January 2024

QIAsure Methylation Test Instructions for Use (Handbook)

Σ 72

For the detection of promoter methylation of the *FAM19A4* and *hsa-mir124-2* genes

Version 1

For Research Use Only (RUO). Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease.

For use with Rotor-Gene® Q 5plex HRM instrument

REF 616015

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

R1 MAT 1134057EN

Sample to Insight

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Intended Use

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The QIAsure Methylation Test 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 QIAsure Methylation Test include bisulfite-converted DNA isolated from specimens collected in the following ways:

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

Intended User

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 Rotor-Gene® Q MDx 5plex HRM system.

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. 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 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 (3, 7, 8, 10, 11, 12, 14). The QIAsure Methylation Test allows the detection of promoter hypermethylation of the genes *FAM19A4* and *hsa-mir124-2* on bisulfite-converted DNA isolated from human genomic DNA using ACTB (human β-actin gene) as an internal sample quality control.

Principle of the Procedure

The QIAsure Methylation Test 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 a reference gene. The kit contains 2 tubes of the QIAsure Master Mix and 2 tubes of the QIAsure Calibrator. The master mix is intended for amplification of bisulfite-converted human genomic DNA. The master mix contains the primers and probes for the target genes and the reference gene, which serves as the internal sample quality control. The calibrator is a linearized plasmid containing sequences of the *FAM19A4*, *hsa-mir124-2*, and ACTB amplicons.

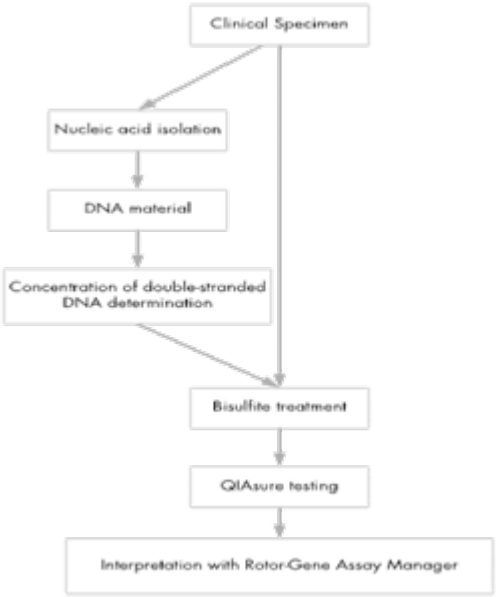


Figure 1. Workflow procedure

The QIAsure assay runs on Rotor-Gene Q MDx instrument and the Rotor-Gene AssayManager® software automatically performs data analysis and interpretation. 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. The QIAsure assay calculates the ΔC_T value as the difference between the C_T value of the *FAM19A4* or *hsa-mir124-2* targets and the C_T value of the reference (ACTB). This ΔC_T is a relative quantitative value for the promoter methylation level of the *FAM19A4* or *hsa-mir124-2* gene. For normalization, the ΔC_T value of a calibrator sample is subtracted from the ΔC_T of the *FAM19A4* or *hsa-mir124-2* targets resulting in a $\Delta\Delta C_T$ value (9). The calibrator is a standardized low-copy plasmid DNA sample with known copy number of the three targets (i.e., *FAM19A4*, *hsa-mir124-2*, and ACTB).

Materials Provided

Kit contents

QIAsure Methylation Test		**72**
Catalog no.		**616015**
Number of reactions		**72**
QIAsure Master Mix (2 tubes)	Brown color tube	
QIAsure Calibrator (2 tubes)	Transparent color tube	
QIAsure Methylation Test Instructions for Use		1

Materials Required but Not Provided

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). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Consumables and reagents for sample preparation for self-collected samples

- Hologic PreservCyt® Solution

Reagents for preparation of samples stored in SurePath™ collection medium

- Buffer AL (QIAGEN catalog 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-Lighting (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 for the Rotor-Gene Q instrument

- Strip tubes and Caps, 0.1 mL (cat. no. 981103)
- Purified water (e.g. molecular biology grade, distilled or deionized)

Equipment

- Adjustable pipets* dedicated for PCR (1–10 µL; 10–100 µL)
- 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* like QIAxpert (QIAGEN, cat. no. 90022340)*

