinoma	121/123	98.4% (94.2–99.8)
Adenosquamous cell carcinoma	42/42	100.0% (91.6 – 100)
Rare cancer histotypes	30/32	93.8% (79.2 – 99.2)
Cancer Histotype not specified	4/4	100% (39.8 – 100.0)

HPV-positive self-collected vaginal brush specimens[2]

The clinical performance of the FAM19A4/miR124-2 methylation test for self-collected vaginal brush specimens to detect cervical intraepithelial neoplasia grade 3 and cervical cancer (i.e., CIN 3+), was assessed by testing 247 high-risk HPV-positive vaginal specimens. For 14 samples (5.7%), the β-actin Cq values were >26.4 and subsequently scored invalid. The samples with valid test results comprised 148 self-collected brush samples of women with ≤CIN 1 after 18 months of follow-up, 24 with CIN 2, 50 with CIN 3, 8 with squamous cell carcinoma, and 3 with adenocarcinoma. DNA was extracted from the vaginal specimens, and 250 ng DNA was used for input in the bisulfite-conversion reaction (EZ DNA methylation kit, ZYMO Research). Of the 250 ng bisulfite-converted DNA, 20% was used in the PCR (corresponding to 50 ng original target DNA/PCR). The FAM19A4/miR124-2 methylation test positivity rates stratified by clinical endpoint are given below (Table 6).

Table 6. FAM19A4/miR124-2 methylation test positivity rates

Clinical endpoint	Fraction	Positivity rate (95% CI)
≤CIN 1	34/148	23.0% (16.9–30.4)
CIN 2	7/24	29.2% (14.6–49.8)
CIN 3	33/50	66.0% (52.0– 77.7)
Squamous cell carcinoma	8/8	100.0% (63.1–100.0)
Adenocarcinoma	3/3	100.0% (29.2–100.0)

Among high-risk HPV-positive self-collected vaginal brush specimens, the sensitivity for CIN 3+ is 72.1% (44/61; 95%CI: 59.7–81.9) and for carcinoma 100% (11/11; 95%CI: 72–100)[#]. [6]

Performance of *FAM19A4* and *hsa-mir124-2* to detect advanced transforming CIN lesions[*]

Host cell promoter methylation analysis specifically detects so-called "advanced" CIN lesions, which harbor a cancer-like methylation profile and have an expected high short-term risk of progression to cancer [7, 8, 15]. The performance of promoter hypermethylation analysis of *FAM19A4* and *hsa-mir124-2* was assessed by testing 29 high-risk HPV-positive specimens of women with advanced transforming CIN 2/3 and 19 high-risk HPV-positive specimens of women with early transforming CIN 2/3. Methylation was particularly associated with advanced disease, scoring all advanced CIN2/3 lesions (100%; 29/29; 95%CI: 88–100) hypermethylation–positive, compared with 47% (9/19; 95%CI: 27–69) of early CIN 2/3 lesions.

[#]) Remark: Hypermethylation of the targets in samples of women harboring advanced CIN lesion and/or cervical cancer might remain undetected due to sampling variability, for example as a result of inadequate sampling.

Robustness*

Robustness of the FAM19A4/miR124-2 methylation test was determined as the agreement between the output of the FAM19A4/miR124-2 methylation test and that of a Research Use Only (RUO) version of the assay. Testing was performed on bisulfite-converted genomic DNA obtained from 10 high-risk HPV-positive cervical samples, 5 of which were previously identified as hypermethylation-negative for both markers and 5 as methylation-positive (e.g., for at least one of the 2 markers). Testing was performed in duplicate in 8 runs by four different operators (one run per operator per day) using two different Lots and three different Rotor-Gene Q MDx instruments, performed in two different laboratories. In total, 16 data points were obtained for each sample (Table 7).

Table 7. Agreement of FAM19A4/miR124-2 methylation test vs. RUO version of the assay

Sample number	RUO result	Agreement Lab 1 compared to RUO	Agreement Lab 2 compared to RUO
1	Neg	100% (8/8)	100% (8/8)
2	Neg	100% (8/8)	100% (8/8)
3	Neg	62.5% (5/8)	62.5% (5/8)
4	Neg	100% (8/8)	100% (8/8)
5	Neg	100% (8/8)	100% (8/8)
Subtotal		92.5% (37/40)	92.5% (37/40)
6	Pos	100% (8/8)	100% (8/8)
7	Pos	100% (8/8)	100% (8/8)
8	Pos	100% (8/8)	100% (8/8)
9	Pos	100% (8/8)	100% (8/8)
10	Pos	100% (8/8)	100% (8/8)
Subtotal		100% (40/40)	100% (40/40)
Total (positive and negative)		96.25% (77/80)	96.25% (77/80)

Four of the five samples previously identified as methylation-negative demonstrated 100% agreement when using the FAM19A4/miR124-2 methylation test in both laboratories. Sample 3 displayed an agreement of 62.5% (5/8) in both laboratories. Observed variation related to *FAM19A4* with levels around the assay cutoff. Overall agreement among the methylation-negative samples was 92.5% (37/40).

All 5 samples previously identified as methylation-positive demonstrated 100% agreement with the reference assay, thus overall agreement was 100% (40/40).

Bisulfite-conversion directly on cervical specimens*

The protocol "Bisulfite-conversion directly on cervical specimens using the EpiTect Fast 96 Bisulfite Kit" was verified against the reference protocol (i.e. bisulfite-conversion with prior sample DNA quantity control) on 119 cervical scrapes followed by the FAM19A4/miR124-2 methylation test. The success rate for bisulfite-conversion directly on cervical samples using 2.5% cervical specimen input was 95.8% (114/119) and increased to 100% after retesting the invalids with 10% cervical specimen input. The agreement in FAM19A4/miR124-2 methylation test result between the bisulfite-conversion protocols was 90.8% (108/119; kappa value 0.75).

