November 2017

therascreen® PITX2 RGQ PCR Kit Handbook

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Version 1

IVD

For in vitro diagnostic use
For use with Rotor-Gene® Q MDx 5plex HRM instrument
For use with QIAamp® DSP DNA FFPE Tissue Kit
For use with EpiTect® Fast DNA Bisulfite Kit

CE

REF 873211

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

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Contents

Intended Use

The *therascreen* PITX2 RGQ PCR Kit is an in vitro methylation-specific real-time PCR test intended for the determination of the percent methylation ratio (PMR) in the pituitary homeobox 2 (PITX2) promoter 2. The test uses bisulfite-converted gDNA from FFPE tissue obtained from high-risk breast cancer patients. The PMR will aid clinicians in the prediction of response to adjuvant anthracycline-based chemotherapy with or without endocrine therapy in high-risk lymph node-positive, estrogen receptor-positive, HER2-negative breast cancer patients.

The product is intended to be used by qualified users, such as technicians and physicians, trained in molecular biology techniques and in vitro diagnostic procedures.

The *therascreen* PITX2 RGQ PCR Kit is used with the QIAGEN® Rotor-Gene Q MDx 5Plex HRM Platform.

Summary and Explanation

The QIAamp DSP DNA FFPE Tissue Kit is used for purification of DNA from FFPE tissue. Pituitary homeobox 2 (PITX2) is a transcription factor induced by the Wnt/β-catenin signaling pathway. PITX2 functions as an effector of Wnt-signaling by recruiting and interacting with β-catenin to increase expression of target genes involved in cell proliferation, migration, tumor progression and chemosensitivity (1–6). Gene expression activity of PITX2 is regulated by methylation within its promoter region by so-called "epigenetic modification". Small molecules, so called "methyl groups", are attached to the DNA base cytosine in the promoter region of a gene. Such a completely or partially methylated gene is downregulated in its activity. In breast cancer, PITX2 has been reported to be both a prognostic marker and predictive marker for response to endocrine or anthracycline-based chemotherapy. Several clinical studies have shown a strong statistical correlation between methylation in the

promoter region of the PITX2 gene and clinical outcome measures such as progression-free survival, metastasis-free survival, disease-free survival and overall survival (7–12).

The *therascreen* PITX2 RGQ PCR Kit is a real-time methylation-specific PCR-based (qMSP) assay. The sample type is bisDNA, i.e., bisulfite-converted genomic DNA (gDNA). gDNA is first purified from formalin-fixed paraffin-embedded (FFPE) tissue obtained from high-risk lymph node-positive, estrogen receptor-positive, HER2-negative breast cancer patients. After bisulfite exposure to distinguish between methylated and unmethylated PITX2, the percent methylation ratio (PMR) of three CpG motifs of the PITX2 gene promoter 2 is quantified by qMSP and calculated by the Rotor-Gene AssayManager® software with Gamma Plug-in and PITX2 Assay Profile. The PMR obtained will provide information to the treating physician about whether a patient is likely to respond to anthracycline-based chemotherapy. If the PMR obtained is equal to or lower than 12, the patient is likely to respond to anthracycline-based chemotherapy. In contrast, if the obtained PMR is higher than 12, an alternative treatment may be proposed, as the patient has a lower probability of responding to anthracycline-based chemotherapy (see "Clinical cut-off validation", page 72).

Principle of the Procedure

The *therascreen* PITX2 RGQ PCR Kit uses real-time PCR (qPCR) for the determination of the percent methylation ratio (PMR) in the PITX2 promoter 2. The sample type for the *therascreen* PITX2 RGQ PCR Kit is bisulfite-converted gDNA. This bisulfite conversion is performed using the EpiTect Fast DNA Bisulfite Kit (QIAGEN, cat. no. 59824 or 59826). The gDNA used for this conversion is purified from FFPE tissue from high-risk breast cancer patients using the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404). The workflow is shown in Figure 1.

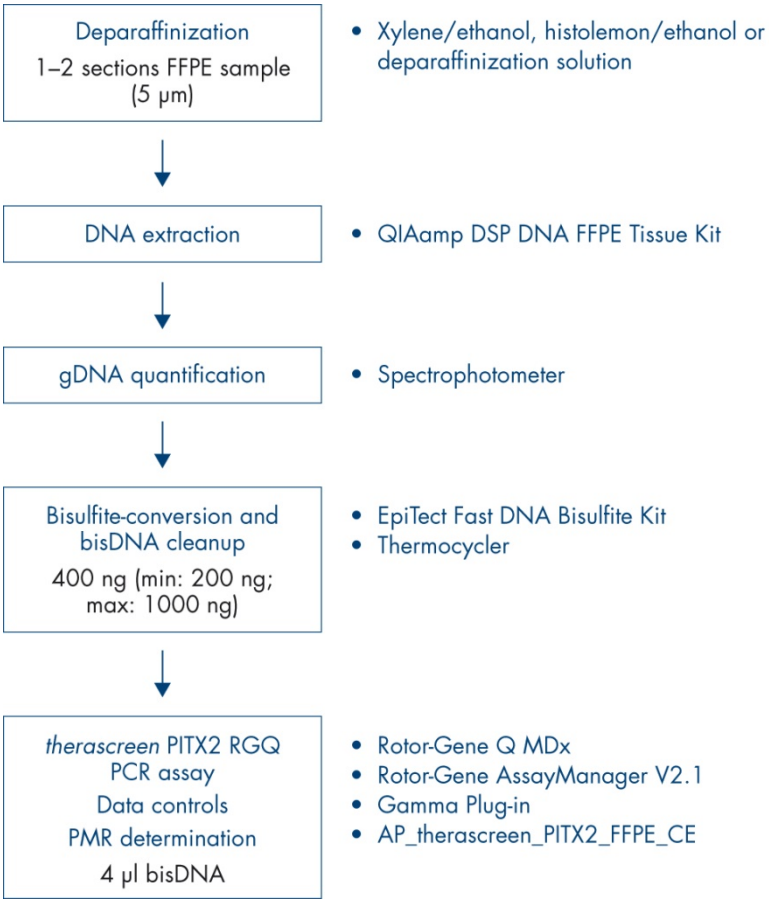

Figure 1. *therascreen* PITX2 RGQ PCR Kit workflow.

The use of qPCR permits the accurate detection of a targeted bisDNA sequence during the exponential phase of the amplification process. qPCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during PCR cycling.

The *therascreen* PITX2 RGQ PCR Kit assay exploits the qPCR oligonucleotide hydrolysis principle of the TaqMan® probes in combination with methylation-unspecific primers (Figure 2, next page). This assay uses one pair of primers amplifying all bisulfite-converted target sequences. Two different signals are obtained from this amplification by using two TaqMan probes labeled with different dyes. These probes, which consist of oligonucleotides labeled with a 5' reporter dye (FAM™ or HEX™) and a downstream 3' dye-free quencher, hybridize to the targeted sequences within the PCR product. One probe is specific for the bisDNA sequences from methylated sequences, dyed with FAM. The other is specific for the bisDNA sequences from unmethylated sequences, dyed with HEX. Analysis by TaqMan qPCR exploits the 5'→ 3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer. During PCR, if the target of interest is present, both forward and reverse primers specifically anneal and flank the annealed probe. The 3' end of the probe is blocked to prevent extension of the probe during PCR (Figure 3, page 10). During the polymerization phase, the 5'→ 3' exonuclease activity of the DNA polymerase cleaves the probe leading to the release of quencher and reporter fluorescence signal emission. The probe fragments are then displaced from the target and polymerization of the strand continues. This process occurs in every cycle and does not interfere with the exponential accumulation of product (Figure 3, page 10). The increase in fluorescence signal is detected only if the target sequence is complementary to the primers and probe and hence amplified during PCR. The PCR cycle at which the fluorescence from a particular reaction crosses the predefined threshold values (given by the *therascreen* PITX2 Assay Package) is defined as the C_T value.

The outcomes of the *therascreen* PITX2 RGQ PCR Kit assay are two C_T values, one for FAM and one for HEX. From the ΔC_T value between both signals, a PMR is calculated (Figure 2, next page). The PMR calculation is based on the following formula (11):

$$PMR = \frac{100}{1 + 2^{C_T FAM - C_T HEX}}$$

The PMR obtained will provide information to the treating physician about whether a patient is likely to respond to anthracycline-based chemotherapy. If the PMR obtained is equal or to lower than 12, the patient is likely to respond to anthracycline-based chemotherapy. In contrast, if the obtained PMR is higher than 12, an alternative treatment may be proposed, as the patient has a lower probability of responding to anthracycline-based chemotherapy.

The turnaround time to execute all tasks, from gDNA purification to data analysis, is less than two working days.

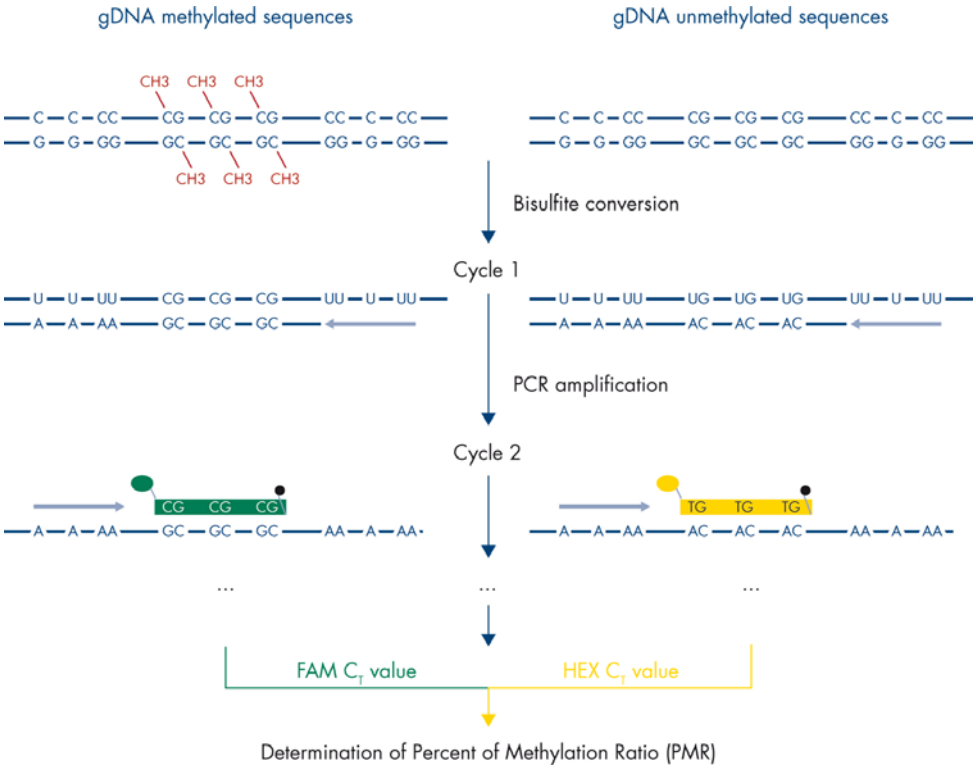

Figure 2. Principle of the *therascreen* PITX2 RGQ PCR Kit assay.

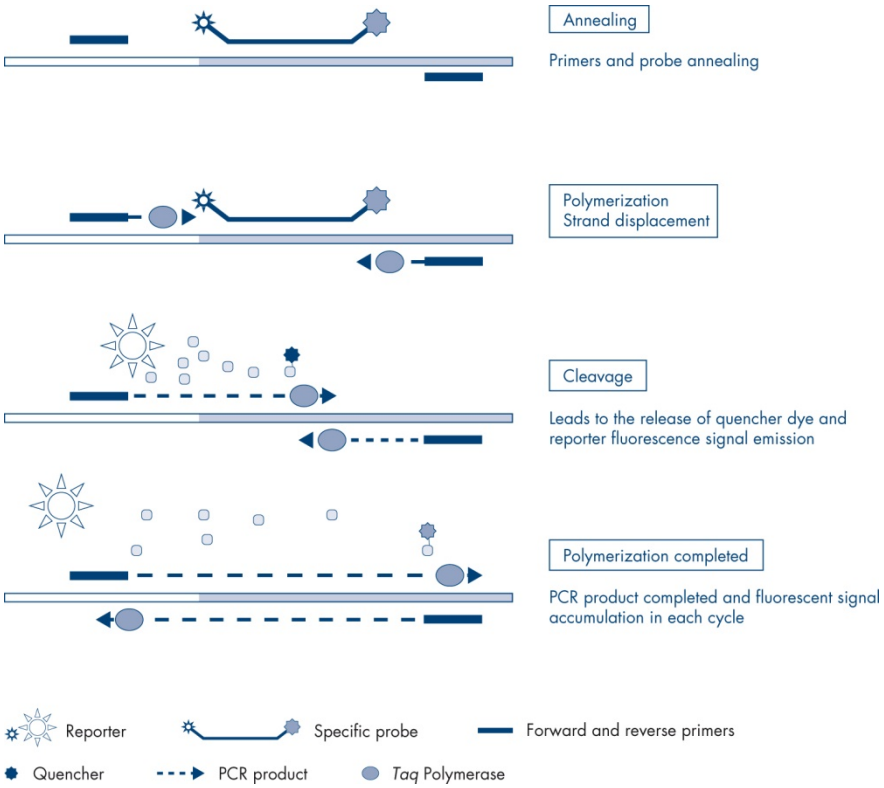

Figure 3. Principle of TaqMan real-time PCR assay.