Equipment for real-time PCR

- Rotor-Gene Q MDx 5plex HRM System (cat. no. 9002033) or Rotor-Gene Q MDx 5plex HRM instrument (cat. no. 9002032)†
- Rotor-Gene Assay Manager Core Application software version 1.0.x (where x is greater than or equal to 4)
- Rotor-Gene AssayManager Epsilon Plug-in installed, version 1.0.x (where x is greater than or equal to 1)
- QIAsure Assay Profile (from file AP_QIAsure_RUO_V1_0_Y.iap) (where Y is equal to or bigger than 1) for application on bisulfite-converted DNA

*Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.
†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 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). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

QIAsure 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.

General precautions

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.

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.
- Avoid DNA or PCR product carryover contamination, which could result in a false-positive signal.
- Always use DNase-free disposable pipet tips with aerosol barriers.
- Reagents of QIAsure assay are optimally diluted. Do not dilute reagents further as this may result in a loss of performance.
- All reagents supplied in the QIAsure 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 QIAsure kit, even from the same batch, as this may affect performance.
- Refer to the Rotor-Gene Q MDx instrument user manual for additional warnings, precautions, and procedures.
- Before the first run of the day, perform a warm-up run for the Rotor-Gene Q MDx 5plex HRM at 95°C for 10 minutes.
- 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.
- Use extreme caution to prevent contamination of the mixes with the synthetic materials that are contained in the PCR reagents.
- Discard sample and assay waste according to your local safety procedures.

Reagent Storage and Handling

Shipping conditions

The QIAsure Methylation Test is shipped on dry ice. If any component of the QIAsure Methylation Test is not frozen upon arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, handbooks, or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**.

Storage conditions

The QIAsure Methylation Test must be stored immediately at –30 to –15°C upon receipt in a constant-temperature freezer and protected from light.

Stability

When stored under the specified storage conditions, the QIAsure Methylation Test is stable until the stated expiration date on 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.

- Gently mix by inverting the tube 10 times and centrifuge all tubes before opening.
- Expiration dates for each reagent are indicated on the individual component labels. Under correct storage conditions, the product will maintain performance for the stability time as long as the same batches of components are used.

Specimen Handling and Storage

All specimens must be treated as potentially infectious material.

Cervical specimens

The QIAsure Methylation Test is for use with bisulfite-converted genomic DNA samples obtained from genomic DNA specimens, for instance cervical specimens and self-collected vaginal brush 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, specimens PreservCyt or SurePath collection medium are stable for 3 months prior to DNA extraction.

Note: Cervical samples in STM may be shipped at 2–30°C for overnight delivery to the testing laboratory, and refrozen at –20°C upon receipt.

Self-collected vaginal brush specimens

The QIAsure Methylation Test can be used for bisulfite-converted genomic DNA samples obtained 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 to –15°C for up to 12 months.

Sample Preparation for Samples Stored in PreservCyt

The QIAsure Methylation Test has been validated for use with bisulfite-converted genomic DNA, for instance obtained from cervical specimens. Bisulfite-conversion of genomic DNA can be performed

i. with prior sample DNA extraction and DNA quality control, or

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.

- **Bisulfite-conversion with prior DNA extraction and DNA quality 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 methods:

  - DNA extraction

    Standard DNA extraction kits (e.g., column-based and magnetic bead-based kits) are compatible with the QIAsure Methylation Test.

  - 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 like QIAxpert (QIAGEN).

- Aliquoting DNA eluate

  Optimal DNA input for bisulfite-conversion ranges from 100 ng to 2 µg, with 200 ng recommended for the bisulfite-conversion. 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 EZ DNA Methylation-Lightning Kit and is performed according to the manufacturer's recommendation.

  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%).

- **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 from QIAGEN. We refer to *Epitect® Fast 96 Bisulfite Conversion Handbook* 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% of the cervical specimen in PreservCyt® collection medium (i.e. 500 µl from 20 ml) and pellet by centrifugation for 10 minutes at minimal 3390 x g. Discard the supernatant leaving the cell pellet at a maximum 20 µl 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.

