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CALR RGQ PCR Kit Handbook

For research use only

Not for use in diagnostic procedures

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



24

REF

674013



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Intended Use

The CALR RGQ PCR Kit is intended for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of products.

We recommend all users of QIAGEN products adhere to the National Institutes of Health (NIH) guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Principle of the Procedure

The CALR RGQ PCR Kit is a real-time PCR test. The kit uses quantitative real-time PCR (qPCR) techniques for the qualitative detection and/or identification of somatic mutations in the region c.1091_1162 (cDNA annotation) of exon 9 in the gene encoding calreticulin (CALR; GenBank® Accession Number CR457070) (1, 2). The kit provides reagents to perform seven separate PCR amplification reactions in the same run for the identification of the two major CALR mutations (Type 1 and Type 2) and the detection of additional minor variants in genomic DNA extracted from human peripheral whole blood.

The use of real-time PCR permits the accurate detection of a targeted DNA sequence during the exponential phase of the amplification process. Real-time PCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during PCR cycling. At present, three main types of qPCR techniques are available: qPCR analysis using SYBR® Green I Dye, qPCR analysis using hydrolysis probes and qPCR analysis using hybridization probes.

This assay exploits the qPCR oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. Another dye-linked oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye (F) and a downstream 3' dye-free quencher (Q), hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes 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 1). During the polymerization phase, the 5'→3' exonuclease

activity of the DNA polymerase cleaves the probe leading to the release of quencher dye 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 (see Figure 1).

The increase in fluorescence signal is detected only if the target sequence is complementary to the primers and probe and hence amplified during PCR.

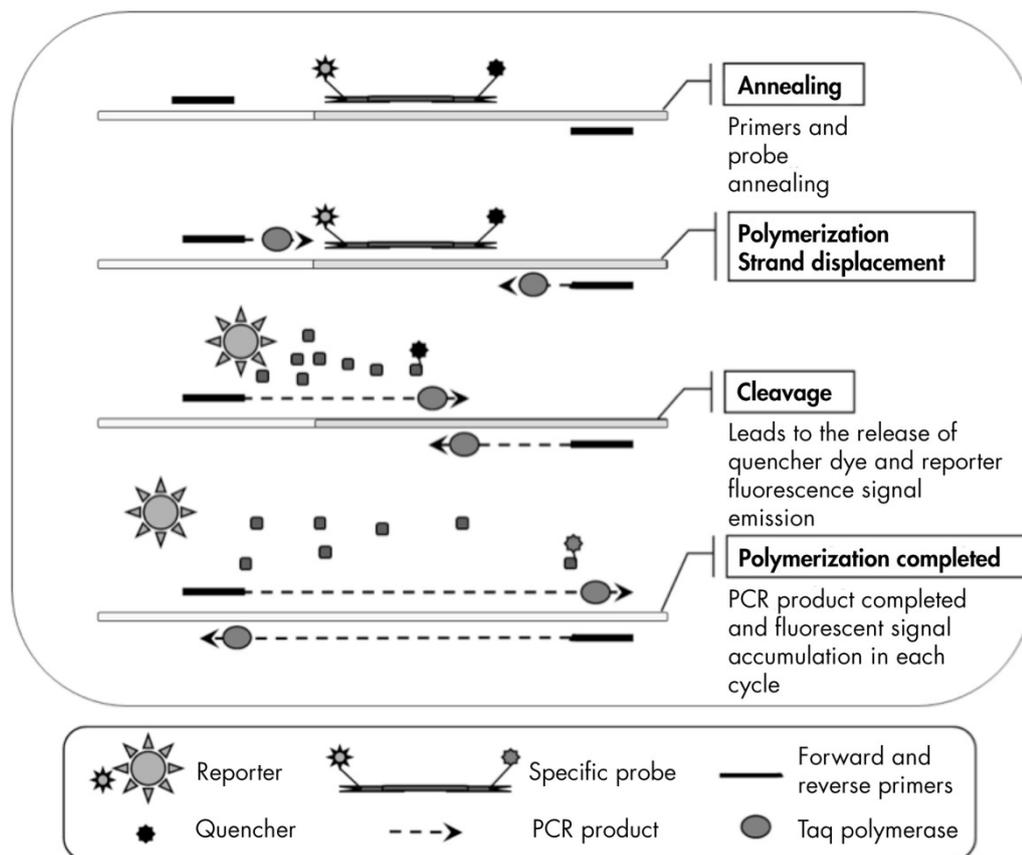


Figure 1. Principle of real-time PCR reactions.