Materials Provided

Kit contents

***therascreen* PITX2 RGQ PCR Kit**		**(8)**
Catalog no.		**873211**
Number of reactions		**8**
Purple	PITX2 RGQ PCR Master Mix	660 µl
Blue	PITX2 RGQ PCR Primer Probe Mix	192 µl
Yellow	PITX2 RGQ PCR Reference 50	12 µl
Orange	PITX2 RGQ PCR Reference Low	12 µl
Green	PITX2 RGQ PCR Negative Control	12 µl
Colorless	PITX2 RGQ PCR NTC	12 µl
–	Instructions For Use (Handbook)	1

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.

Make sure that instruments have been checked and calibrated according to the manufacturer's recommendations. Make sure that all kits reagents have not expired and have been transported and stored under the correct conditions.

Reagents

- Ethanol (molecular grade 96–100%)

  Note: Do not use denatured alcohol as this contains other substances such as methanol or methylethylketone.

Equipment

- Thermomixer, heated orbital incubator, heating block, or water bath capable of incubation at 56°C and 90°C.

  Note: Consider the tube form requirements of the thermomixer to select the appropriate tube size (e.g., 2 or 1.5 ml tubes)

- Adjustable pipets* dedicated for PCR (1–10 µl; 10–100 µl; 100–1000 µl)

  A minimum of two sets of pipets is recommended, one for preparation and distribution of PCR reaction mixes and one for bisDNA and controls handling including PCR template loading.

- Nuclease-free, aerosol-resistant, sterile PCR pipet tips with hydrophobic filters (pipet tips with aerosol barriers are recommended for preventing cross-contamination).
- 1.5 ml or 2 ml microcentrifuge tubes (1.5 ml tubes, available from Eppendorf, cat. no. 0030120.086 or Sarstedt, cat. no. 72.690)
- Benchtop centrifuge with rotor for 0.5 ml, 1.5 ml, and 2.0 ml reaction tubes (able to reach 20,000 x *g*)
- Vortexer
- Spectrophotometer, e.g., NanoDrop® instrument or QIAxpert® (QIAamp plug-in: total nucleic acid measurement)†
- Disposable gloves

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

† This is not a complete list of suppliers.

Optional reagents for workflow control

- One vial containing one section (15 or 20 µm) of KRAS G13D Reference Standard (Horizon Discovery, cat. no. HD216).

For manual DNA purification

- QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404)
- Deparaffinization Solution (cat. no. 19093) or xylene or histolemon (Carlo Erba, cat. no. 454911)

  Important: Deparaffinization Solution, xylene or histolemon are not supplied with the QIAamp DSP DNA FFPE Tissue Kit and must be ordered separately.

Additional For bisulfite conversion

- EpiTect Fast DNA Bisulfite Kit (cat. no. 59824 or 59826)
- 0.2 ml reaction tubes or 8-well strips
- 0.2 ml tubes microcentrifuge
- Thermal cycler with heated lid (since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure)

For PCR on Rotor Gene Q MDx

- Rotor-Gene Q MDx 5plex HRM (cat. no. 9002032) and provided accessories
- Rotor-Gene AssayManager software version 2.1.x (where x = 0 or higher)
- Gamma Plug-in version 1.0.x (where x = 0 or higher) for Rotor-Gene AssayManager v2.1
- therascreen_PITX2_FFPE_CE Assay Profile V1.0.x (where x = 1 or higher)
- Loading Block for 72 x 0.1 ml Tubes (cat. no. 9018901)
- 72-Well Rotor (cat. no. 9018903)
- Adaptor Locking Ring 72-Well Rotor (cat. no. 9018904)
- Rotor Holder (cat. no. 9018908)

- Strip Tubes and Caps, 0.1 ml, for the Rotor-Gene Q MDx (cat. no. 981103 or 981106)
- Ice (or a cooling block)

Warnings and Precautions

For in vitro diagnostic use

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.

For safety information regarding Deparaffinization Solution, xylene-ethanol, histolemon-ethanol, the QIAamp DSP DNA FFPE Tissue Kit or EpiTect Fast DNA Bisulfite Kit, please refer to the respective handbooks. For safety information regarding instruments, see the relevant instrument user manual.

General precautions

Use of qPCR tests requires good laboratory practices, including traceability, maintenance of equipment dedicated to molecular biology and compliance with applicable regulations and relevant standards.

This kit is intended for in vitro diagnostic use. Reagents and instructions supplied in this kit have been tested for optimal performance.

- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Discard samples and assay waste according to your local safety procedures.
- Reagents for the *therascreen* PITX2 RGQ PCR Kit are diluted optimally. Do not dilute reagents further as this may result in a loss of performance.
- Do not use reaction volumes (reaction mix plus sample) of less or more than 20 µl.
- Quality control procedures at QIAGEN employ functional kit release testing for each individual kit batch. Therefore, do not mix reagents from different batches, as this may affect performance.
- The *therascreen* PITX2 entire workflow requires transfer of samples in different tubes therefore make sure that the traceability of the samples is well maintained at each step.
- Make sure that the PITX2 assay profile and required Rotor-Gene AssayManager v2.1 Gamma plug-in are installed.
- Refer to the Rotor-Gene Q MDx User Manual and Rotor-Gene AssayManager v2.1 Core Application User Manual for additional warnings, precautions and procedures.
- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Thaw all *therascreen* PITX2 RGQ PCR components and samples in a fridge, on ice, on a cooling block or at room temperature for as long as necessary.

  Note: If thawed at room temperature, check regularly whether the material has thawed, especially the PITX2 RGQ PCR Master Mix (MMx) as it contains dNTPs that are sensitive to temperature.

  Note: PITX2 RGQ PCR PPM should be protected from light as it contains dye nucleotides.

  Note: Repeated thawing and freezing should be avoided and should not exceed a maximum of four freeze–thaw cycles.
- Prepare all reactions (reaction mix plus sample) on ice or in a cooling block.
- Do not use expired or incorrectly stored components.
- Reaction mixes may be altered if exposed to light.

- Do not swallow any reagent.
- Use individual, dedicated pipets for setting up reaction mix and adding templates.
- Do not open the Rotor-Gene Q MDx instrument until the run is finished.
- Do not open Rotor-Gene Q MDx tubes after the run is finished. Discard tubes according to your local safety procedures.
- Caution must be observed to ensure correct sample testing with emphasis on wrong sample entry, loading error and pipetting error.
- Make sure the samples are handled in a systematic way to ensure correct identification.
- Use extreme caution to prevent contamination of the reaction mix with the materials that are contained in the PITX2 RGQ PCR Reference 50 and PITX2 RGQ PCR Reference Low Control reagents.
- Use extreme caution to prevent DNA or PCR product carryover contamination resulting in a false-positive signal.
- Use extreme caution to prevent contamination by DNase, which may cause degradation of the template DNA.

We therefore recommend the following:

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials) and wear disposable gloves when performing the assay.
- Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.

  Prepare PCR reaction mix with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrices (DNA, plasmid or PCR products) are introduced. In this same area, add PITX2 RGQ PCR NTC to the appropriate tube (Figure 4, page 36), but close this tube after loading of all other controls and samples to assess cross-contamination. Add samples to be tested, PITX2 RGQ PCR Reference 50, PITX2 RGQ PCR Reference Low and PITX2 RGQ PCR Negative Control in a separate room with specific material (pipets, tips, etc.).

Reagent Storage and Handling

Shipping conditions

The *therascreen* PITX2 RGQ PCR Kit is shipped on dry ice. If any component of the *therascreen* PITX2 RGQ PCR Kit is not frozen on arrival, if the outer packaging has been opened during transit or the shipment does not contain a packing note or the reagents, please contact QIAGEN Technical Services or local distributors (visit **www.qiagen.com**).

Storage conditions

The *therascreen* PITX2 RGQ PCR Kit should be stored immediately upon receipt at –30°C to –15°C in a constant temperature freezer and protected from light.

For storage and handling information relating to Deparaffinization Solution, xylene-ethanol, histolemon-ethanol, the QIAamp DSP DNA FFPE Tissue Kit or EpiTect Fast DNA Bisulfite Kit, refer to respective kit handbooks.

Stability

When stored under the specified storage conditions, the *therascreen* PITX2 RGQ PCR Kit is stable until the stated expiration date.

Once opened, reagents can be stored in their original packaging at –30 to –15°C until the expiration date shown on the packaging. Repeated thawing and freezing should be avoided and should not exceed a maximum of four freeze–thaw cycles.

For stability information relating to Deparaffinization Solution, xylene-ethanol, histolemon-ethanol, the QIAamp DSP DNA FFPE Tissue Kit or EpiTect Fast DNA Bisulfite Kit, refer to respective kit handbooks.

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

Specimen Handling and Storage

The *therascreen* PITX2 RGQ PCR Kit is for use with bisDNA samples. The purified and bisulfite-converted DNA is from FFPE tumor tissue taken from primary lesions of high-risk lymph node-positive, estrogen receptor-positive, HER2-negative breast cancer patients. Fix tissue samples in formalin as per laboratory protocol (10% neutral buffered formalin is generally accepted) as quickly as possible after surgical removal.

- Tissue specimen should be fixed in 4–10% formalin as quickly as possible after surgical removal or core biopsy.
- Ideally, use a fixation time of 14–24 hours (longer fixation times lead to more severe DNA fragmentation, resulting in poor performance in qPCR/qMSP assays).
- Thoroughly dehydrate samples prior to embedding (residual formalin can inhibit proteinase K digest).
- Sections 5 µm thick must be cut from the paraffin block.
- For sections that have a tumor area <100 mm^2, it is recommended to process two sections to increase the total tumor area to at least 100 mm^2.
- Label, handle, and store tumor specimens, blocks, sections, and samples ready for purification in a controlled fashion according to local procedures.
- Transport and store FFPE blocks and sections at room temperature. Sections may be used rapidly for DNA purification.

- DNA purified using the QIAamp DSP DNA FFPE Tissue Kit may be stored at 2–8°C for short-term storage up to 24 hours or at –30 to –15°C if long-term storage is required.
- Bisulfite-converted DNA using the EpiTect Fast DNA Bisulfite Kit can be stored at –30 to –15°C for at least 9 months without decrease in quality or conversion. Further investigations into long-term storage are ongoing. Contact QIAGEN for more information.
- The workflow control KRAS G13D Reference Standard section (Horizon Discovery, cat. no. HD216) can be stored at room temperature for 36 months from the date of manufacture.

Procedure

Genomic DNA purification and preparation

The *therascreen* PITX2 RGQ PCR Kit has been validated in combination with QIAGEN Deparaffinization Solution (cat. no. 19093) for FFPE section deparaffinization, the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for gDNA purification and the EpiTect Fast DNA Bisulfite Kit (cat. no. 59824 or 59826) for gDNA bisulfite conversion.

FFPE section deparaffinization can be performed using Deparaffinization Solution, xylene-ethanol or histolemon-ethanol (equivalency of these three deparaffinization methods was proven during product development).

If the Deparaffinization Solution (cat. no. 19093) is used start with the procedure "FFPE section deparaffinization with QIAGEN Deparaffinization Solution" page 21.

If xylene-ethanol or histolemon-ethanol is used, go directly to the procedure, "Manual gDNA purification with the QIAamp DSP DNA FFPE Tissue Kit" on page 23.

Optional: To assess whether purification and bisulfite conversion performed correctly, a workflow control can be used. The workflow control validated for the *therascreen* PITX2 RGQ PCR Kit workflow is the KRAS G13D Reference Standard section (Horizon Discovery, cat. no. HD216).

Make sure that gDNA purification reagents have not expired and have been transported and stored under the correct conditions. Do not use expired or incorrectly stored components.

Starting material

Starting material for DNA purification should be freshly cut sections of FFPE tissue, they can be kept overnight at room temperature if required. Up to two sections, each with a thickness of 5 µm and a total surface area above 100 mm^2, must be used as starting material for gDNA purification.

FFPE section deparaffinization with QIAGEN Deparaffinization Solution

IMPORTANT: If deparaffinization is performed with xylene-ethanol or histolemon-ethanol, proceed with "Manual gDNA purification with the QIAamp DSP DNA FFPE Tissue Kit" on page 23.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Equilibrate all buffers to room temperature; equilibrate Deparaffinization Solution to 20–25°C.
- Deparaffinization Solution is not supplied with the QIAamp DSP DNA FFPE Tissue Kit and must be ordered separately.

Things to do before starting

- Preheat a thermomixer or heated orbital incubator to 56°C for use in steps 4 and 8. If a thermomixer or heated orbital incubator is not available, a heating block or water bath can be used instead.
- If Buffer AL or Buffer ATL contain precipitates, dissolve them by heating to 70°C with gentle agitation.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.

Procedure (for up to two sections)

1. Using a scalpel, trim excess paraffin off the sample block. Cut into sections 5 µm thick.

   Note: If the sample surface has been exposed to air, discard the first 2–3 sections.
2. Immediately place the section(s) in a 1.5 ml or 2 ml microcentrifuge tube (not supplied).
3. Add 160 µl Deparaffinization Solution and vortex vigorously for 10 seconds.