- **Bisulfite-conversion directly on direct 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% of the cervical specimen in PreservCyt collection medium (i.e. 500 µL 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

Specimens stored in SurePath need to be pretreated before DNA extraction. The QIAsure Methylation Test has been validated for use with bisulfite-converted genomic DNA from these specimens according to the procedure outlined below.

- Take 2.5% of the cervical sample
- 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 like QIAxpert (QIAGEN).
- 200 ng DNA is recommended for bisulfite conversion, if 200 ng is not available use the maximum input volume. Perform the conversion with the EZ DNA Methylation Kit or EZ DNA Methylation-Lightning Kit (Zymo) according manufacturer's instruction. Use 2.5 µL eluate for the QIAsure Methylation Test.

General recommendations for bisulfite-conversion

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

The input in the QIAsure reaction is 2.5 µL of bisulfite-converted DNA.

If the internal sample quality control is negative (i.e., ACTB C_T values are >30), 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 ACTB C_T 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 specimen 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%* 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, up to 5 days at –25 to –15°C, and up to 3 months below –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.

*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.

Protocol: QIAsure Methylation Test PCR in the Rotor-Gene Q 5plex HRM instrument

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 5-plex HRM at 95°C for 10 minutes.
- Rotor-Gene AssayManager v1.0 enables automated interpretation of the PCR results. The QIAsure kit must be run on the Rotor-Gene Q MDx instrument using the Rotor-Gene AssayManager v1.0. Take time to familiarize yourself with the Rotor-Gene AssayManager v1.0 (cat. no. 9022739), and Epsilon Plug-In, and refer to the user manuals for both.
- The Rotor-Gene AssayManager v1.0 Assay Profile to be used is:
    - QIAsure RUO Assay Profile (from file AP_QIAsure_RUO_V1_0_Y.iap

Things to do before starting

- Rotor-Gene AssayManager software version v1.0.x (where x is greater than or equal to 4) must be installed on the computer connected to the Rotor-Gene Q. For details about the installation of the Rotor-Gene AssayManager v1.0 Core Application software, refer to *Rotor-Gene AssayManager v1.0 Core Application User Manual*.

*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.

- The QIAsure Methylation Test requires a specific plug-in, named the "Epsilon Plug-in" (version 1.0.1 or higher). This plug-in can be downloaded from the QIAGEN web page: **www.qiagen.com/rotor-gene-assaymanager/resources**. This plug-in must be installed on a computer that already Rotor-Gene AssayManager v1.0.x (where x is greater than or equal to 4) installed.
- The QIAsure Methylation Test requires an assay specific profile to run with the Rotor-Gene AssayManager v1.0 software. This Assay Profile contains all parameters needed for cycling and analyzing the experiment. The QIAsure Assay Profile is:
    - QIAsure RUO Assay Profile (from file AP_QIAsure_RUO_V1_0_Y.iap). The profiles can be downloaded from the QIAsure Methylation Test web page: **www.qiagen.com/shop/qiasure-methylation-test-kit-eu**. The Assay Profile needs to be imported in Rotor-Gene AssayManager software.

      Note: The QIAsure Methylation Test can only run if certain configuration settings in the Rotor-Gene AssayManager v1.0 are programmed.

For system-wide process safety, the following required configuration settings must be set for the closed mode:

- Material number required
- Valid expiry date required
- Lot number required

Installation of Epsilon Plug-in and importing assay profile

The installation and importing of the Epsilon Plug-in and the assay profile are detailed in the *Rotor-Gene AssayManager Core Application User Manual* and the *Epsilon Plug-In User Manual*.

- Download both the Epsilon Plug-in and the latest version of the QIAsure assay profile from the QIAGEN website.
- To start the installation process, double-click **EpsilonPlugin.Installation.msi**, then follow the installation instructions. For a detailed description of the process, refer to the section **Installing Plugins** in the *AssayManager Core Application User Manual*.

  Note: For system-wide process safety, select the Settings tab and ensure that the boxes for **Material number required**, **Valid expiry date required**, and **Lot number required** are checked for the closed mode (section Work list). If these are not enabled, check the boxes to enable.
- After successful installation of the plugin, a person with administrator rights for the Rotor Gene AssayManager software will need to import the AP_QIAsure_V1_0_Y.iap assay profile as follows.