Identification of the two major *CALR* mutations

To identify Type 1 and Type 2 *CALR* mutations, an allele-specific amplification is achieved by ARMS (Allele Refractory Mutation System) technology which exploits the specific hybridization of primers to a complementary sequence and the ability of the DNA polymerase to distinguish between a match and a mismatch at the 3' end of a PCR primer.

When the PCR primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low-level background amplification occurs (Figure 2).

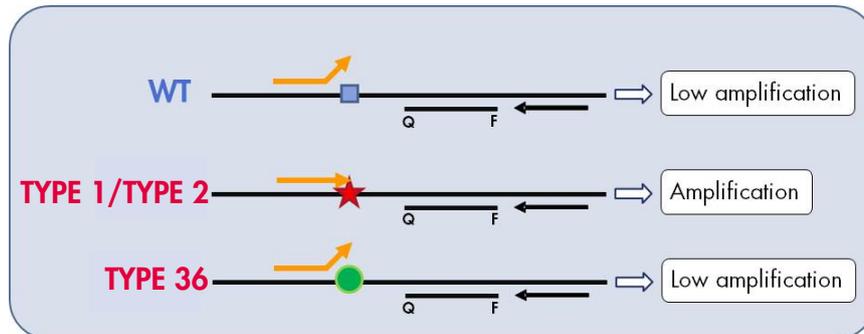


Figure 2. Identification of Type 1 and Type 2 *CALR* mutations by ARMS PCR. WT: wild-type; Q – F: BHQ[®] – FAM[™] double-dye probe; ⇄ forward primer (orange) and reverse primer (black).

Detection of minor variants of *CALR* mutations

For the detection of minor variants of *CALR* mutations, primers and probes are combined in the reaction mixes with an additional oligonucleotide that is 3'-blocked with the addition of a phosphate group (a so-called CLAMP oligonucleotide). The CLAMP oligonucleotide is specific to a wild-type targeted sequence and, when annealed, inhibits elongation of the PCR product (PCR clamping). When the PCR template contains the wild-type sequence, the CLAMP hybridizes before the PCR primer and there is no or low extension by the DNA polymerase. When a mutated target sequence is present, the CLAMP does not hybridize or hybridizes poorly, the PCR primer binds and amplification proceeds (Figure 3).

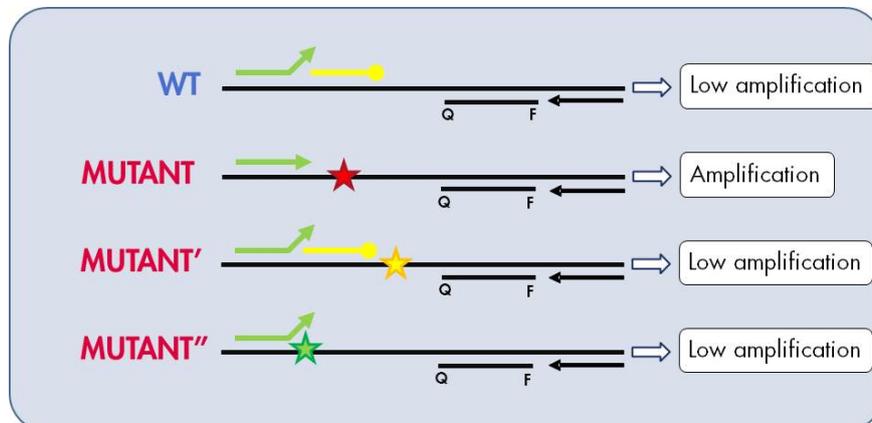


Figure 3. Detection of *CALR* minor mutations. WT: wild-type; Q – F: BHQ – FAM double-dye probe; ⇄ forward primer (green) and reverse primer (black); —○: 3'-phosphate oligonucleotide (CLAMP oligonucleotide; yellow).

Internal amplification control (IAC) in all reaction mixes

To validate and control the qPCR reaction in the presence of human genomic DNA (gDNA) template, each CALR reaction mix includes primers and probe to detect an endogenous sequence of the *ABL1* human gene. This control sequence is amplified in a multiplex PCR reaction of all CALR mutant and wild-type DNA and is labeled with hexachlorofluorescein (HEX™) to distinguish it from the fluorescein amidite (FAM) labeled amplicons in the mutation reactions. For both probes, the quencher is Black Hole Quencher® (BHQ-1).