   Centrifuge briefly to collect the sample in the bottom of the tube.
4. Incubate at 56°C for 3 minutes, and then allow to cool at room temperature (15–25°C).
5. Add 180 µl Buffer ATL, and mix by vortexing.
6. Centrifuge for 1 minute at 11,000 x *g* (10,000 rpm). Two phases appear (blue and clear).
7. Add 20 µl proteinase K to the lower, clear phase by pushing the pipet through the upper phase. Mix gently by pipetting up and down.
8. Incubate at 56°C ± 3°C for ≥1 hour (or until the sample has completely lysed).
9. Incubate at 90°C ± 5°C for 1 hour ± 5 minutes.

   The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA.

   Note: If using only one heating block, leave the sample at room temperature (15–25°C) after the 56°C incubation in step 8, until the heating block has reached 90°C for step 9.
10. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.
11. Transfer the lower, clear phase into a new 2 ml microcentrifuge tube (not provided).

    Note: Do not transfer any blue phase.
12. Continue with step 14 of "Manual gDNA purification with the QIAamp DSP DNA FFPE Tissue Kit" on page 23.

Manual gDNA purification with the QIAamp DSP DNA FFPE Tissue Kit

Manual gDNA purification is performed with the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) according to the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C)

Things to do before starting

- Equilibrate all buffers to room temperature.
- Set a thermomixer or heated orbital incubator to 56°C for use in step 12.
- If a thermomixer or heated orbital incubator is not available, a heating block or water bath can be used instead.
- If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.

Procedure

Note: If using QIAGEN Deparaffinization solution, steps 1 to 14 should be replaced by the procedure described in "FFPE section deparaffinization with QIAGEN Deparaffinization Solution" on page 21.

1. Using a scalpel, trim excess paraffin off the sample block.
2. Cut 1 to 2 sections 5 µm thick to reach at least 100 mm^2 tumor surface (see "Starting material", page 21).

   If the sample surface has been exposed to air, discard the first 2–3 sections.
3. Immediately place the sections in a 1.5 or 2 ml microcentrifuge tube (not provided).

4. Add 1 ml xylene or histolemon to the sample. Close the lid and vortex vigorously for ≥10 seconds.
5. Centrifuge at full speed for 2 minutes ± 30 seconds at room temperature.
6. Remove the supernatant by pipetting. Do not remove any of the pellet.
7. Add 1 ml ethanol (96–100%) to the pellet, and mix by vortexing.

   The ethanol extracts residual xylene from the sample.
8. Centrifuge at full speed for 2 minutes ± 30 seconds at room temperature.
9. Remove the supernatant by pipetting. Do not remove any of the pellet.

   Carefully remove any residual ethanol using a fine pipet tip.
10. Open the tube and incubate at 15–40°C. Incubate for 10 minutes ± 1 minute or until all residual ethanol has evaporated.
11. Resuspend the pellet in 180 µl Buffer ATL. Add 20 µl proteinase K, and mix by vortexing.
12. Incubate at 56°C ± 3°C for ≥1 hour (or until the sample has been completely lysed).
13. Incubate at 90°C ± 5°C for 1 hour ± 5 minutes.

    The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 90°C.
14. Briefly centrifuge the tube to remove drops from the inside of the lid.

    Note: If using Deparaffinization Solution, continue with step 15.
15. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then, add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.

    It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the QIAamp procedure.

16. Briefly centrifuge the tube to remove drops from the inside of the lid.

17. Carefully transfer the entire lysate to the QIAamp MinElute® column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at approximately 6000 x *g* for ≥1 minute. Place the QIAamp MinElute column in a clean 2 ml collection tube (provided), and discard the collection tube containing the flow-through.

    If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

18. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at approximately 6000 x *g* for ≥1 minute. Place the QIAamp MinElute column in a clean 2 ml collection tube (provided), and discard the collection tube containing the flow-through.

19. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at approximately 6000 x *g* for ≥1 minute. Place the QIAamp MinElute column in a clean 2 ml collection tube (provided), and discard the collection tube containing the flow-through.

    Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

20. Centrifuge at full speed (approximately 20,000 x *g*) for ≥3 minutes to dry the membrane completely.

    This step is necessary, since ethanol carryover into the eluate may inhibit the qPCR reactions performed.

21. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 50 µl Buffer ATE to the center of the membrane.

22. Close the lid and incubate at room temperature (15–25°C) for 5 minutes. Centrifuge at full speed (approximately 20,000 x *g*) for ≥1 minute.

Quantitation of DNA

Buffer ATE used for elution in gDNA purification kits contains the preservative sodium azide. Sodium azide absorbs at 260 nm and therefore a blank measurement with Buffer ATE should be performed to calibrate the spectrophotometer.

The concentration of DNA is determined by measuring absorbance at 260 nm following the instrument procedure using QIAGEN's QIAxpert, for example (QIAamp plug-in: total nucleic acid measurement) or a NanoDrop instrument*. Absorbance readings at 260 nm should fall between 0.1 and 1.0 to be accurate. An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml (A_{260} = 1 = 50 µg/ml). The total amount of DNA purified (ng) = concentration of DNA (ng/µl) x volume of sample (µl).

Note: If using the QIAamp plug-in, an internal ATE-blank spectra is automatically subtracted from the OD values, so no additional blank ATE sample is required with this configuration.

Ideally, a minimal gDNA concentration is 10 ng/µl† but samples as low as 5 ng/µl may be processed with risk of "Low input" invalid results.

gDNA bisulfite conversion using the EpiTect Fast DNA Bisulfite Kit

This protocol enables bisulfite conversion of DNA amounts of 200, 400 or up to 1000 ng (measured using OD_{260} nm measurement) in a volume of up to 40 µl. The recommended DNA input per reaction of bisulfite conversion is 400 ng. However, in case of low DNA yield, DNA inputs as low as 200 ng can be used and in case of retest due to a "Low input" flag in the qPCR analysis (see "Flags", page 57), 1000 ng or as close to this amount as possible should be used.

* This is not a complete list of possible spectrophotometers for OD_{260} nm measurement.

† 10 ng/µl to get 400 ng gDNA input (recommended input) for bisulfite conversion as maximum volume of gDNA for conversion is 40 µl.

Note: The gDNA input is referred to gDNA quantification with OD 260 measurement (e.g., using a NanoDrop or QIAxpert with QIAamp plug-in for total nucleic acid measurement).

Starting material

- Genomic DNA should be used for bisulfite treatment without any previous restriction digest step.

Important points before starting

- Make sure that bisulfite conversion reagents have not expired and have been transported and stored under the correct conditions. Do not use expired or incorrectly stored components.
- DNA Protect Buffer should turn from green to blue after addition to the DNA-Bisulfite Solution mixture, indicating sufficient mixing and correct pH for the bisulfite conversion reaction, incorrect pH could impact the fixation of the converted DNA on the column.
- Perform all centrifugation steps at room temperature (15–25°C).
- Bisulfite Solution can be stored at room temperature (15–25°C) for at least 6 months.
- White precipitates may form in the Buffer BD-ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the MinElute DNA spin column.

Things to do before starting

- Prepare the kit reagents as described in the "Preparation of reagents" section of the *EpiTect Fast Bisulfite Conversion Handbook*.
- Equilibrate samples and buffers to room temperature.
- **Optional**: Set a thermomixer, heating block, or heated orbital incubator to 60°C to dissolve the Bisulfite Solution.

Handling of MinElute DNA spin columns

Due to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling MinElute DNA spin columns to avoid cross-contamination between sample preps:

- Carefully pipet the sample or solution into the MinElute DNA spin column without wetting the rim of the column. Avoid touching the MinElute DNA spin column membrane with the pipet tip.
- Always change pipet tips between liquid transfers. We recommend the use of aerosol-barrier pipet tips.
- Open one MinElute DNA spin column at a time, and take care to avoid generating aerosols.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

Centrifugation

- MinElute DNA spin columns will fit into most standard 1.5–2 ml microcentrifuge tubes. A set of 2 ml collection tubes is supplied for the dry centrifugation step.
- All centrifugation steps should be carried out at room temperature (15–25°C).
- Process MinElute DNA spin columns in a microcentrifuge.
- Always close MinElute DNA spin columns before placing them in the microcentrifuge.
- For efficient parallel processing of multiple samples, we recommend filling a rack with the collection tubes into which the MinElute DNA spin columns can be transferred after centrifugation. Collection tubes can be used several times.

Procedure

1. Thaw DNA to be used in the bisulfite-conversion reactions. Make sure the Bisulfite Solution is completely dissolved.

   Note: If necessary, heat the Bisulfite Solution to 60°C and vortex until all precipitates are dissolved again.

   Note: Do not place dissolved Bisulfite Solution on ice.

2. Prepare the bisulfite reactions in 200 µl PCR tubes (not provided) according to Table 1, next page. Add each component in the order listed.

   Note: The combined volume of DNA and RNase-free water must total 40 µl.

   Note: To determine the appropriate volume for the gDNA input of interest use the following formula:

$$\text{gDNA volume required for a bisulfite conversion (µl)} = \frac{\text{Input of interest (ng)}}{\text{Average gDNA concentration (ng/µl)}}$$

   Note: When using the *therascreen* PITX2 RGQ PCR Kit, the "Low concentration" protocol from the *EpiTect Fast Bisulfite Conversion Handbook* must always be used, even with 1000 ng input, as the concentration of gDNA purified from FFPE samples is usually low.

   Note: The bisulfite mix should be immediately vortexed for 5 seconds after adding the DNA Protect Buffer to protect samples from degradation.

Table 1. Bisulfite reaction components

Component	Volume per reaction (µl)
DNA	Variable* (maximum 40 µl)
RNase-free water	Variable*
Bisulfite Solution	85
DNA Protect Buffer	15
Total volume	**140**

* The combined volume of DNA and RNase-free water must total 40 µl.

3. Close the PCR tubes and mix immediately the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25°C).

   Note: DNA Protect Buffer should turn from green to blue after addition to the DNA-Bisulfite Solution mixture, indicating sufficient mixing and correct pH for the bisulfite conversion reaction, or DNA binding to the MinElute DNA spin column.

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 2, next page.

   The complete cycle should take approximately 30 minutes.

   Note: If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.

Table 2. Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	10 min	60°C
Denaturation	5 min	95°C
Incubation	10 min	60°C
Hold	Indefinite*	20°C

* Converted DNA can be left in the thermal cycler overnight without any loss of performance.

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.

   IMPORTANT: Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. It is important to use PCR tubes that close tightly.

   Note: Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Cleanup of bisulfite-converted DNA

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.

   Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

7. Add 310 µl Buffer BL to each sample. Mix the solution by vortexing and then centrifuge briefly.
8. Add 250 µl ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 seconds, and centrifuge briefly to remove the drops from inside the lid.

9. Place the necessary number of MinElute DNA spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube from step 8 into the corresponding MinElute DNA spin column.
10. Centrifuge the spin columns at maximum speed for 1 minute. Discard the flow-through, and place the spin columns back into the collection tubes.
11. Add 500 µl Buffer BW (wash buffer) to each spin column, and centrifuge at maximum speed for 1 minute. Discard the flow-through, and place the spin columns back into the collection tubes.
12. Add 500 µl Buffer BD (desulfonation buffer) to each spin column, and incubate for 15 minutes at room temperature (15–25°C).

    If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

    IMPORTANT: The bottle containing Buffer BD should be closed immediately after use to avoid acidification from carbon dioxide in air.

    Note: It is important to close the lids of the spin columns before incubation.
13. Centrifuge the spin columns at maximum speed for 1 minute. Discard the flow-through, and place the spin columns back into the collection tubes.
14. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 minute. Discard the flow-through and place the spin columns back into the collection tubes.
15. Repeat step 14 once.
16. Add 250 µl ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 minute.
17. Place the spin columns into new 2 ml collection tubes (provided), and centrifuge the spin columns at maximum speed for 1 minute to remove any residual liquid.
18. Place the spin columns with open lids into a clean 1.5 ml microcentrifuge tube (not provided) and incubate the columns for 5 minutes at 60°C in a heating block. This step ensures the evaporation of any remaining liquid.

19. Add 15 µl Buffer EB (elution buffer) directly onto the center of each spin-column membrane and close the lids gently.

    Note: Do not elute with less than 15 µl buffer as the eluate volume would be too small to proceed with the qPCR step.

20. Incubate the spin columns at room temperature for 1 minute.

21. Centrifuge for 1 min at 15,000 x *g* (12,000 rpm) to elute the DNA.

    Note: We recommend storing purified DNA at 2–8°C for up to 24 hours. When storing purified DNA for longer than 24 hours, we recommend storage at –30 to –15°C.

Protocol: qPCR on Rotor-Gene Q MDx 5plex HRM instrument

The *therascreen* PITX2 RGQ PCR Kit must be run on the Rotor-Gene Q MDx 5plex HRM instrument* using automated interpretation of results with Rotor-Gene AssayManager v2.1.

Take time to familiarize yourself with the Rotor-Gene Q MDx instrument and with the Rotor-Gene AssayManager v2.1 software before starting the protocol. See the user manuals for the instrument, Rotor-Gene AssayManager v2.1, and the Gamma Plug-in for details.

Important note: If you are using the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in and the assay profile for the first time, please refer to the section "Installation of the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in and importing the assay profile" on page 49 for installation instructions. If the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in and the assay profile are already installed and imported in your computer, continue with the instructions below:

Setting up the qPCR

The *therascreen* PITX2 RGQ PCR Kit contains products to test eight samples in a maximum of three runs.