1. Click the icon  to open the Rotor-Gene AssayManager software.

   The Rotor-Gene AssayManager login window opens (see Figure 2 below).

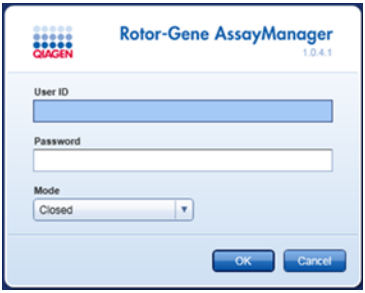
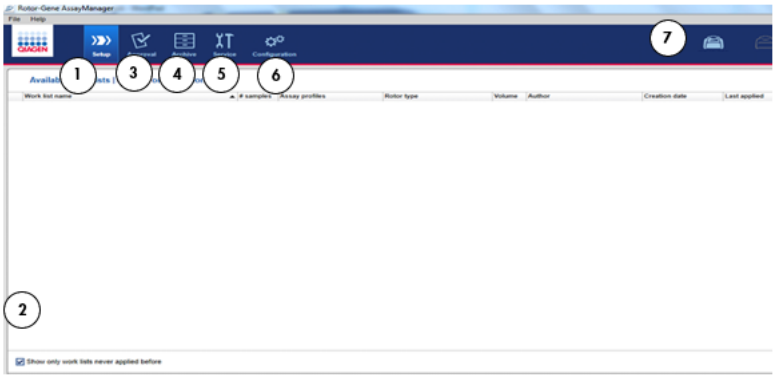


Figure 2. Rotor-Gene AssayManager login screen.

2. Login to Rotor-Gene AssayManager using your user ID and password. Do not change the Closed mode. Click **OK**.

   The Rotor-Gene AssayManager screen opens.



1 - Setup tab. This tab enables you to manage and apply work lists.

2 - Checking applied work lists shows new work lists only

3 - Approval tab. This tab enables you to find previous experiments (runs).

4 - Archive tab. This tab enables you to find old experiments (runs) that were already approved.

5 - Service tab. This tab shows a report of an audit trail of each file generated by the software.

6 - Configuration tab. This tab enables configuration of all software parameters.

7 - Rotor-Gene Q icons:

 Not connected

 Connected

3. Select the configuration environment.
4. Select the Assay Profiles tab.
5. Click **Import**.

6. Select the **AP_QIAsure_RUO_V1_0_Y.iap** to be imported in the dialog box, then click **Open**.
7. After the assay profile is succesfully imported, it can be used in the Setup environment.

   Note: The same version of an assay profile cannot be imported twice.

Sample processing on Rotor-Gene Q MDx instruments with 72-rotor

Up to 70 bisulfite-converted DNA samples can be tested within the same run (experiment), besides a calibrator and no template control. The schematic in Table 1 below provides an example of the loading block or rotor setup for a run with the QIAsure Methylation Test. Numbers denote positions in the loading block and indicate final rotor position.

Table 1. Plate and rotor setup for a run with the QIAsure kit on Rotor-Gene Q instrument

Strip	Tube position	Sample name	Strip	Tube position	Sample name	Strip	Tube position	Sample name
1	1	Calibrator	7	25	Sample 23	13	49	Sample 47
	2	NTC		26	Sample 24		50	Sample 48
	3	Sample 1		27	Sample 25		51	Sample 49
	4	Sample 2		28	Sample 26		52	Sample 50
2	5	Sample 3	8	29	Sample 27	14	53	Sample 51
	6	Sample 4		30	Sample 28		54	Sample 52
	7	Sample 5		31	Sample 29		55	Sample 53
	8	Sample 6		32	Sample 30		56	Sample 54
3	9	Sample 7	9	33	Sample 31	15	57	Sample 55
	10	Sample 8		34	Sample 32		58	Sample 56
	11	Sample 9		35	Sample 33		59	Sample 57
	12	Sample 10		36	Sample 34		60	Sample 58

Table 1. Plate and rotor setup for a run with the QIAsure kit on Rotor-Gene Q instrument (continued)