Materials Provided

Kit contents

CALR RGQ PCR Kit		(24)
Catalog number		674013
Number of reactions		24
Color	Identity	Volume
Yellow	CALR Reaction Mix Type 1	850 μ l
Yellow	CALR Reaction Mix Type 2	850 μ l
Purple	CALR Reaction Mix CLAMP 1	850 μ l
Purple	CALR Reaction Mix CLAMP 2	850 μ l
Purple	CALR Reaction Mix CLAMP 3	850 μ l
Purple	CALR Reaction Mix CLAMP 4	850 μ l
Purple	CALR Reaction Mix CLAMP 5	850 μ l
Green	CALR Wild-Type Control	145 μ l
Red	CALR Mutant Control	145 μ l
Mint	Taq DNA Polymerase	85 μ l
White	TE Buffer	1.9 ml

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.

- Dedicated pipets (adjustable) (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 DNA handling including PCR template loading.

- Nuclease-free, aerosol-resistant, sterile PCR pipet tips with hydrophobic filters
- 1.5 ml or 2.0 ml nuclease-free PCR tubes
- Disposable gloves
- Vortex mixer
- Spectrophotometer

Additional equipment and materials for manual DNA extraction

- QIAamp® DSP DNA Blood Mini Kit (cat. no. 61104)
- Ethanol (96-100%)

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

- Heating block for lysis of samples at 56°C
- Benchtop centrifuge with rotor for 0.5 ml/1.5 ml/2.0 ml reaction tubes (capable of attaining 13,000–14,000 rpm)

Additional equipment and materials for automated DNA extraction

- QIAasympphony® SP instrument (cat. no. 9001297), software version 4.0 or higher, and provided accessories including Blood_200_V7_DSP protocol
- Tube Insert 3b (cat. no. 9242083)
- QIAasympphony DSP DNA Mini Kit (cat. no. 937236)
- Sample Prep Cartridges, 8-well (cat. no. 997002)
- 8-Rod Covers (cat. no. 997004)
- Filter-Tips, 1500 µl (cat. no. 997024)
- Filter-Tips, 200 µl (cat. no. 990332)

- Elution Microtubes CL (cat. no. 19588)
- Tip disposal bags (cat. no. 9013395)
- Microtubes 2.0 ml Type H (Sarstedt®, cat. no. 72.694)

Additional equipment and materials for PCR on Rotor Gene Q

- Rotor-Gene Q 5plex HRM (cat. no. 9001580), software version 2.3 or higher, and provided accessories
- Loading Block for 72 x 0.1 ml Tubes (cat. no. 9018901)
- 72-Well Rotor (cat. no. 9018903)
- 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 (cat. no. 981103 or 981106)
- Ice or a cooling block

Warnings and Precautions

Warnings

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 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 relating to the extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) and QIASymphony DSP DNA Mini Kit (cat. no. 937236), please refer to the respective handbooks.

WARNING Risk of personal injury



Do not add bleach or acidic solutions to the sample preparation waste.

Buffers in the reagent cartridge of the QIASymphony DSP DNA Mini Kit contain guanidine salts which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilled, clean with suitable laboratory detergent and water. If the spilled liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Precautions

Use of real-time PCR tests requires good laboratory practices, including maintenance of equipment dedicated to molecular biology and compliance with applicable regulations and relevant standards.

This kit is intended for research use only. Not for use in diagnostic procedures. 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 CALR 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 than 25 μ l.
- 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 performances.
- Refer to the *Rotor-Gene Q User Manual* for additional warnings, precautions and procedures.
- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Do not use expired or incorrectly stored components.
- Reaction mixes may be altered if exposed to light.
- Use extreme caution to prevent contamination of the mixes with the materials that are contained in the CALR Mutant Control and CALR Wild-Type Control reagents.
- Use extreme caution to prevent DNA or PCR product carry-over contamination resulting in a false positive signal.
- Use extreme caution to prevent contamination by DNase, which might cause degradation of the template DNA.
- Use individual, dedicated pipets for setting up reaction mixes and adding templates.
- Do not open the Rotor-Gene Q instrument until the run is finished.
- Do not open Rotor-Gene Q 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.

We therefore recommend the following:

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials) and wear 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 pre-PCR master 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 TE Buffer in NTC tubes and close them. Add samples to be tested, CALR Mutant Control and CALR Wild-Type Control reagents in a separate room with specific material (pipets, tips, etc.).

Reagent Storage and Handling

The CALR RGQ PCR Kit is shipped on dry ice. If any component of the CALR 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).