Things to do before starting

- Cool a Loading Block 72 x 0.1 ml Tubes for 10 minutes in a deep-freezer or for at least 1 hour at fridge temperature.

* If applicable, 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.

- Thaw all *therascreen* PITX2 RGQ PCR Kit components and samples in a fridge, on ice, on a cooling block or at room temperature for as long as necessary.

  Note: If thawed at room temperature, check regularly whether the material is already thawed, especially the PITX2 RGQ PCR MMx as it contains dNTPs that are sensitive to temperature.

  Note: PITX2 RGQ PCR PPM should be protected from sunlight as it contains dye nucleotides.

- Place the thawed products on ice, on a cooling block or in fridge until placing them back into –30 to –15°C after use.

  Note: *therascreen* PITX2 RGQ PCR Kit components can be kept at 2–8°C and protected from light for maximum of 6 hours if used several times on the same day.

  Note: *therascreen* PITX2 RGQ PCR Kit components can be used for a maximum four freeze–thaw cycles.

- Clean the bench area that is dedicated for the PCR mix preparation to reduce the risk of template or nuclease contamination.

- Vortex the tubes (10–12 seconds) and then centrifuge them briefly, before use. Except PITX2 RGQ PCR MMx which is mixed by pipetting up and down as it contains *Taq* Polymerase.

Procedure

1. Prepare PITX2 qPCR reaction mix **on ice** (or using a cooling block) in a 1.5 or 2 ml tube (not provided) according to the number of samples to be processed.

   The pipetting scheme for the preparation of PITX2 reaction mix, shown in Table 3 (next page), is calculated to achieve final reaction volumes of 20 µl after addition of 4 µl bisDNA sample or control. Extra volume is included to compensate for pipetting errors and to allow preparation of sufficient reaction mix for four samples tested in duplicate, plus four controls. If fewer samples are tested, the reaction mix can be prepared accordingly. Remember to allow for the extra volume to compensate for pipetting errors (one extra well for up to 10 wells and two extra wells for up to 20 wells).

Table 3. Preparation of the *therascreen* PITX2 RGQ PCR reaction mix

Component	1 reaction (µl)	Example for a plate of 12 wells: 12+2 extra reactions (µl)*
PITX2 RGQ PCR Master Mix	10	140
PITX2 RGQ PCR Primer Probe Mix	6	84
Total volume of qPCR reaction mix (µl)	16	224
qPCR reaction mix distribution	16 µl per tube	
Sample distribution	4 µl per tube	
Total volume of qPCR reaction	20 µl	

* An extra reaction volume is included to compensate for pipetting error: one extra well for up to 10 wells and two extra wells for up to 20 wells.

2. Vortex (10–12 seconds) and briefly centrifuge the PITX2 qPCR reaction mix. Place the qPCR strip tubes on a pre-cooled Loading Block 72 and dispense 16 µl of the PITX2 qPCR reaction mix per strip tube following the example of loading block setup shown in Figure 4.

   Note: It is recommended to dispense the 16 µl of the reaction mix by reverse pipetting.

1	REF50	9	Sample 3	17	NA	25	NA	33	NA	41	NA	49	NA	57	NA	65	NA
2	REFlow	10	Sample 3	18	NA	26	NA	34	NA	42	NA	50	NA	58	NA	66	NA
3	NC	11	Sample 4	19	NA	27	NA	35	NA	43	NA	51	NA	59	NA	67	NA
4	NTC	12	Sample 4	20	NA	28	NA	36	NA	44	NA	52	NA	60	NA	68	NA
5	Sample 1	13	NA	21	NA	29	NA	37	NA	45	NA	53	NA	61	NA	69	NA
6	Sample 1	14	NA	22	NA	30	NA	38	NA	46	NA	54	NA	62	NA	70	NA
7	Sample 2	15	NA	23	NA	31	NA	39	NA	47	NA	55	NA	63	NA	71	NA
8	Sample 2	16	NA	24	NA	32	NA	40	NA	48	NA	56	NA	64	NA	72	NA

Figure 4. Loading block setup for an experiment with the *therascreen* PITX2 RGQ PCR Kit, testing four samples. The numbers denote positions in the loading block and indicate final rotor position. The positions of the controls are set in the PITX2 assay profile and cannot be changed. If controls are not placed as indicated, the automated result analysis cannot be performed. **REF50**: PITX2 RGQ PCR Reference 50; **REFlow**: PITX2 RGQ PCR Reference Low; **NC**: PITX2 RGQ PCR Negative Control, **NTC**: PITX2 RGQ PCR NTC (NTC); **Sample 1 to 4**: bisDNA samples, **NA**: Empty well.

3. Vortex (10–12 seconds) and briefly centrifuge bisDNA samples, PITX2 RGQ PCR Reference 50 (Ref50), PITX2 RGQ PCR Reference Low (RefLow), PITX2 RGQ PCR Negative Control (NC) and PITX2 RGQ PCR NTC (NTC).

4. Add 4 µl sample or control material into its corresponding tube according to the setup in Figure 4 to obtain a total volume of 20 µl. Mix gently 5 times by pipetting up and down.

   Note: Be careful to change tips between each tube to avoid false-positive results from contamination by any nonspecific template.

5. Close all tubes and check that no bubbles are present at the bottom of the tubes.
6. Return all the *therascreen* PITX2 RGQ PCR Kit components and samples to the appropriate storage conditions to avoid any material degradation.

Preparing the Rotor-Gene MDx

It is highly recommended to launch the run as soon as possible after the preparation, however, if the plate is prepared but cannot be launched directly (due to instrument unavailability), it is possible to store the plate at 2–8°C and protected from light up to 24 hours (see "In-use timeframe", page 72).

7. Place a 72-Well Rotor on the Rotor-Gene Q MDx rotor holder.
8. Fill the rotor with strip tubes previously prepared according to the assigned positions, starting at position 1, as shown in Figure 5.
9. Complete empty positions with empty, closed tubes to fill the rotor entirely.

   Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions (important for run validity and traceability of sample) as shown in Figure 5.

   Note: Always keep the four controls (REF50, REFlow, NC and NTC) in positions 1 to 4 so that gain optimization (performed on tube position 1) is always performed on the same amplification. Make sure controls are loaded in the right order for the automated analysis of the controls (an inversion of controls will invalidate the run by the PITX2 assay profile).

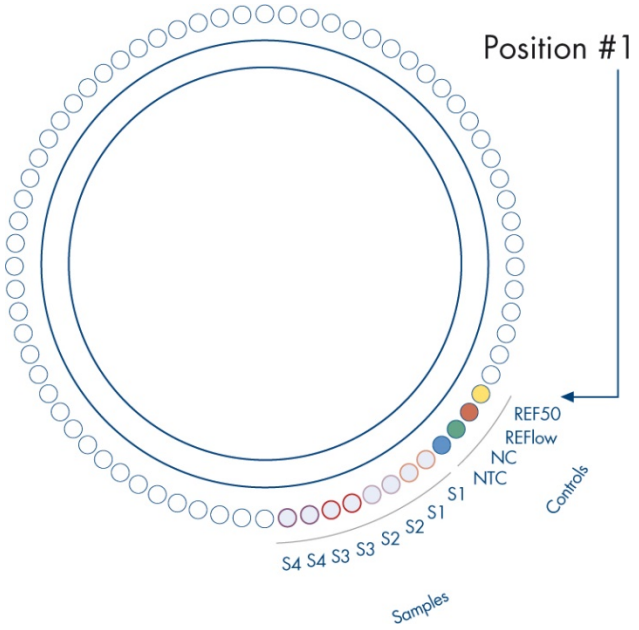

Figure 5. Rotor setup for an experiment with the *therascreen* PITX2 RGQ PCR Kit. **REF50**: PITX2 RGQ PCR Reference 50; **REFlow**: PITX2 RGQ PCR Reference Low; **NC**: PITX2 RGQ PCR Negative Control, **NTC**: PITX2 RGQ PCR NTC (NTC); **S1 to S4**: bisDNA samples. **Note**: All of the remaining positions ○ should be filled with empty tubes.

10. Attach the locking ring.
11. Load the Rotor-Gene Q MDx instrument with the rotor and locking ring. Close the instrument lid.

Creating a work list and starting the qPCR run

Note: The work list can be created and saved before preparing the samples or when the experiment is set up on the instrument, as described in this handbook.

12. Switch on the Rotor-Gene Q MDx instrument.
13. Open the Rotor-Gene AssayManager software by clicking the icon:  . The Rotor-Gene AssayManager window opens (Figure 6).

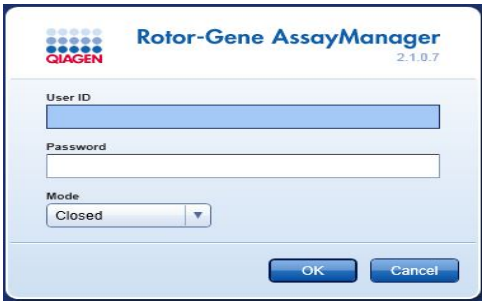

Figure 6. Rotor-Gene AssayManager login screen.

14. Log in as a user with the "Operator" role in the closed mode. Click "OK". The Rotor-Gene AssayManager screen opens (Figure 7, next page).
15. Check that the RGQ is correctly detected to the software before launching the run.
16. Select the "Setup" tab.

    Note: The overall functionalities of the Setup environment and of "Creating/Editing a Work List" are described in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.
17. Click "New work list" (Figure 7).

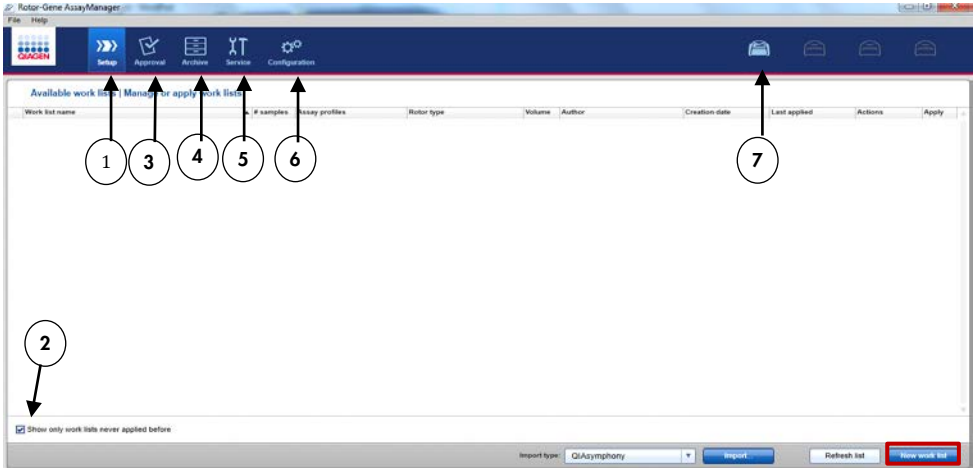

Figure 7. Description of the different tabs present in the RGAM software.

1 Setup tab. This tab allows managing or applying work lists.

2 Checking applied work lists. Shows new work lists only. An "applied work list" was already performed.

3 Approval tab. This tab enables you to find previous experiments.

4 Archive tab. Allows you to find old experiments that were already approved.

5 Service Tab. Shows a report of an audit trail of each file generated by the software.

6 Configuration tab. Allows configuration of all software parameters

7 Rotor-Gene Q MDx (RGQ) icons:

 Not connected  Connected

18. Select the PITX2 assay profile from the list of available assay profiles (Figure 8).

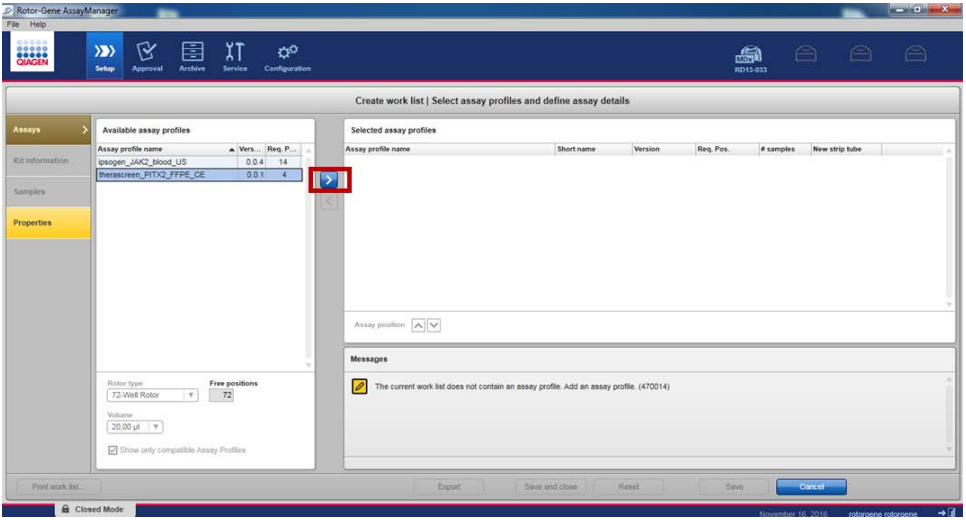

Figure 8. Assay profile import.

19. Transfer the selected assay profile to the list of selected assay profiles by clicking on the arrow (to the right of the assay profile name). The assay profile should now be displayed in the selected assay profiles list (Figure 8).