Strip	Tube position	Sample name	Strip	Tube position	Sample name	Strip	Tube position	Sample name
4	13	Sample 11	10	37	Sample 35	16	61	Sample 59
	14	Sample 12		38	Sample 36		62	Sample 60
	15	Sample 13		39	Sample 37		63	Sample 61
	16	Sample 14		40	Sample 38		64	Sample 62
5	17	Sample 15	11	41	Sample 39	17	65	Sample 63
	18	Sample 16		42	Sample 40		66	Sample 64
	19	Sample 17		43	Sample 41		67	Sample 65
	20	Sample 18		44	Sample 42		68	Sample 66
6	21	Sample 19	12	45	Sample 43	18	69	Sample 67
	22	Sample 20		46	Sample 44		70	Sample 68
	23	Sample 21		47	Sample 45		71	Sample 69
	24	Sample 22		48	Sample 46		72	Sample 70

⚠ Tubes must be inserted into the rotor as indicated in Table 1 on the previous page. The automated analysis set in the Assay Profile is based on this organization.

If a different layout is used, aberrant results will be obtained.

Note: Fill all unused positions with empty tubes.

PCR on Rotor-Gene Q instruments with 72-tube rotor

Before the first run of the day, perform a warm-up run for Rotor-Gene Q MDx 5plex HRM at 95°C for 10 minutes.

1. Create a work list for the sample to be processed, as follows:
    a. Power ON the Rotor-Gene Q MDx 5plex HRM instrument.
    b. Open the Rotor-Gene AssayManager and log in as a user with an Operator role in closed mode.
    c. Click **New work list** in the work list manager (Setup environment).
    d. Select the QIAsure assay profile **AP_QIAsure_RUO_V1_0_Y.iap** from the list of available assay profiles.
    e. Click **Move** to transfer the applicable assay profile to the **Selected assay profiles** list.

       The assay profile is now displayed in the Selected assay profiles list.
    f. Enter the number of samples in the corresponding field.
    g. Enter the following QIAsure kit information, which is printed on the lid of the box:
        - **Material number**: 1133306
        - **Valid expiry date using this format**: YYYY-MM-DD
        - **Lot number**
    h. Select the **Samples** step.

       A list with the sample details is shown on the AssayManager screen. This list represents the expected layout of the rotor.
    i. Enter the sample identification number in the list. You can enter any optional sample information as a comment for each sample.
    j. Select the **Properties** step and enter a work list name in the **Work list name** field (see Figure 3 on the facing page).

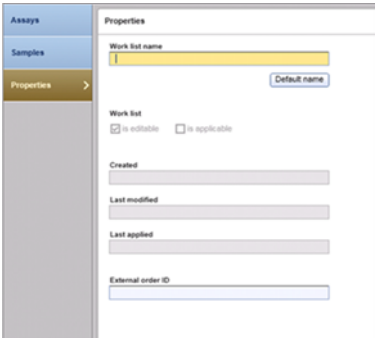


Figure 3. Properties.

k. Check the **is applicable** box and click **Apply.**

l. Save the work list.

The work list can be printed, which may help with the preparation and setup of the PCR. To print the work list, click **Print work list**. The sample details are included as part of this work list.

Note: The work list can be created once the run is set in the instrument, or the work list can be saved before adding the samples into the instrument.

2. Set up the QIAsure run.

- To minimize the risk for PCR reaction contamination, it is strongly recommended that a PCR-cabinet with UV-irradiation capability is used.
- Dispensing of the QIAsure 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.

Note: Change tips between each tube to avoid any nonspecific template or reaction mix contamination which may lead to false-positive results.

a. Thaw the QIAsure Master Mix and QIAsure Calibrator completely, and protect the QIAsure Master Mix from light whenever possible.