The CALR RGQ PCR Kit should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. When stored under the specified storage conditions, the CALR 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 stated expiration date shown on the packaging. Repeated thawing and freezing should be avoided.

For storage and handling information relating to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) or QIASymphony DSP DNA Mini Kit (cat. no. 937236), please 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

Whole blood

The CALR RGQ PCR Kit is for use with genomic DNA samples extracted from whole blood specimens anti-coagulated with 2K-EDTA. Whole blood may be stored as follows:

- At 2°C to 8°C for no more than 96 hours
- At 15°C to 25°C for no more than 96 hours
- Frozen at –30°C to –15°C for no more than 1 month

Genomic DNA samples

Genomic DNA may be stored at 2°C to 8°C for 1 week post-extraction or at –30°C to –15°C if long term storage is required.

Genomic DNA Extraction and Preparation

The CALR RGQ PCR Kit has been validated in combination with the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) for manual extraction or the QIAAsymphony SP instrument in combination with the QIAAsymphony DSP DNA Mini Kit (cat. no. 937236) for automated extraction.

Make sure that gDNA extraction reagents have not expired and have been transported and stored under the correct conditions.

Manual gDNA extraction with the QIAamp DSP DNA Blood Mini Kit

Manual gDNA extraction is performed with the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) according to the *QIAamp DSP DNA Blood Mini Kit Handbook*.

Things to do before starting

- Equilibrate blood samples to room temperature (15–25°C), and make sure that they are well homogenized.
- Prepare the Lysis Buffer
If a precipitate has formed in Lysis Buffer (AL), dissolve by incubating at 56°C.
- Prepare QIAGEN Protease
Add 1.2 ml Protease Solvent (PS) to the vial of lyophilized QIAGEN Protease (QP) and mix carefully. To avoid foaming, mix by inverting the vial several times. Make sure that the QP is completely dissolved.
Note: Once dissolved in PS, QP is stable for up to 2 months when stored at 2–8°C. To prolong the life of the protease, storage at –20°C is recommended, but repeated freezing and thawing should be avoided. For this reason, storage of aliquots of QP is recommended.
- Prepare Wash Buffer 1
Using a measuring cylinder, add 25 ml ethanol (96–100%) to the bottle containing 19 ml Wash Buffer 1 (AW1) concentrate. Store reconstituted AW1 at room temperature (15–25°C).
Note: Always mix reconstituted AW1 by inverting the bottle several times before starting the procedure.

- Prepare Wash Buffer 2

Using a measuring cylinder, add 30 ml ethanol (96–100%) to the bottle containing 13 ml Wash Buffer 2 (AW2) concentrate. Store reconstituted AW2 at room temperature (15–25°C).

Note: Always mix reconstituted AW2 by inverting the bottle several times before starting the procedure.

- Prepare the Elution Buffer

One bottle of Elution Buffer (AE) is provided with the kit. To prevent contamination of AE, we strongly recommend using pipet tips with aerosol barriers when pipetting AE from the bottle and replacing the cap of the bottle immediately afterwards.

Equilibrate Buffer AE to room temperature (15–25°C).

- Set a heating block to 56°C for use in step 4.

Procedure

1. Pipet 20 µl of protease QP into a lysis tube (LT).

Note: Check the expiration date of the reconstituted protease before use.

2. Add 200 µl of blood sample to the lysis tube.

3. Add 200 µl of Lysis Buffer (AL) to the lysis tube, close the lid and mix using a pulse-vortex for 15 seconds and centrifuge briefly.

Note: To ensure efficient lysis, it is essential that the sample and AL are mixed thoroughly to yield a homogenous solution.

Note: Since Buffer AL has a high viscosity, be sure to add the correct volume of AL by pipetting carefully and by using a suitable pipet.

Important: Do not add QP directly to Buffer AL.

4. Incubate at 56°C (±1°C) for 10 minutes (±1 minute).

5. Centrifuge the lysis tube for about 5 seconds at full speed to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96–100%) to the lysis tube, close the lid and mix thoroughly using a pulse-vortex for ≥15 seconds.

7. Centrifuge the lysis tube for ≥5 seconds at full speed to remove any drops of liquid from the inside of the lid.

8. Carefully apply the entire lysate from step 7 to the QIAamp Mini spin column without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.

Note: If processing several samples, open only one lysis tube at a time.