20. In the "Assays" tab, complete the yellow fields: Number of samples (up to 8) in accordance with your plate setup (Figure 9).

    Note: The number of samples does not correspond to the number of wells and does not include controls. Samples are tested in duplicates; therefore, one sample corresponds to two wells. For example, the number of samples to be inserted is 4 for the plate of 12 wells presented in Figure 4 (page 36).

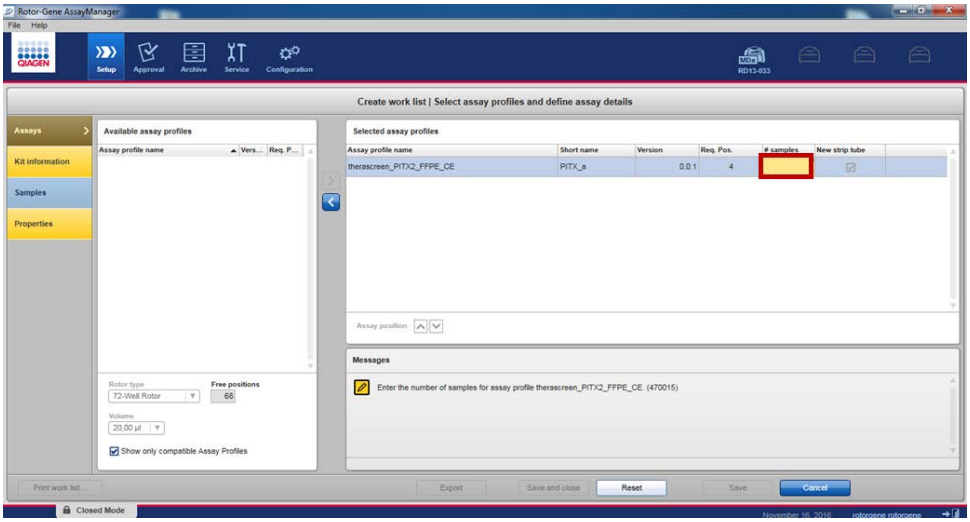

Figure 9. Inserting the number of samples.

21. Select the "Kit Information" tab. Insert the kit information by either selecting "Use kit bar code" (and scan the bar code) or selecting "Enter kit information manually" and inserting manually the kit information found on the label of the *therascreen* PITX2 RGQ PCR Kit box:

    MAT Material number

    Expiry date

    LOT Lot number

22. Select the "Samples" tab. A list with the sample details is shown. This list represents the expected layout of the rotor.

23. Enter the sample identification as well as any optional sample information as a comment for each sample (Figure 10).

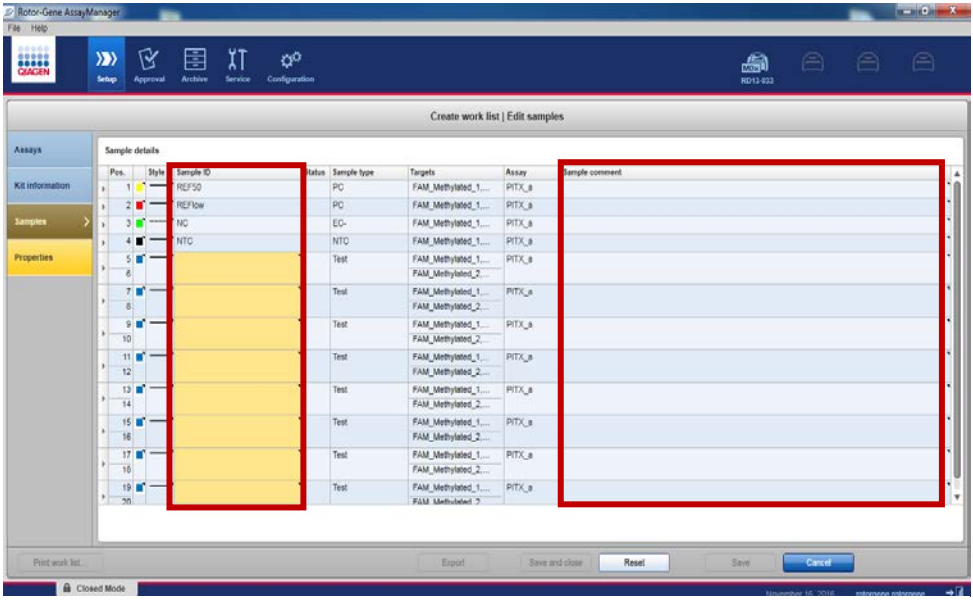

Figure 10. Sample setting.

24. Select “Properties” and enter a work list name (Figure 11).

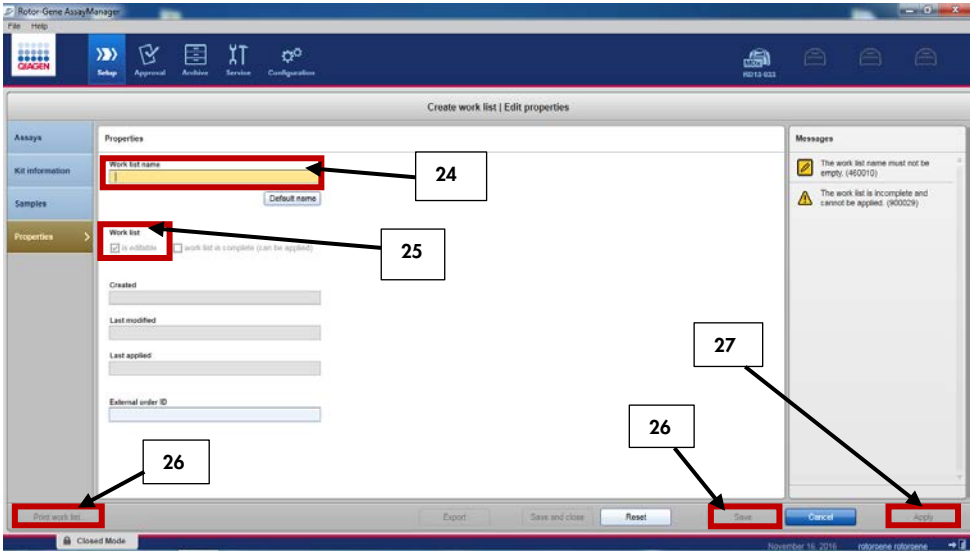

Figure 11. Creation of the worklist.

25. Enable the check box “worklist is complete (can be applied)”.
26. Save the work list.

    Optional: Press “Print work list” to print the work list. Printing the work list may help with the preparation and setup of the run. The sample details are included as part of the work list.
27. Select the corresponding work list from the work list manager and click “Apply”. Alternatively, if the work list is still open, click “Apply”.

    Note: Check that the Rotor-Gene Q MDx is correctly detected by the software before launching the run.
28. Enter the experiment name.
29. Select the cycler to be used in “Cycler Selection”.

    Note: A Rotor-Gene Q MDx 5plex HRM cycler must be used.

30. Check that the locking ring is correctly attached and confirm on the screen that the locking ring is attached.
31. Click “Start run”. The qPCR run should start.

Release and report qPCR results

The general functionality of the Approval environment is described in the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

After a run has finished and the cycler has been released, the experiment will be stored in the internal database. The analysis of the acquired data is performed automatically according to the rules and parameter values defined by the assay profile.

Note: The user role “Approver” is required to approve a run.

1. When the run has finished, click on “Finish run” to analyze and export data.

   Note: Until this step is completed, the experiment is not saved in the internal database.
2. After clicking “Finish run”, enter the password and click “Release and go to approval” (Figure 12).

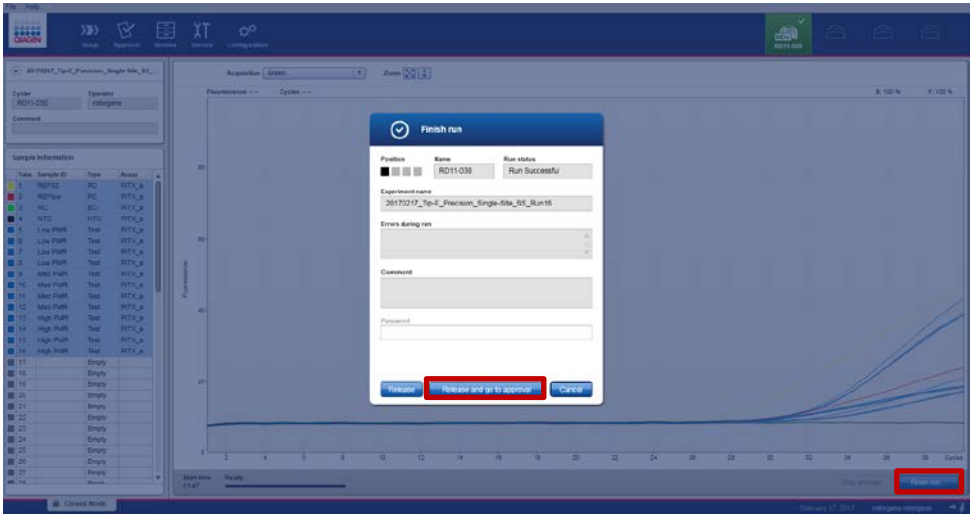

Figure 12. Finalization of the run.

For users logged in with the "Approver" role, click "Release and go to approval".

For users logged in with the "Operator" role, click "Release".

If "Release and go to approval" was clicked, the results for the experiment are displayed in the "Approval" environment.

If "Release" was clicked by a user with the "Operator" role, someone with an "Approver" role must log in and select the "Approval" environment.

Note: In the "Approval" tab, experiments can be analyzed by shifting between each tab (i.e., experiment, assay, audit, trail, run control results).

3. Check the amplification curves for each sample, tick the first box on the right side of the "flags" column (the box becomes green) (Figure 13).

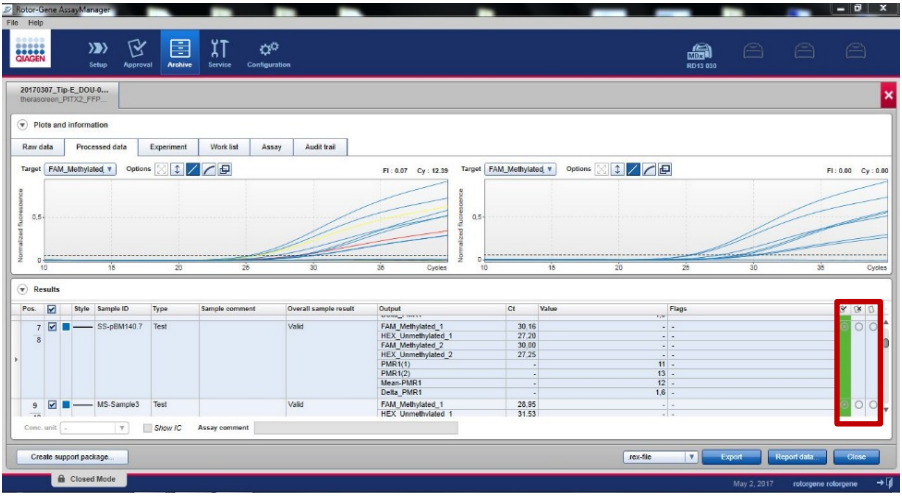

Figure 13. Amplification Curve checking.

4. Click “Release/report data” (at the bottom right of the window) to create a .pdf report and to save the LIMS file (a copy is automatically saved in C:\Documents and settings\AllUsers\Documents\QIAGEN\RotorGeneAssayManager\Export\Reports).
5. Close the pdf file and return to the Rotor-Gene AssayManager. Click “OK” each time it is asked.
6. Go to the “Archive” tab to export the .rex file. Check that “start date” and “end date” are correct and click “apply filter”. Select the experiment to export then click on “Show assays” (Figure 14).

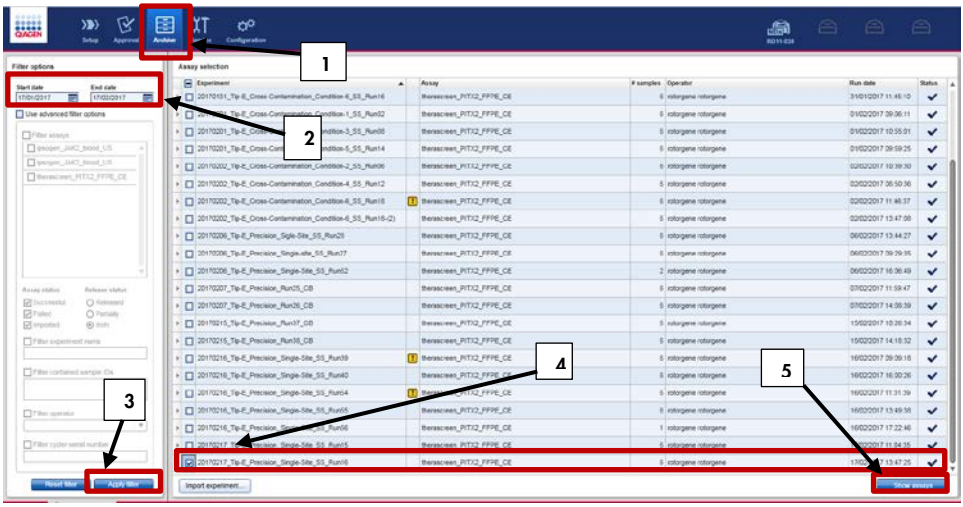

Figure 14. Export of the run data.