   Note: Do not exceed 30 minutes for the thawing step, to avoid any material degradation.

b. Mix gently by inverting 10 times, then briefly centrifuge before use.

c. Dispense 17.5 µL of the ready-to-use QIAsure Master Mix into the appropriate strip tubes. Reaction setup may be done at room temperature.

d. Return the QIAsure Master Mix to the freezer to avoid any material degradation.

e. Transport tubes to separate area to dispense the assay controls and bisulfite-converted samples.

f. Add 2.5 µL of water to the no template control (NTC) to position 2 (see Table 1 on page 24 above). Mix gently by pipetting up and down.

g. Add 2.5 µL of QIAsure Calibrator to position 1 (see Table 1 on page 24 above). Mix gently by pipetting up and down and close the tube with a cap.

h. Add 2.5 µL of bisulfite-converted DNA to the corresponding tube. Mix gently by pipetting up and down.

i. Once a set of 4 tubes have been filled, cap the tubes.

   Note: The PCR tubes can be stored for 30 minutes between pipetting samples into the PCR tubes and start of the experiment in the machine at 2–8°C in the dark.

j. Return the QIAsure Calibrator to the freezer to avoid any material degradation.

   Note: Change tips between each tube to avoid any nonspecific template or reaction mix contamination, which may lead to false-positive results.

3. Prepare Rotor-Gene Q MDx and start run (experiment) as follows:
    a. Place a 72-well rotor on the rotor holder.
    b. Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Table 1 on page 24, with empty capped strip tubes placed into all unused positions.

       Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions as shown in Table 1 on page 24.
    c. Attach the locking ring.
    d. Load the Rotor-Gene Q MDx instrument with the rotor and locking ring, and close the instrument lid.
    e. Within the Rotor-Gene AssayManager v1.0 software, either select the corresponding work list from the work list manager and click **Apply**, or if the work list is still open, click **Apply**.

       Note: If the work list for the run is not created, log in to Rotor-Gene AssayManager v1.0 and follow Step 1 before proceeding.
    f. Enter the run (experiment) name.
    g. Select the cycle to be used in the **Cycler selection** list.
    h. Check correct attachment of locking ring and confirm on the screen that the locking ring is attached.
    i. Click **Start experiment**.

       The QIAsure Methylation Test run starts.
4. After the run is finished, click **Finish run**.
5. Release and approve the run.

- For users logged in with an Approver role, click **Release and go to approval**.
- For users logged in with an Operator role, click **Release**.

6. Release results.

   - If **Release and go to approval** is clicked, the results for the experiment are displayed.
   - If **Release** is clicked by a user with user role, someone with an Approver role needs to log in and select the Approval environment.
   - Select the filter options and click **Apply** to filter the assay to be approved.
   - Review results and approve the results of each test sample.

   In the Results table, scroll to the sample to be approved. Every sample result to be approved has three radio buttons at the dedicated row end.

   You can **Accept** or **Reject** the result of a sample.

   Note: A result automatically set to INVALID by Rotor-Gene AssayManager cannot be converted to a valid result anymore, even if the result is rejected.

   Optional: Enter a comment in the **Sample comment** column.

   - Click **Release/Report** data.
   - Click **OK**. The report is generated in Adobe Portable Document format (.pdf) and automatically stored in the predefined folder. The default folder path is **QIAGEN** > **Rotor-Gene AssayManager** > **Export** > **Reports**.

     Note: This path and folder can be changed in the Configuration environment.

   - Go to the Archive tab to export the .rex file corresponding to the raw data. Find your experiment using the filter options ,and click **Show assays**. Then, click on **Export .rex file** and click **OK** to save. The software automatically saves the .rex file in this predefined folder: **QIAGEN** > **Rotor-Gene AssayManager** > **Export** > **Experiments**.

Note: This path and folder can be changed in the tab Specify the .rex file export destination.

Note: For troubleshooting, a support package from the run is required. Support packages can be generated from the approval or archive environment. See the *Rotor-Gene AssayManager Core Application User Manual*, Troubleshooting, "Creating a support package" at **www.qiagen.com/rotor- gene- assaymanager/resources**. In addition, the audit trail from the time of incident ±1 day may be helpful. The audit trail can be retrieved in the Service environment (*Rotor-Gene AssayManager Core Application User Manual*).

7. Unload the Rotor-Gene Q MDx instrument, and discard the strip tubes according to your local safety regulations.

Interpretation of Results

The analysis is entirely automated.

