September 2023

PreCursor-M Gold

Instructions For Use

For the assessment of hypermethylation of the genes

LHX8 and *ASCL1*

FOR RESEARCH USE ONLY (RUO)

NOT FOR USE IN DIAGNOSTIC PROCEDURES

Self-screen B.V., Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

REF QHR14100

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Contents

Intended Use

FOR RESEARCH USE ONLY (RUO). The result shall not be used for clinical diagnosis nor patient management. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease.
PreCursor-M Gold is a multiplex real-time methylation-specific PCR assay for the detection of promoter hypermethylation of the human genes *LHX8* and *ASCL1* in bisulfite-converted DNA prepared from human sample material.

This product is developed to be used on Rotor-Gene Q 5plex instruments from Qiagen (i.e. MDx, 5plex and 5plex HRM).

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, promoter hypermethylation of the genes *LHX8* (*LIM Homeobox 8*) and/or *ASCL1 (Achaete-Scute Family BHLH Transcription Factor 1)* has been detected [2-6]. Host-cell promoter methylation is specifically present in 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 and can be detected in different sampling types [3, 7, 8, 10, 11, 12, 14, 15 16]. The PreCursor-M Gold assay allows the detection of promoter hypermethylation of the genes *LHX8* and *ASCL1* on bisulfite-converted DNA prepared from human sample material using *ACTB* (human *β-actin* gene) as an internal sample quality control.

PreCursor-M Gold uses primers and probes specific for methylated DNA of the two human target genes *LHX8* and *ASCL1*. The sample reference is a methylation-unspecific PCR for the gene beta actine (*ACTB*) and is used for normalization and quality control. 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 C_T value (cycle threshold) 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 ΔΔCt values of the target genes *LHX8* and *ASCL1.* First the ΔCt value is calculated by deducting the Ct value of the sample reference gene *ACTB* from the Ct value of the sample target gene. For normalization, the ΔCt value of the calibrator is deducted from the ΔCt of the sample target genes resulting in a ΔΔCt value. The calibrator contains a standard concentration of low-copy DNA fragments of the three targets of the assay, *LHX8* and *ASCL1*, and *ACTB*.

Materials Provided

	Volume per tube (at least)
PreCursor-M Gold Master Mix	1080 µl
PreCursor-M Gold Calibrator	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 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)

Consumables and reagents for the Rotor-Gene Q 5 plex

- Strip tubes and Caps, 0.1 ml (QIAGEN, cat.no. 981103)
- 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

Equipment for real-time PCR

- Rotor-Gene Q 5plex HRM System (cat. no. 9002033 or 9002032)[1]

1 Rotor-Gene Q 5plex HRM instrument with a production date of January 2010 or later. The production date can be obtained from the serial number on the back of the instrument. The serial number is in the format “mmyynnn“ where “mm“ indicates the production month in digits, “yy“ indicates the last two digits of the production year, and “nnn“ indicates the unique instrument identifier.

Warnings and Precautions

For in Research 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 Gold 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 Gold are optimally diluted. Do not dilute reagents further as this may result in a loss of performance.
- All reagents supplied in the PreCursor-M Gold 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 Gold kit, even from the same batch, as this may affect performance.
- Refer to the Rotor-Gene Q 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 Gold is shipped on dry ice. 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 Gold 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 by placing them at room temperature for 15 minutes. Mix by gently vortexing and centrifuge the tubes before opening.

Sample preparation and handling

The PreCursor-M Gold PCR is for use with bisulfite-converted genomic DNA samples obtained from human specimens, for instance cervical specimens and self-collected vaginal brush specimens. Validated collection media for cervical specimens (scrapes) is PreservCyt® collection medium. Optimal storage temperature of the clinical specimens is 2–8°C upon arrival at the lab. Under these storage conditions, specimens in PreservCyt collection medium are stable for 3 months prior to DNA extraction. 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 Gold is compatible with bisulfite-converted genomic DNA derived from human specimens, for instance cervical specimens. Our recommendations are outlined below.

The 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 Gold (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 depending on the cellularity of the sample.
  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%).

General recommendations for bisulfite-conversion

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

The input in the PreCursor-M Gold reaction is 5 µL of bisulfite-converted DNA.

If the internal sample quality control is negative (i.e., ACTB Ct values are >28), the specimen bisulfite-converted DNA preparation resulted in material of insufficient quantity and/or quality and is considered invalid. To reach an ACTB Ct that is within the valid range repeat bisulfite-conversion reaction with a higher input of specimen DNA and/or repeat DNA isolation with a higher input of specimen.

Bisulfite-converted DNA can be stored up to 24 hours at 2–8°C and up to 3 months at –15°C to -70°C. 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.