7. Export the .rex file (the file is saved in C:\Documents and settings\AllUsers\Documents \QIAGEN\RotorGeneAssayManager\Export\Experiments).

   Note: The software automatically generated a LIMS file in C:\Documents and settings\All Users\Documents\QIAGEN\RotorGeneAssayManager\Export\LIMS

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

   Note: A support package from the run is required for assistance with troubleshooting by QIAGEN Technical Support. Support packages can be generated from the "Approval" or "Archive" environments. For more information, see "Creating a support package" in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

   In addition to the support package, the audit trail from ±1 day of the time of an incident might be helpful. The audit trail can be retrieved in the "Service" environment. For more information, see the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

Installation of the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in and importing the assay profile

Rotor-Gene AssayManager v2.1 software must be installed on the computer connected to the Rotor-Gene Q MDx. The software can be downloaded from "Operating Software" under the "Product Resources" tab at the Rotor-Gene AssayManager v2.1 product page: **www.qiagen.com/Products/Rotor-GeneAssayManager_v2_1.aspx**.

For details regarding the installation of the Rotor-Gene AssayManager v2.1 core software, please refer to the *Rotor-Gene AssayManager v2.1 Core Application User Manual*. For details about additional software on connected computers, please refer to the *Rotor-Gene AssayManager v2.1 Quick-Start Guide*.

For automatic interpretation of results using the *therascreen* PITX2 RGQ PCR Kit with Rotor-Gene AssayManager v2.1, the latest Gamma Plug-in must be installed to your Rotor-Gene AssayManager v2.1. See the "Product Resources" at the Rotor-Gene AssayManager v2.1 product page: **www.qiagen.com/Products/Rotor-GeneAssay Manager_v2_1.aspx** to access the latest version of the plug-in.

The *therascreen* PITX2 RGQ PCR Kit also requires an assay profile. The assay profile contains all parameters needed for cycling and analyzing the PITX2 assay. These parameters are locked for the run. The PITX2 assay profile (AP_therascreen_PITX2_FFPE_CE) corresponds to an ".iap" file that can be downloaded from the *therascreen* PITX2 RGQ PCR Kit product page: **www.qiagen.com/shop/detection-solutions/personalized-healthcare/therascreen-pitx2-rgq-pcr-kit-ce/** on the "Product Resources" tab under "Protocol Files". The assay profile must be imported in Rotor-Gene AssayManager v2.1 software.

Details regarding installation of the Gamma Plug-in and the importing of the assay profile into the Rotor-Gene AssayManager v2.1 software are as follows.

1. Download the Gamma Plug-in from **www.qiagen.com**.
2. Start the installation process by double-clicking on GammaPlugin.Installation.msi file, and following the installation instructions. For a detailed description of this process, please refer to the section "installing plug-ins" in the *AssayManager Core Application User Manual*.
3. After successful installation of the plug-in, a person with administrator rights for the Rotor Gene AssayManager software will need to import the required Assay Profile as follows:
4. Go to windows explorer, and save the AP in the following file: "C:\Documents and Settings\All Users\Documents \QIAGEN\Rotor-GeneAssayManager\Import\ AssayProfiles".
5. Open the Rotor-Gene AssayManager software by clicking the  icon.
6. Login to Rotor-Gene AssayManager with your user ID and password. Do not change the "Closed mode". Click "OK". The Rotor-Gene AssayManager screen opens.
7. Select the configuration environment (Figure 15).

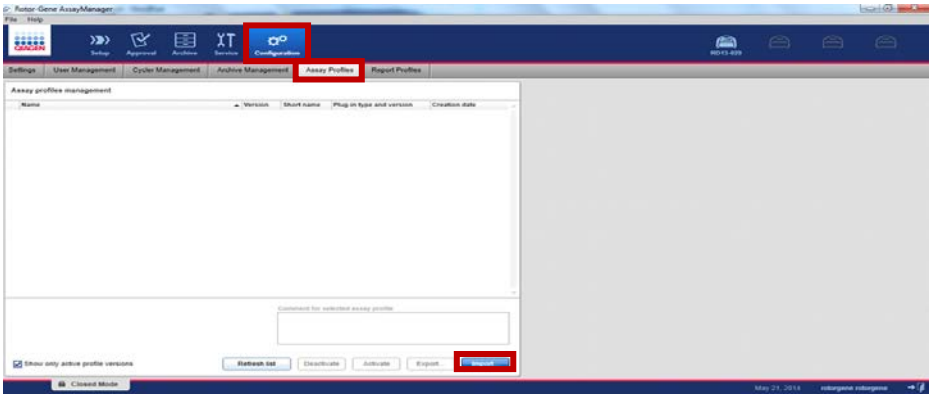

Figure 15. Configuration tab.

8. Select the tab “Assay Profiles”.
9. Click “Import”.
10. Select the Assay Profile AP_therascreen_PITX2_FFPE_CE_V1.0.x.iap (where x = 1 or higher) to be imported in the dialog and click “Open”.
11. Once the Assay Profile has been successfully imported, it can be used in the “Setup” environment.

Interpretation of Results

Data analysis

Analysis of the *therascreen* PITX2 RGQ PCR Kit results for each control and sample is performed automatically by the Rotor-Gene AssayManager v2.1 associated with the Gamma Plug-in v1.0 and the PITX2 assay profile hereafter referred as the PITX2 Assay Package.

The PITX2 Assay Package analyzes amplification curves, and may invalidate non-conforming curves, depending on their shape and noise amplitude. If this is the case, a flag will be associated with the invalidated curve. Warning flags can also be displayed for non-invalidating curve anomalies (see Flag list and details in the section "Flags" page 57).

To determine assay validity, PITX2 Assay Package also analyzes the run controls, i.e., PITX2 RGQ PCR Reference 50 (REF50), PITX2 RGQ PCR Reference Low (REFlow), PITX2 RGQ PCR Negative Control (NC) and PITX2 RGQ PCR NTC (NTC). Validity for each control is based on compliance of C_T and/or PMR values with pre-defined specifications (see "Overall sample results", page 55 and "Flags" page 57).

Note: If at least one control is invalid, the results obtained for all test samples are considered invalid and no PMR results are displayed.

PITX2 Assay Package also analyzes the samples by checking the validity of the duplicates and the validity of the input (see "Overall sample results", page 55 and "Flags" page 57). Finally, a PMR value with no digits is assigned to the samples by meaning of the two PMR results obtained for each sample replicate. The PMR obtained for each patient sample will provide information to the treating physician about whether a patient is likely to respond to anthracycline-based chemotherapy. If the PMR obtained is equal to or lower than 12, the patient is likely to respond to anthracycline-based chemotherapy. In contrast, if the obtained PMR is higher than 12, an

alternative treatment may be proposed, as the patient has a lower probability of responding to anthracycline-based chemotherapy (Figure 16).

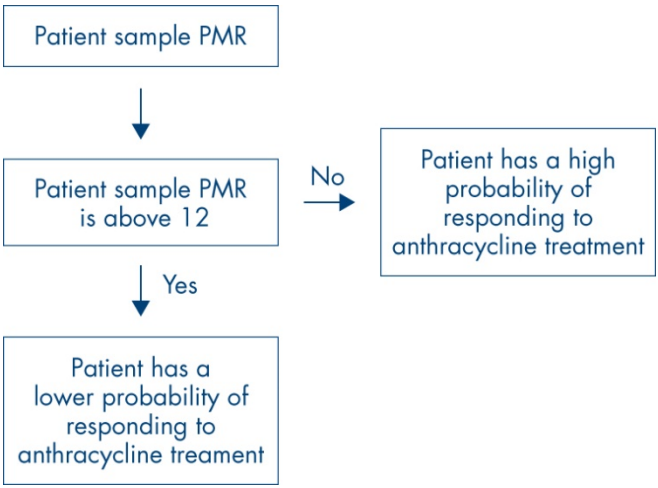

Figure 16. Interpretation of PMR results of patient samples for the *therascreen* PITX2 RGQ PCR Kit.

The results of the test samples, automatically analyzed and set by the PITX2 Assay Package, must be approved and released by a user logged in with the role of "Approver". Sample results to be approved have three additional approval buttons at the end of the dedicated row. These buttons are used to interactively accept or reject the sample results. For further information, please refer to the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

Workflow Control note: The sample HD216 (workflow control) should give a PMR value between 30 and 50. If this PMR is obtained with this workflow control, both gDNA purification and bisulfite conversion step can be validated.

In case of invalid results, refer to "Troubleshooting guide", page 61.

Retests

In case of invalid results, retests are required. If the assay is invalid, i.e., one of the four controls invalid, the entire run including all tested samples should be retested. If the assay is valid but one or several samples are invalid, the invalid sample(s) should be retested after investigating the type of failure (see "Flags", page 57, Table 6 and Table 7, pages 58–59). A workflow of the retest procedure is presented in Figure 17.

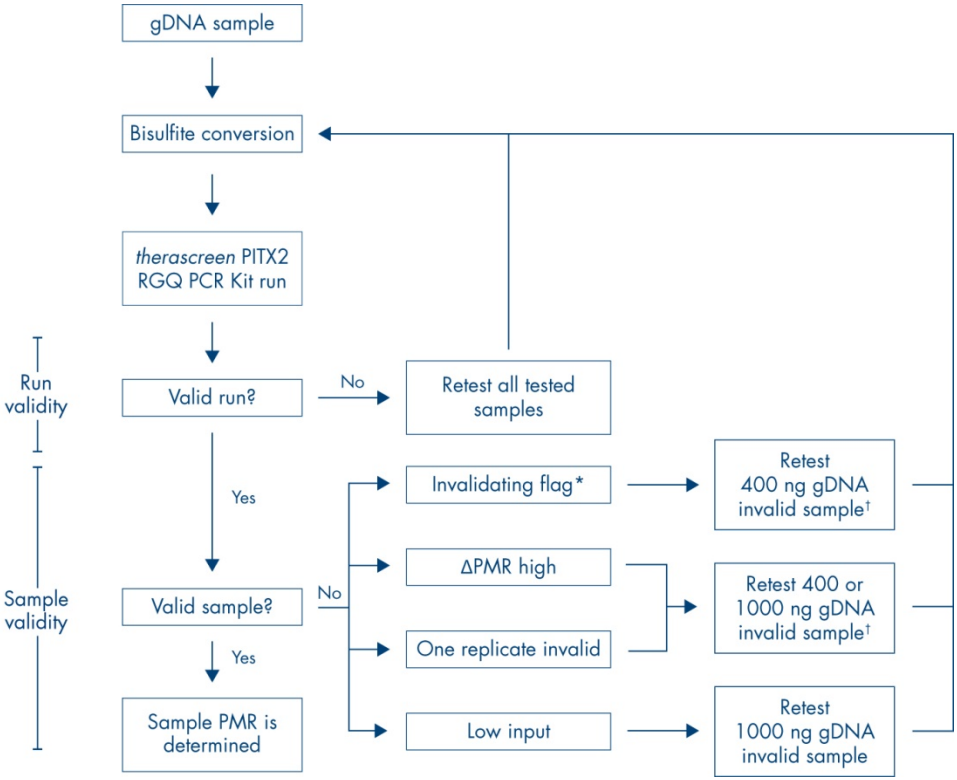

Figure 17. Retest workflow for the *therascreen* PITX2 RGQ PCR Kit.

* See Table 6 and Table 7, pages 58–59.

† An input of 200 ng can be used if not enough gDNA is available; however, the risk of invalid result due to "low input" flag is higher.

Results display

Targets and combined targets

The results for each reaction of the *therascreen* PITX2 RGQ PCR Kit are displayed under the following target and combined target names:

- "FAM_Methylated_1": Green channel results for all controls and for the replicate 1 of test samples.
- "FAM_Methylated_2": Green channel results for the replicate 2 of test samples.
- "HEX_Unmethylated_1": Yellow channel results for all controls and for the replicate 1 of test samples.
- "HEX_Unmethylated_2": Yellow channel results for the replicate 2 of test samples.
- "PMR": These targets are combined targets; the corresponding result takes into account the validity of controls. These targets are shown for all controls and test samples if valid.
- "Mean_PMR": These targets are combined targets; the corresponding result takes into account the validity of controls. These targets are shown for all test samples if valid.

Overall sample results

The conclusion of the analysis for each control and sample is displayed in the "Overall Sample Result" column of the report (Table 4).

Table 4. Overall sample results and actions

Overall sample result	Sample type	Description	Action
Valid	REF50, REFlow, NC, NTC and test sample*	Control or test sample is valid	N/A
Invalid†	REF50, REFlow, NC, NTC and test sample	Control is invalid	Repeat the entire run
Invalid	Test sample	test sample is invalid	Set up new run to repeat the invalid sample(s)
Invalid, one replicate invalid	Test sample	If one of the target (FAM_Methylated_1, FAM_Methylated_2, HEX_Unmethylated_1 or HEX_Unmethylated_2) is invalid, the sample is considered as invalid	Set up new run to repeat the invalid sample(s)
Invalid, delta PMR high‡	Test sample	If the delta PMR value between the first replicate and the second replicate is above a specific value§, the sample is considered as invalid	Set up new run to repeat the invalid sample(s)

* Interpretation of the test sample valid PMR result is explained previously (see Figure 16).