Rotor-Gene AssayManager v1.0 first analyzes amplification curves, and may invalidate nonconforming curves, depending on their shape and noise amplitude. If this is the case, a flag will be associated with the invalidated curve (see Table 2).

Rotor-Gene AssayManager v1.0 will then analyze the run controls.

- Calibrator
- NTC

Note: The report generated at the end of the run shows the results obtained on run controls with invalidating flags in front of invalid data.

If all of the controls in the run conform, then Rotor-Gene AssayManager will analyze the unknown samples.

Table 2 shows the invalidating sample flags that may be assigned to an individual tube during the analysis by Rotor-Gene AssayManager v1.0, along with an explanation of what this flag means.

Table 2. Invalidating sample flags and description of terms

Flag	Behavior	Description
ABOVE_ACCEPTED_RANGE	Invalid	The target value is higher than the defined range. This can be a CT, endpoint-fluorescence, concentration, or calculated value, e.g., mean CT or ΔCT.
ASSAY_INVALID	Invalid	The assay is invalid because at least one external control is invalid.

Table 2. Invalidating sample flags and description of terms (continued)

Flag	Behavior	Description
BELOW_ACCEPTED_RANGE	Invalid	The target value is lower than the defined range. This can be a CT, endpoint-fluorescence, concentration, or calculated value, e.g., mean CT or ΔCT.
CONSECUTIVE_FAULT	Invalid	Target that was used for calculation of this target is invalid.
CURVE_SHAPE_ANOMALY	Invalid	The raw data amplification curve shows a shape that deviates from the established behavior for this assay. There is a high likelihood of incorrect results or a result misinterpretation.
FLAT_BUMP	Invalid	The raw data amplification curve shows a shape like a flat bump deviating from the established behavior for this assay. There is a high likelihood of incorrect results or result misinterpretation (e.g., wrong CT value determination).
IN_ACCEPTED_RANGE	Valid	NTC shows signal CT values above 36 for target ACTB.
INVALID_CALCULATION	Invalid	Calculation for this target failed.
MULTIPLE_THRESHOLD_CROSSING	Invalid	The amplification curve crosses the threshold more than once. An unambiguous CT cannot be determined.
NO_BASELINE	Invalid	No start value for the initial baseline has been found.
NO_CT_DETECTED	Variable	No CT is detected for this target.
NO_VALUE	Invalid	The target has no value but it is expected to have one. This value does not have to be in certain range. This can be a CT endpoint-fluorescence, concentration, or calculated value (e.g., mean CT or ΔCT).
NORM_FACTOR_ALTERATION	Warning	Deviation during the normalization procedure. The amplification curve is displayed with a default normalization; results should be manually checked for correctness.

Table 2. Invalidating sample flags and description of terms (continued)

Flag	Behavior	Description
OTHER_TARGET_INVALID	Invalid	Another target for the same sample is invalid.
SATURATION	Invalid	The raw data fluorescence is saturating strongly before the inflection point of the amplification curve.
SATURATION_IN_PLATEAU	Warning	The raw data fluorescence is saturating in the plateau phase of the amplification curve.
SPIKE	Warning	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the CT is determined.
SPIKE_CLOSE_TO_CT	Invalid	A spike is detected in the amplification curve close to the CT.
STEEP_BASELINE	Invalid	A steeply rising baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_BASELINE_DIP	Invalid	A strong drop in the baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_NOISE	Invalid	Strong noise outside the growth phase of the amplification curve detected.
STRONG_NOISE_IN_GROWTH_PHASE	Invalid	Strong noise is detected in the growth (exponential) phase of the amplification curve.
UNCERTAIN	Variable	Results from the AUDAS are conflicting with results from the core analysis. An unambiguous automatic assessment of data validity is not possible.
UNEXPECTED_CT_DETECTED	Variable	A CT value is detected for a target that should not amplify.
UNEXPECTED_VALUE	Invalid	The target has a value, but it is not an expected value. This can be a CT, endpoint-fluorescence, concentration, or calculated value, e.g., mean CT or ΔCT.