*) determined on Rotor-Gene® Q MDx 5plex instrument

The protocol “Bisulfite-conversion directly on cervical specimens using the QIAsymphony Bisulfite Kit” was verified against the reference protocol (i.e. bisulfite-conversion with prior sample DNA quantity control) on 120 cervical scrapes followed by the FAM19A4/miR124-2 methylation test. The success rate for bisulfite-conversion directly on cervical samples using 2.5% cervical specimen input was 94.2% (113/120) compared to 97.5% (117/120) with the reference protocol. The agreement in FAM19A4/miR124-2 methylation test result between the bisulfite-conversion protocols was 94.7% (107/113; kappa value 0.88).

Equivalence performance study between MIC qPCR and Rotor-Gene Q MDx 5-plex cyclers

The performance of the PreCursor-M+ on the Mic qPCR cycler was compared to the performance on the Rotor-Gene Q MDx 5-plex HRM (RGQ). The limit of detection (LOD) was determined on serial dilution series of gBlock dilution series containing all three amplicon sequences (i.e., β-actin, *FAM19A4*, and *hsa-mir124-2*; range 750,000 to 0.25 copies per PCR). In total 6 experiments were performed on 3 different days on 2 different Mic cyclers resulting in 10 measurements per dilution. The LOD on the Mic qPCR cycler for the three targets was highly comparable to that of the RGQ being equal or at maximum one dilution step (~3 fold) higher. The performance on clinical samples was determined on bisulfite-converted DNA of 100 cervical scrapes. The success rate of the PreCursor-M+ on the Mic qPCR cycler was 97% (97/100) and on the RGQ 96% (96/100). The agreement in PreCursor-M+ methylation test result between the cyclers was 95.8% (92/96; kappa value 0.90).

References

1. Costello, J.F., and Plass, C. (2001) Methylation matters. J. Med. Genet. 38, 285–303.
2. Wilting, S.M., et al. (2010) Methylation-mediated silencing and tumour suppressive function of *hsa-mir124* in cervical cancer. Mol. Cancer 9, 167.
3. De Strooper, L.M., et al., (2014) Methylation analysis of the *FAM19A4* gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. Cancer Prev. Res. 7, 1251–7.
4. De Strooper, L.M., et al. (2014) CADM1, MAL and *mir124-2* methylation analysis in cervical scrapes to detect cervical and endometrial cancer. J. Clin. Pathol. 67, 1067–71.
5. De Strooper, L.M., et al. (2016) Comparing the performance of *FAM19A4* methylation analysis, cytology and HPV 16/18 genotyping for the detection of cervical (pre)cancer in high-risk HPV-positive women of a gynecologic outpatient population (COMETH study). Int. J. Cancer 138, 992–1002.
6. De Strooper, L.M., et al. (2016) Validation of the *FAM19A4*/*mir124-2* DNA methylation test for both lavage- and brush-based self-samples to detect cervical (pre)cancer in HPV-positive women. Gynecol. Oncol. 141, 341–7.
7. Bierkens, M. et al. (2013) CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease. Int. J. Cancer 133, 1293–9.
8. Steenbergen, R.D.M. et al. (2014) Clinical implications of (epi)genetic changes in HPV-induced precancerous lesions. Nat. Rev. Cancer 14, 395–405.
9. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–8.
10. De Strooper, L.M. et al. (2018) Cervical cancer risk in HPV-positive women after a negative FAM19A4/miR124-2 methylation test: A post hoc analysis in the POBASCAM trial with 14 year follow-up. Int. J. Cancer 143, 1541-1548.
11. Vink, F.J. et al. (2020). FAM19A4/miR124-2 methylation in invasive cervical cancer: A retrospective cross-sectional worldwide study. Int J Cancer. 147, 1215-1221.
12. Kremer, W.W. et al. (2022). Clinical Regression of High-Grade Cervical Intraepithelial Neoplasia Is Associated With Absence of FAM19A4/miR124-2 DNA Methylation (CONCERVE Study). J. Clin. Oncol. doi: 10.1200/JCO.21.02433.
13. Bonde, J. et al. (2020). Methylation markers FAM19A4 and miR124-2 as triage strategy for primary human papillomavirus screen positive women: A large European multicenter study. Int J Cancer. 1-1014.
14. Vink et al, IJC , 2021 - Classification of high-grade cervical intraepithelial neoplasia by p16ink4a, Ki-67, HPV E4 and FAM19A4/miR124-2 methylation status demonstrates considerable heterogeneity with potential consequences for management

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Use by
	In vitro diagnostic medical device
	CE-IVD marked symbol
	Contains reagents sufficient for <N> reactions
	Catalog number
	Lot number
	Material number
	Components
	Contains
	Number
Rn	R is for revision of the Instructions for Use (Handbook) and n is the revision number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Protect from light
	Consult instructions for use
	Caution

Contact information

For technical assistance and more information, please contact Self-screen or our local distributors.

Manufacturer: Self-screen B.V., Plesmanlaan 125, 1066CX, Amsterdam, The Netherlands.
info@self-screen.nl

Distributor: Fujirebio Europe N.V. , Technologiepark 6, 9052 Gent, Belgium.
Tel. +32 9 329 13 29; customer.support@fujirebio-europe.com

Revision History

Revision	Changes
1	First version
2	Changed to new address Self-screen B.V.. Added protocol and performance data of EZ DNA Methylation Lightning Kit (ZYMO D5030) and QIAsymphony Bisulfite kit; Sample preparation section was updated for handling of PreservCyt and SurePath samples; Added the protocol of SurePath collection medium; Updated clinical performance section.
3	Typo's in reference to other assays have been corrected.