† When controls are invalid, the invalid C_T values and the PMR results are displayed between square brackets for information.

‡ When a sample is invalidating by a high delta PMR, the C_T values and the PMR results of both replicates and the mean PMR are displayed for information. However, the sample should be retested to obtain a valid result.

§ Specific value is varying according to the PMR value obtained for each sample (see Table 5, next page).

Table 5. Delta PMR criteria

Mean-PMR	Delta PMR duplicates
0–1	≤1
1–5	≤5
5–10	≤7
10–15	≤9
15–35	≤13
35–65	≤15
65–85	≤18
85–100	≤6

Flags

Flags are displayed to give additional information about the obtained results, in particular about invalid results. Unproblematic anomalies can be flagged by a warning flag that does not lead to an invalid result. For universal flags included in the Gamma Plug-in, also refer to the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

The automated analysis of the *therascreen* PITX2 RGQ PCR Kit assay may provide both assay-specific flags (Table 6, next page) and general flags (Table 7, page 59).

Table 6. Assay-specific flags

Assay-specific flag	Sample type	Description	Action
Assay-specific flags for controls			
BELOW_ACCEPTED_RANGE	REF50, REFlow	The PMR result is below the accepted range (<36 for REF50, <2 for REFlow).	Repeat the entire run
ABOVE_ACCEPTED_RANGE	REF50, REFlow	The PMR result is above the accepted range (>65 for REF50, >13 for REFlow).	Repeat the entire run
NO_SIGNAL	REF50	The C_T value of the target FAM_Methylated_1 or/and HEX_Unmethylated_1 is >32	Repeat the entire run
NO_SIGNAL	REFlow	The C_T value of the target HEX_Unmethylated_1 is >32	Repeat the entire run
Assay-specific flags for test samples			
PMR_ABOVE_OR_EQUAL_92*	Test sample	The PMR result is above the Limit of Detection determined for the probe targeting former unmethylated sequences. This flag is not invalidating, it is a warning flag.	None
PMR_BELOW_OR_EQUAL_4*	Test sample	The PMR result is below the Limit of Detection determined for the probe targeting former methylated sequences. This flag is not invalidating, it is a warning flag.	None
LOW_INPUT_RETEST_NEEDED	Test sample	The C_T value of the target FAM_Methylated_1 and HEX_Unmethylated_1 or FAM_Methylated_2 and HEX_Unmethylated_2 are >32.5	Increase the gDNA input in the bisulfite conversion and repeat the run

* As PMR results are given with no digit but the software is calculating PMR with digit, the Limit of detection flag can be present or not for value at the PMR limit, i.e., 4 and 92. Indeed, flag are present from PMR results >92 and <4, thus for example a PMR results at 4.1 or 91.8 rounded respectively at 4 and 92 will not be flag as below or above the limit of detection respectively.

Note: All flags shown above are invalidating except the two related to the Limit of Detection. When replicates are invalid, the C_T values are displayed between square brackets for information but the invalid PMR result is not displayed. And the mean PMR of both replicate is not displayed.

Table 7. General flags

General flag	Behavior	Description	Action
CONSECUTIVE_FAULT	Invalid	A target that was used for calculation of this target is invalid.	Repeat the sample or the run if it is due to invalidity in a control.
ASSAY_INVALID	Invalid	The assay is invalid because at least one control is invalid	Repeat the entire run.
ANALYSIS_FAILED	Invalid	Assay is set to invalid because the analysis has failed due to various reasons.	Contact QIAGEN Technical Services
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 misinterpretation of results.	Repeat the sample or the run if it is observed in a control.
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 misinterpretation of results (e.g., wrong C_T value determination).	Repeat the sample or the run if it is observed in a control.
INVALID_CALCULATION	Invalid	Calculation for this target failed.	Repeat the sample or the run if it is observed in a control.
LOW_FLUORESCENCE_ CHANGE	Warning	The percentage fluorescence change for this sample relative to the sample tube with the largest fluorescence change is lower than a defined limit.	None
LOW_REACTION_ EFFICIENCY	Warning	The reaction efficiency for this sample has not reached a defined limit.	None
MULTIPLE_THRESHOLD_ CROSSING	Invalid	The amplification curve crosses the threshold more than once. An unambiguous C_T cannot be determined.	Repeat the sample or the run if it is observed in a control.
NO_BASELINE	Invalid	No initial baseline has been found. The subsequent analysis cannot be performed.	Repeat the sample or the run if it is observed in a control.
RUN_FAILED	Invalid	Assay is set to invalid due to a problem with the cycler or the cycler connection.	Repeat the entire run.
RUN_STOPPED	Invalid	Assay is set to invalid because the run has been stopped manually.	Repeat the entire run.

General flag	Behavior	Description	Action
SATURATION	Invalid	The raw data fluorescence is saturating strongly before the inflection point of the amplification curve.	Repeat the sample or the run if it is observed in a control.
SATURATION_IN_ PLATEAU	Warning	The raw data fluorescence is saturating in the plateau phase of the amplification curve.	None
SPIKE	Warning	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the C_T is determined.	None
SPIKE_CLOSE_TO_CT	Invalid	A spike is detected in the amplification curve close to the C_T.	Repeat the sample or the run if it is observed in a control.
STEEP_BASELINE	Invalid	A steeply rising baseline for the raw data fluorescence is detected in the amplification curve.	Repeat the sample or the run if it is observed in a control.
STRONG_BASELINE_DIP	Invalid	A strong drop in the baseline for the raw data fluorescence is detected in the amplification curve.	Repeat the sample or the run if it is observed in a control.
STRONG_NOISE	Invalid	Strong noise is detected outside the growth phase of the amplification curve.	Repeat the sample or the run if it is observed in a control.
STRONG_NOISE_IN_ GROWTH_PHASE	Invalid	Strong noise is detected in the growth (exponential) phase of the amplification curve.	Repeat the sample or the run if it is observed in a control.
WAVY_BASE_ FLUORESCENCE	Invalid	Wavy baseline for the raw data fluorescence detected in the amplification curve.	Repeat the sample or the run if it is observed in a control.

Note: For samples replicates presenting an invalidating flag the C_T values are displayed between square brackets for information but the invalid PMR result is not displayed. And the mean PMR of both replicate is not displayed.

Troubleshooting guide

This troubleshooting guide may be helpful in solving any problems that may arise in the PITX2 promoter 2 PMR determination using the *therascreen* PITX2 RGQ PCR Kit. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

For troubleshooting information relating to Deparaffinization Solution (cat. no. 19093), the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) and EpiTect Fast DNA Bisulfite Kit (cat. no. 59824 or 59826), please refer to the respective kit handbooks.

For troubleshooting information relating to the Rotor-Gene Q MDx instrument and Rotor-Gene AssayManager v2.1 software, please refer to the respective user manuals.

		Comments and suggestions
Low yield of gDNA		
	The purified gDNA amount is below the 400 ng recommended for performing the *therascreen* PITX2 RGQ PCR Kit workflow	An input of 200 ng can be used, however the risk of an invalid result due to "low input" flag is higher.
Assay invalid due to REFlow and/or REF50 invalid(s)		
a)	One component of the reaction mix not added	Check that the reaction mix has been prepared correctly (Table 3, page 36). Check that all components of the qPCR reaction mix have been added. Repeat the PCR run.
b)	Error in Rotor-Gene Q MDx instrument	Check instrument maintenance logs. For example, lens misalignment may lead to higher background. If lens alignment is not part of your maintenance plan, please contact QIAGEN Technical Services for more information and potential intervention.
c)	Error in Rotor-Gene Q MDx instrument accessories	The 72-Well Rotor may be incorrectly locked. Repeat the PCR run.

		Comments and suggestions
d)	Reaction mix has degraded	Kit has been frozen/thawed more than four times, or the kit contents were not stored at –30°C to –15°C, or the PPM or reaction mix were not protected from light. Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit. Repeat the PCR run.
e)	Strip tube and/or sample ID inversion	Check the pipetting scheme and setup of the reaction. Repeat the PCR run.
f)	Controls missing or loaded in an incorrect position	Make sure that the correct control is loaded in the correct position.
g	Poor mixing of control samples	The thawing of the controls was incomplete before loading or the mixing of controls with the reaction mix (pipetting up and down) was not performed correctly. Repeat the PCR run.
h)	Incorrect pipetting volume	Check the pipetting scheme and setup of the reaction. Check that a 4 µl volume of control and a 16 µl volume of qPCR reaction mix have been added. Perform a visual inspection of all pipetted volumes. Check the pipets and if necessary, recalibrate them before repeating the qPCR step.
i)	Inefficient tube closing	The tube was not efficiently capped, leading to evaporation during the qPCR run.
Assay invalid due to no-template control (NTC) or negative control (NC) invalid(s)		
a)	Cross-contamination or contamination of reagents	Always handle samples, kit components and consumables in accordance with recommended practices to prevent carryover of contamination. Make sure tips are changed between pipetting different reagents or when loading different tubes. Prepare the qPCR reaction mix with dedicated material (pipets, tips, etc.). Prepare the qPCR reaction mix and NTC reaction in a dedicated area where no DNA matrices (DNA, plasmid or PCR products) are introduced. Repeat the PCR run.
b)	One component of the reaction mix not added	Check that the reaction mix has been prepared correctly (Table 3, page 36). Check that all components of the qPCR reaction mix have been added. Repeat the PCR run.
c)	Strip tube and/or sample ID inversion	Check the pipetting scheme and setup of the reaction. Repeat the qPCR run.
d)	Reaction mix or probes have degraded	Store kit contents at –30°C to –15°C and protect the PPM tube from light. Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit. Repeat the PCR run.

		Comments and suggestions
e)	Incorrect amplification curve (artefacts)	Check the corresponding amplification for unusual curves (e.g., straight line). Repeat the qPCR run.
Invalid sample due to "Low input" flag		
a)	FFPE block conditions	Check transport/storage conditions of the used FFPE block.
b)	FFPE block preparation	Make sure sample was fixed in 4–10% formalin. Check that one to two sections of 5 µm thickness were cut to reach 100 mm^2 of tissue surface to ensure sufficient cells.
c)	Pipetting volume may be incorrect	Check the pipetting scheme and setup of the reaction. Check that a 4 µl volume of sample and a 16 µl volume of qPCR reaction mix have been added. Perform a visual inspection of all pipetted volumes. Check the pipets and if necessary, recalibrate them before repeating the qPCR step.
d)	gDNA purification or bisulfite conversion failed	Check whether the workflow control gave expected results. Make sure that the *therascreen* PITX2 RGQ PCR Kit workflow protocol was followed, as described above. Make sure that kits are not expired, reagents are correctly prepared (e.g., ethanol added, avoid transferring precipitates to the MinElute DNA spin column). Check room temperature is not below 15°C during manipulation to avoid crystallization of buffers. Check transport and storage conditions. Repeat the entire workflow.
e)	Strip tube and/or sample ID inversion	Check the pipetting scheme or if an empty tube is at the correct position and check the setup of the reaction. Repeat the PCR run.
f)	Poor gDNA sample quality	Repeat with more material. Up to 1000 ng DNA input measured using an OD 260 nm method can be used.
g)	Inefficient tube closing	The tube was not efficiently capped, leading to evaporation during the qPCR run.
h)	Sample not loaded	Make sure the sample was loaded in both wells.
Invalid sample due to "Delta PMR high"		
a)	Reaction mix has degraded	Store kit contents at –30°C to –15°C and protect the reaction mixes from light. Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit. Repeat the PCR run.

		Comments and suggestions
b)	Pipetting volume may be incorrect	Check the pipetting scheme and setup of the reaction. Check that a 4 µl volume of control/sample and a 16 µl volume of qPCR reaction mix have been added. Perform a visual inspection of all pipetted volumes. Check the pipets and if necessary, recalibrate them before repeating the qPCR step.
c)	Strip tube and/or sample ID inversion	Check the pipetting scheme and setup of the reaction. Repeat the PCR run.
d)	The amplification curve may be incorrect	Check the corresponding amplification plot for unusual curves. Repeat the invalid sample.
e)	Late signal due to low amount leading to more variable PMR results	Repeat with more material. Up to 1000 ng DNA input measured using an OD 260 nm method can be used.
f)	Poor mixing of control samples	The thawing of the control was incomplete before loading or the mixing of control with the reaction mix (pipetting up and down) was not performed correctly. Repeat the PCR run.
g)	Inefficient tube closing	The tube was not efficiently capped, leading to evaporation during the qPCR run.

Invalid sample due to "one replicate invalid"

a)	Not enough material (close to the limit)	Repeat with more material. Up to 1000 ng DNA input measured using an OD 260 nm method can be used.
b)	Pipetting volume may be incorrect	Check the pipetting scheme and setup of the reaction. Check that a 4 µl volume of control/sample and a 16 µl volume of qPCR reaction mix have been added. Perform a visual inspection of all pipetted volumes. Check the pipets and if necessary, recalibrate them before repeating the qPCR step.
c)	Strip tube and/or sample ID inversion	Check the pipetting scheme and setup of the reaction. Repeat the PCR run.
d)	The amplification curve may be incorrect	Check the corresponding amplification plot for unusual curves. Repeat the PCR run.
e)	Poor mixing of control samples	The thawing of the control was incomplete before loading or the mixing of control with the reaction mix (pipetting up and down) was not performed correctly. Repeat the PCR run.
f)	Inefficient tube closing	The tube was not efficiently capped, leading to evaporation during the qPCR run.