Table 2. Invalidating sample flags and description of terms (continued)

Flag	Behavior	Description
UPSTREAM	Variable	Sample status was set to Invalid or Unclear by an upstream process (e.g., QIAsymphony). **Note**: For samples that are flagged as unclear, the behavior of Rotor-Gene AssayManager is defined in the "Configuration" environment of the AssayManager software. "Invalid" flags from upstream processes always result in an invalid corresponding sample in Rotor-Gene AssayManager.
WAVY_BASE_FLUORESCENCE	Invalid	Wavy baseline for the raw data fluorescence detected in the amplification curve.

- If all of the controls in the run are valid, then the Rotor-Gene AssayManager v1.0 will analyze the unknown samples. In the sample, a minimal amount of bisulfite-converted DNA must be present for analysis of the hypermethylation levels of the targets *FAM194* and *hsa-miR124-2*. This is indicated by the C_T value of the housekeeping gene ACTB, which must be ≤30 for a sample to be validated by the Rotor-Gene AssayManager.
- The ΔΔCT values for *FAM19A4* and *hsa-mir124-2* will then be calculated and reported.

  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.
- 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 delayed signal for the targets. Therefore, for samples with an ACTB Ct value ≤23, it is advised to repeat the PCR with 5 times less input of converted DNA by diluting the sample in water.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support** (for contact information, visit **www.qiagen.com).**

For troubleshooting information related to Rotor-Gene AssayManager, refer to the *Rotor-Gene AssayManager Core Application User Manual*.

Comments and suggestions

General handling

Sample DNA concentration too low for bisulfite conversion		
a.	Check DNA extract	Repeat DNA extraction with more concentrated clinical sample

Sample is scored invalid; the amplification of ACTB 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 reactions has optimal performance for DNA input ranging from 100 ng to 2 µg
c.	For the Bisulfite-conversion directly on cervical specimen protocol, 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		
a.	Insufficient mixing	Mix sample and reaction mix by pipetting (approximately 10 times per tube). Repeat sample.

Positive control is invalid: the amplication 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 consumbles 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 positives on the fluorescence curve.

Absent or low signals in sample, but the control run is okay	
a. Inhibitory effects	Always check that 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.

If the problem persists, contact QIAGEN Technical Service.

Limitations

The QIAsure Methylation Test reagents may exclusively be used for Research Use Only (RUO). Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of the disease.

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.

Reagents and instructions supplied in this kit have been validated for optimal performance.

The QIAsure Methylation Test is to be used by laboratory professionals trained in the use of the Rotor-Gene Q MDx instruments and Rotor-Gene AssayManager v1.0.

The product is to be used by personnel specially instructed and trained in the techniques of real-time PCR only.

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

Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

All reagents supplied in the QIAsure Methylation Test are intended to be used solely with the other reagents supplied in the same kit. This may otherwise affect performance.

The QIAsure Methylation Test is validated for instance obtained from cervical specimens collected and stored in PreservCyt, SurePath, or STM media and from self-collected vaginal brush specimens that have been stored in PreservCyt upon arrival in the laboratory.

Only the Rotor-Gene Q MDx has been validated for use with the QIAsure Methylation Test PCR assay.

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 not indicated in this Instructions for Use.

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Symbols

The following symbols appear in the instructions for use or on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Material number (i.e., component labeling)
	Global Trade Item Number
	Contains
	Component
	Number
Rn	R is for revision of the Instructions for Use and n is the revision number

Symbol	Symbol definition
	Temperature limitation
	Manufacturer
	Consult instructions for use
	Caution

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**)

Ordering Information

Product	Contents	Cat. no.
QIAsure Methylation Test	For 72 reactions: 2 Master Mixes, 2 calibrators	616015
RELATED PRODUCTS		
Rotor-Gene Q MDx		
Rotor-Gene Q MDx 5plex HRM instrument	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9002033
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9002032
Rotor-Gene Q MDx instruments		
Loading Block 72 x 0.1ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Rotor-Gene Q AssayManager - for routine testing with Rotor-Gene Q MDx instruments		
Rotor-Gene AssayManager	Software for routine testing in combination with the Rotor-Gene Q and QIAsymphony RGQ instruments; single license software for installation on one computer	9022739

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit Instructions for Use. QIAGEN kit Instructions for Use are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Revision	Description
R1, January 2024	First version

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