9. Close the lid of the QIAamp Mini spin column, and centrifuge at approximately 6000 x g (8000 rpm) for 1 minute.

10. Place the QIAamp Mini spin column in a clean wash tube (WT) and discard the tube containing the filtrate.
Note: If the lysate has not completely passed through the membrane after centrifugation at $6000 \times g$ (8000 rpm), centrifuge again at full speed (up to $20,800 \times g$) for 1 minute.
Note: If the lysate still does not pass through the membrane during centrifugation, discard the sample and repeat the isolation and purification with new sample material.
11. Carefully open the QIAamp Mini spin column, and add 500 μ l Buffer AW1 without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.
12. Close the lid of the QIAamp Mini spin column, and centrifuge at approximately $6000 \times g$ (8000 rpm) for 1 minute.
13. Place the QIAamp Mini spin column in a clean wash tube, and discard the tube containing the filtrate.
14. Carefully open the QIAamp Mini spin column, and add 500 μ l Buffer AW2 without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.
15. Close the lid of the QIAamp Mini spin column, and centrifuge at full speed (approximately $20,000 \times g$, or 14,000 rpm) for 1 minute.
16. Place the QIAamp Mini spin column in a clean wash tube and discard the tube containing the filtrate.
17. Centrifuge at full speed (approximately $20,000 \times g$, or 14,000 rpm) for 3 minutes to dry the membrane completely.
18. Place the QIAamp Mini spin column in a clean elution tube (ET) and discard the wash tube containing the filtrate.
19. Carefully open the lid of the QIAamp Mini spin column and apply 50 to 200 μ l Buffer AE to the center of the membrane.
Note: Lower elution volumes increase the final DNA concentration in the eluate significantly, but slightly reduce the overall DNA yield.
20. Close the lid and incubate at room temperature (15–25°C) for 1 minute.
21. Centrifuge at approximately $6000 \times g$ (8000 rpm) for 1 minute to elute the DNA.
22. Store the gDNA sample under appropriate conditions.
23. Discard used sample tubes, plates and waste according to your local safety regulations.

Automated gDNA extraction with the QIASymphony DSP DNA Mini Kit

Automated gDNA extraction is performed with the QIASymphony SP instrument in combination with the QIASymphony DSP DNA Mini Kit (cat. no. 937236). Follow the instructions in the *QIASymphony DSP DNA Kit Handbook*. Select the **Blood_200_V7_DSP** protocol on the QIASymphony

Note: The following features of the protocol are specific to extraction of gDNA from blood for analysis with the CALR RGQ PCR Kit:

- Transfer 300 µl of whole blood into a microtube (2.0 ml Type H, Sarstedt, cat. no. 72.694).
- Elution volume and output position is 100 µl for the whole blood protocol.

Important points before starting

- Whole blood total volume to be extracted is 200 µl (plus 100 µl dead volume).
- Ensure that you are familiar with operating the QIASymphony SP. Refer to the user manuals supplied with your instrument for operating instructions.
- Optional maintenance is not mandatory for instrument function but is highly recommended to reduce risk of contamination.
- Before using a reagent cartridge for the first time, check that Buffers QSL1 and QSB1 do not contain a precipitate.

If necessary, remove the troughs containing Buffers QSL1 and QSB1 from the reagent cartridge and incubate for 30 minutes at 37°C with occasional shaking to dissolve precipitate. Make sure to replace the troughs in the correct positions.

If the reagent cartridge is already pierced, make sure that the troughs are sealed with Reuse Seal Strips and incubate the complete reagent cartridge for 30 minutes at 37°C with occasional shaking in a water bath.

- Try to avoid vigorous shaking of the reagent cartridge (RC) otherwise foam may be generated leading to liquid-level detection problems.

Things to do before starting

- Before starting the procedure, ensure that the magnetic particles are fully resuspended. Vortex the trough containing the magnetic particles vigorously for at least 3 minutes before first use.
- Make sure that the piercing lid is placed on the reagent cartridge and that the lid of the magnetic-particle trough has been removed or, if using a partially used reagent cartridge, make sure the Reuse Seal Strips have been removed.
- Make sure to open the enzyme tubes.
- If samples are bar-coded, orientate samples in the tube carrier so that the bar codes face the bar code reader at the left side of the QIASymphony SP.