		Comments and suggestions
g)	One well of both sample wells not loaded	Make sure the sample was loaded in both wells.
Run failure due to inconsistent fluorescence signal in controls and/or samples (across all tubes)		
	Error in Rotor-Gene Q MDx instrument accessories	Check instrument maintenance logs. The 72-Well Rotor may be faulty.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *therascreen* PITX2 RGQ PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Quality control of the complete kit has been performed on a Rotor-Gene Q MDx 5plex HRM instrument. This kit is manufactured according to ISO 13485 standard. Certificates of analysis are available on request at **www.qiagen.com/support**.

Limitations

The kit is intended for professional use. The system performance has been established using formalin-fixed paraffin-embedded (FFPE) breast cancer tissues only.

The *therascreen* PITX2 RGQ PCR Kit is validated only for FFPE tissue from high-risk estrogen receptor-positive, HER2-negative, lymph node-positive breast cancer patients.

The product is to be used only by qualified users, such as technicians and physicians, who are trained in molecular biology techniques and in vitro diagnostic procedures.

This kit should be used following the instructions given in this handbook, in combination with validated instruments listed in “Materials Required but Not Provided”, page 11.

All reagents supplied in the *therascreen* PITX2 RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same kit.

Attention should be paid to expiration dates printed on the box label. Do not use expired components.

The *therascreen* PITX2 RGQ PCR Kit is validated only for use with Deparaffinization Solution (cat. no. 19093), or xylene-ethanol or histolemon-ethanol, the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) and the EpiTect Fast DNA Bisulfite Kit (cat. no. 59824 or 59826).

Only the Rotor-Gene Q MDx 5plex HRM (for PCR) has been validated.

Any off-label use of this product and/or modification of the components will void QIAGEN’s liability.

Any diagnostic must be generated in conjunction with other clinical or laboratory findings.

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

Performance Characteristics

Where biological samples were used in all the studies in this section, the deparaffinization step prior to gDNA extraction was performed using QIAGEN Deparaffinization Solution. Please note, however, that equivalency between Deparaffinization Solution and xylene or histolemon has been demonstrated.

Limit of blank

Limit of blank (LoB) was determined based on the data point corresponding to the lower and upper 95% percentile of the results obtained with PMR 0 and PMR 100 samples, respectively, as described in CLSI/NCCLS EP17-A2 (14). Tested samples correspond to artificial samples generated with different copy numbers (100, 200, 500 and 750 copies) of non-target plasmid (target of the other probe) in a background of unconverted gDNA. The LoB results are based on 64 and 63 measurements for the probes targeting former methylated sequences and 64 and 61 measurements for the probes targeting former unmethylated sequences, per batch, using two different kit pilot batches. The LoB results are summarized in Table 8.

Table 8. Summary of the limit of blank results

	PMR 0 samples		PMR 100 samples	
	Measured LoB, PMR	**Final LoB, PMR**	**Measured LoB, PMR**	**Final LoB, PMR**
Batch 1	0		99	
Batch 2	0	0	98	98

Limit of detection

Following the Probit approach described in the CLSI/NCCLS EP17-A2 (14), the limit of detection (LoD) is the PMR value at which 95% of measurements exceed the LoB. LoD was determined for each probe at the minimal gDNA input of 200 ng and at the recommended gDNA input of 400 ng using two different *therascreen* PITX2 RGQ PCR Kit pilot batches. Three samples were per tested input (200 ng and 400 ng) and for each probe. These samples were produced at different total amplifiable copy numbers i.e., 50, 100 and 150 copies for 200 ng gDNA input and 100, 200 and 300 copies for 400 ng gDNA input. Therefore, 60 samples were produced in total for the LoD study. Tested samples correspond to artificial samples produced from mixes of target and non-target plasmids (giving five different theoretical PMR levels per sample) in a background of unconverted gDNA. For each probe tested at each input, the LoD results are obtained from at least 20 measurements per *therascreen* PITX2 RGQ PCR Kit pilot batch for each PMR level of each sample. The LoD for low PMR samples is 4 and for high PMR samples is 92 (Table 9).

Table 9. Summary of the limit of detection results

Sample	Input (ng)	Batch	LOD value	Lower limit	Upper limit
Low PMR sample	200	Batch 1	3	3	5
	200	Batch 2	3	3	4
	400	Batch 1	3	2	6
	400	Batch 2	4	3	6
High PMR sample	200	Batch 1	92	92	92
	200	Batch 2	>92	N/A	N/A
	400	Batch 1	94	93	95
	400	Batch 2	95	93	95

N/A: Not applicable.

DNA input

Five different gDNA inputs (50, 100, 200, 400 and 1000 ng) were tested, each presenting seven different PMR levels (0, 5, 10, 25, 40, 50 and 75). The maximal gDNA input was defined at 1000 ng for technical reasons, as a higher quantity would be difficult to obtain in real-life situation. The gDNA input range acceptable for the *therascreen* PITX2 RGQ PCR Kit was determined by Deming regression using one *therascreen* PITX2 RGQ PCR Kit pilot batch and one Rotor-Gene Q MDx instrument.

The study demonstrated:

- The recommended gDNA input to be used with the *therascreen* PITX2 RGQ PCR Kit is 400 ng of gDNA
- The minimal acceptable gDNA input is 200 ng gDNA and the maximal gDNA input is 1000 ng.
- The minimal gDNA input should be tested only if the recommended input cannot be reached, as the risk of obtaining an invalid result due to a low input is increased, leading to a high chance of retest. The maximal gDNA input is recommended to be tested when a gDNA input of 400 ng gives an invalid PMR result, for example, due to a low input flag.

Linearity

The linearity study was conducted in accordance with the CLSI/NCCLS EP6-A (15). The linearity of the *therascreen* PITX2 RGQ PCR Kit was determined on seven samples at different levels of PMR (0, 5, 10, 25, 40, 50 and 75) prepared from five different gDNA inputs (including 200, 400 and 1000 ng). The study was performed using one *therascreen* PITX2 RGQ PCR Kit pilot batch on one Rotor-Gene Q MDx instrument by one operator. The study showed that linearity is confirmed with samples for which PMR is between 5 and 50 at the acceptable gDNA inputs (i.e., 200–1000 ng).

Repeatability and reproducibility

Repeatability and reproducibility of the *therascreen* PITX2 RGQ PCR Kit were determined in the course of a single-site precision study and a multi-site precision study, both conducted in accordance with the CLSI/NCCLS EP5-A3 (16), see Table 10 and Table 11. The precision studies were performed on three biological samples giving a very low, low and high PMR (9, 16 and 77, respectively). In the single-site precision study the sources of variability were assessed on 23 non-consecutive working days by three operators using three different *therascreen* PITX2 RGQ PCR Kit pilot batches and three Rotor-Gene Q MDx instruments. Two measurements per sample were obtained on each run. Two identical runs per day were performed with at least two hours between runs. The run time has varied throughout the work day, maintaining at least two hour separation between runs to introduce more randomness into the testing. The multi-site precision study was conducted at three different sites where a single operator used a single *therascreen* PITX2 RGQ PCR Kit pilot batch on a single Rotor-Gene Q MDx instrument. Five measurements per sample were obtained on each run. One run per day was performed at each site alternating between morning and afternoon.

Table 10. Summary of the single-site precision study results

	Source of variability (%)						
Sample	**Intra-run**	**Run**	**Batch**	**Instrument**	**Operator**	**Day**	**Total**
Very low PMR	12.29	4.20	0.00	12.54	0.00	9.49	20.39
Low PMR	19.99	0.00	0.00	3.09	0.00	8.04	21.76
High PMR	3.90	0.00	0.00	0.00	0.00	1.64	4.23

Table 11. Summary of the multi-site precision study results

	Source of variability (%)			
Sample	**Inter-run**	**Day**	**Site**	**Total**
Very low PMR	13.90	8.43	4.72	16.93
Low PMR	28.72	0.00	0.00	28.72
High PMR	4.25	0.00	1.77	4.61

Interfering substances

The Interfering Substances Study was conducted in accordance with CLSI/NCCLS EP7-A2 (17). The final concentration of each substance used throughout the sample preparation workflow was first evaluated (taking into account the dilution effect at each step). Based on the relevance of the final concentration of each substance into the starting material for *therascreen* PITX2 RGQ PCR Kit (i.e., bisDNA), all potential interfering substances were tested using one *therascreen* PITX2 RGQ PCR Kit pilot batch. Results have not shown any interfering impact from the substances used throughout the workflow of the *therascreen* PITX2 RGQ PCR Kit (Table 12).

Table 12. Interfering substances tested

Substance tested	Final volume tested in 30 µl
Deparaffinization Solution	1.4×10^{-15}
Histolemon	2.10×10^{-20}
Ethanol (96–100%)	0.50
Bisulfite Solution	7.2×10^{-09}
DNA Protect Buffer	2.26×10^{-10}
Buffer BL	3.44×10^{-08}
Buffer BW	0.1102
Buffer BD	0.002

Cross-contamination

The cross-contamination between negative and positive samples was assessed using one *therascreen* PITX2 RGQ PCR Kit pilot batch and two Rotor-Gene Q MDx instruments. Six conditions were tested using NTC and/or Negative Control as negative samples with or without a bisDNA sample, giving a low PMR as positive sample. The cross-contamination was evaluated at 1.3%.

In-use timeframe

The maximal timeframe between the plate preparation and the launch of the qPCR run was determined using one *therascreen* PITX2 RGQ PCR Kit pilot batch and one artificial samples generated from target and non-target plasmids giving a medium PMR. The maximal acceptable time frame is 24 hours; however, it is recommended to launch the *therascreen* PITX2 RGQ PCR Kit qPCR run as soon as possible after preparing the plate (i.e., after loading all samples to be tested).

Clinical cut-off validation

Prospective analysis was performed to validate the *therascreen* PITX2 RGQ PCR Kit clinical cut-off with FFPE tissue from 145 high-risk lymph node-positive, estrogen receptor-positive, HER2-negative breast cancer patients. Specimens included in the study were archived FFPE tissue that met the following criteria:

- Histologically confirmed invasive breast cancer
- Primary tumor stage pT1, pT2 and pT3
- Histologically confirmed lymph node involvement (≥N1)
- Standard adjuvant anthracycline-based chemotherapy
- No dose-dense therapy
- No other primary systemic chemotherapy (no additional taxanes), except hormonal therapy

PMR was measured for each sample using the final kit format and handbook instructions.

Disease-free survival (DFS) was the primary endpoint and defined as the time from primary surgery to the first documented DFS event. The date of primary surgery was considered as the follow-up index date. DFS events included recurrence of cancer (local disease recurrence or distant metastasis), secondary malignancies considered life-threatening and death of any cause. For patients who died without recurrence of cancer, competing risks analysis according to Fine and Gray was applied (13).

Analysis was performed for DFS follow-up time censored at 10 years. Survival curves were calculated according to the incidence function (13). The PITX2 pre-defined cut-off value of PMR 12 demonstrated a statistical significant differentiation between the two groups for the primary endpoint DFS with a significance level $p<0.05$ (two-sided; alpha value). Therefore, the methylation state of the PITX2 promoter assessed with *therascreen* PITX2 RGQ PCR Kit assay has shown predictive value for anthracycline-based chemotherapy in high-risk lymph node-positive, estrogen receptor-positive, HER2-negative breast cancer patients.

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
 <N>	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	CE mark for European conformity
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
Rn	Revision of the Handbook where n is the revision number
	Manufacturer
	Consult instructions for use
	Keep away from sunlight
	Caution

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Product	Contents	Cat. no.
***therascreen* PITX2 RGQ PCR Kit — for the determination of the percent methylation ratio (PMR) in the PITX2 promoter 2**		
therascreen PITX2 RGQ PCR Kit (8)	For 8 reactions: PITX2 RGQ PCR Master Mix, PITX2 RGQ PCR Primer Probe Mix, PITX2 RGQ PCR Reference 50, PITX2 RGQ PCR Reference Low, PITX2 RGQ PCR Negative Control and PITX2 RGQ PCR NTC	873211
Rotor-Gene Q MDx Platform, software and accessories		
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and HRM 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 5plex HRM System	Real-time PCR cycler and HRM 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

Product	Contents	Cat. no.
Rotor-Gene AssayManager v2.1	Software for routine testing in combination with Rotor-Gene Q and Rotor-Gene Q MDx instruments.	9024203
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
72-Well Rotor	For holding Strip Tubes and Caps 0.1 ml; requires Locking Ring 72 Well Rotor	9018903
Locking Ring 72-Well Rotor	For locking Strip Tubes and Caps, 0.1 ml in the 72-Well Rotor	9018904
Rotor Holder	Metal free-standing holder for assembling tubes and Rotor-Discs® into rotors	9018908
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Related products		
QIAamp DSP DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Buffers, Collection tubes	60404
Deparaffinization Solution (16 ml)	16 ml Deparaffinization Solution	19093

Product	Contents	Cat. no.
EpiTect Fast DNA Bisulfite Kit (200)	For 200 DNA conversion: Bisulfite solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59826
EpiTect Fast DNA Bisulfite Kit (50)	For 50 DNA conversion: Bisulfite solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59824

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