Procedure for the Rotor-Gene Q PCR intrument

Important points before starting

- Take time to familiarize yourself with the Rotor-Gene Q 5plex instrument before starting the protocol. See the instrument user manual.
- Before the first run of the day, perform a warm-up run for Rotor-Gene Q 5plex instrument at 95°C for 10 minutes.

qPCR Reaction Setup

Up to 70 bisulfite-converted DNA samples can be tested within the same run (experiment), besides a calibrator and no template control.

1. Thaw the PreCursor-M Gold master mix and calibrator completely.
2. Briefly vortex and spin down prior to pipetting.
3. Dispense 15 µL of ready-to-use PreCursor-M Gold master mix into the appropriate tubes of the tube-strips (at maximum 72 tubes per Rotor-Gene-Q run). Reaction setup can be done at room temperature.
4. Add 5 µL of bisulfite-converted DNA to the appropriate tubes containing the Precursor-M Gold master mix.
5. Add 5 µL of water for the No Template Control (NTC) reaction to tube position 2.
6. Add 5 µL of Precursor-M Gold Calibrator to tube position 1.
7. Close the tubes by pressing the caps on the tubes.
8. Place tube strips into 72-well Rotor, empty spaces should be filled with empty or dummy tube strips with caps as counterweight.
9. Lock the tubes by attaching the locking ring and place the Rotor in the Rotor-Gene-Q machine.
10. Close the lid of the Rotor-Gene-Q machine.

Rotor-Gene Q PCR machine set up

Before the first run of the day, perform a warm-up run for the Rotor-Gene Q PCR machine at 95°C for 10 minutes.

11. Start the Rotor-Gene-Q Series Software.
12. Select Rotor type: '72-well rotor' and 'Locking ring attached' and click next-button.
13. At operator, enter initials, click next button.
14. At volume enter 20 µL, and click next-button.
15. Click 'Edit profile'-button and define the real-time PCR cycling conditions as follows:

Temperature	Time	Cycles	Acquiring Fluorescence
95 °C	5 minutes	1	No
95 °C	15 seconds	40	No
61 °C	60 seconds		Yes*

*Acquiring to cycling A on Green, Orange, and Yellow
Green= LHX8; Orange=β-actin; Yellow=ASCL1

16. Click OK and then click 'Gain optimization'.
17. Click 'Optimise Acquiring'. Check tube position is set to position 1 for all dyes (e.g. Gain optimization must be performed on the Calibrator reaction tube).
18. Check the box for 'Perform optimisation before 1st acquisition'.
19. Click 'Close' followed by 'Next'.
20. Click 'Start run'
    Optional: Click "Save Template" to add the run template to the list.
21. Click 'Edit samples' to enter sample names etc. (this can also be done after the run is completed).

Interpretation of results

Acquiring Ct values

22. After the run is completed go to 'Analysis tool window', select 'Cycling A. Green', and click 'OK', then select 'Cycling A. Orange', and click 'OK' , and finally select 'Cycling A. Yellow', and click 'OK'
23. Enter the analysis settings for the three dyes as follows:

Color	Target	Threshold	Slope correction	Take off adjustment
Orange	ACTB	0,03	Yes	12 to 40
Green	LHX8	0,03	Yes	10 to 40
Yellow	ASCL1	0,04	Yes	10 to 40

24. Select the wells to be used for analysis.
25. Check the fluorescence data for each reaction for each dye for typical amplification curves (logarithmic increase of fluorescence). If the baseline does not provide satisfactory results, the sample can be repeated and interpret the results accordingly.
26. Save the run as an 'Excel analysis sheet' (located under 'File' in toolbar) to export the results as an Excel CSV file.

Validation criteria for successful analysis

27. The PCR run is valid when it complies with below criteria

	ACTB Ct	LHX8 Ct	ASCL1 Ct
NTC	>40 (no Ct value)	>40 (no Ct value)	>40 (no Ct value)
Calibrator	26 - 30	26 - 30	26 – 30

28. The Ct value of *ACTB* of a sample should be ≤ 28 for a valid result.

Calculating ΔΔCt values

29. Use the below formula to calculate ΔΔCt values of the target genes *LHX8* and *ASCL1.* Lower ΔΔCt values correspond with higher level of methylation of the target gene.

$$\Delta\Delta Ct_{\text{target gene}} = (Ct_{\text{target gene sample}} - Ct_{\textit{ACTB}\text{ sample}}) - (Ct_{\text{target gene calibrator}} - Ct_{\textit{ACTB}\text{ calibrator}})$$

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) Check the bisulfite-converted eluate	Repeat bisulfite conversion, when necessary a higher DNA input can be used.
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.
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) 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	

Comments and suggestions	
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 Gold reagents are for Research Use Only.

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 Gold is to be used by laboratory professionals trained in the use of the Rotor-Gene Q cycler.

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

Do not use components beyond their expiration date.

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

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.

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Use by
 <N>	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

Revision History

Revision	Changes
1	First version
2	Changed to new address Self-screen B.V.; updated Materials required but not provided section; added verified bisulfite-conversion kits; Sample preparation section was updated; updated Limitations section; added reference section.