Procedure

1. Close all drawers and the hood.
2. Switch on the QIASymphony SP; wait until the **Sample Preparation** screen appears and the initialization procedure has finished.
The power switch is located at the bottom-left corner of the QIASymphony SP.
3. Log on to the instrument.
4. Select the protocol to be run.
Choose the **Select All** button and select **DNA Blood** then **Blood_200_V7_DSP** for whole blood samples.
5. Ensure the "Waste" drawer is prepared properly. Perform an inventory scan of the "Waste" drawer, including the tip chute and liquid waste container. Replace the tip disposal bag if necessary.
6. Load the required elution rack into the "Eluate" drawer.
Do not load a 96-well plate onto "Elution slot 4".
Only use "Elution slot 1" with the corresponding cooling adapter.
When using a 96-well plate, make sure that the plate is in the correct orientation as incorrect placement may cause sample mix-up in downstream analysis.
7. Load the required reagent cartridge(s) and consumables into the "Reagents and Consumables" drawer.
Note: Make sure that pipetting tips are correctly fixed.
8. Perform an inventory scan of the "Reagents and Consumables" drawer.

9. Transfer 300 µl of the whole blood sample to be extracted into a microtube (2.0 ml Type H) and place this into the 3B 2 ml adapter on the tube sample carrier.

Load the sample tubes into the "Sample" drawer.

10. Using the touchscreen, enter the required information for each batch of samples to be processed:

- Sample information: Change the default tube format by choosing **Select All** and selecting **Sarstedt reference 72.694** from the **Tube Insert** sheet.
- Confirm the selected protocol: **Blood_200_V7_DSP**.
- Elution volume and output position: Select **100 µl** for the whole blood protocol.

Note: After information about the batch has been entered, the status changes from **LOADED** to **QUEUED**. As soon as one batch is queued, the **Run** button appears.

11. Start the run by pressing the **Run** button.

12. Read and confirm the message that appears.

Note: We recommend waiting beside the instrument until it has performed liquid level detection of the internal control tubes and the QIAasympphony SP carrier status changes to **RUNNING**.

Note: Do not pause or stop the run during processing (unless an emergency occurs), as this will lead to the samples being flagged as "unclear".

Note: It is possible to continuously load samples and add them to this run (until reagents are loaded). Press the **Run** button to start the purification procedure.

13. At the end of the protocol run, the status of the batch changes from **RUNNING** to **COMPLETED**.

Retrieve the elution rack containing the purified nucleic acids from the "Eluate" drawer.

We recommend removing the eluate plate from the "Eluate" drawer immediately after the run has finished. Depending on temperature and humidity, elution plates left in the QIAasympphony SP after the run is completed may experience condensation or evaporation.

14. Export the QIAasympphony SP result file: this report is generated for each elution plate.

14a. Insert the USB stick into one of the USB ports at the front of the QIAasympphony SP.

14b. Click on the **Tools** button.

14c. Select **File Transfer**.

14d. On the **In-/Output Files** tab, select **Results Files** and click **Transfer**.

Keep the name of file export, in the following format:

yyyy-mm-dd hh:mm:ss_Elution rack ID.

15. Check the **Validity of result** column for each sample on the QIAAsymphony SP result file.

- Valid and unclear status: proceed to DNA qualification and quantification.
- Invalid status: sample is rejected. Reprocess the extraction step.

16. If a reagent cartridge is only partially used, seal it with the provided Reuse Seal Strips and close tubes containing proteinase K with screw caps immediately after the end of the protocol run to avoid evaporation.

17. Discard used sample tubes, plates, and waste according to your local safety regulations.

18. Clean the QIAAsymphony SP.

Follow the maintenance instructions in the user manuals supplied with your instrument. Make sure to clean the tip guards regularly to minimize the risk of cross-contamination.

19. Close the instrument drawers and switch off the QIAAsymphony SP.

In general, magnetic particles are not carried over into eluates. If any eluate shows black particles, the magnetic particles can be removed as follows:

- Apply the tube containing the DNA to a suitable magnetic separator (e.g., QIAGEN 12-Tube Magnet, cat. no. 36912) until the magnetic particles are separated.
- If DNA is in microplates, apply the microplate to a suitable magnetic separator (e.g., QIAGEN 96-Well Magnet Type A, cat. no. 36915) until the magnetic particles are separated.
- If no suitable magnetic separator is available, centrifuge the tube containing the DNA for 1 minute at full speed in a microcentrifuge to pellet any remaining magnetic particles.

Quantification and determination of purity of DNA

Elution buffers used in gDNA extraction kits contain the preservative sodium azide. Sodium azide absorbs at 260 nm and therefore a blank measurement should be performed to calibrate the spectrophotometer. Depending on the extraction protocol, the elution buffer should be used as the blank.

- The A_{260}/A_{280} ratio must be ≥ 1.7 . Smaller ratios usually indicate protein contamination or the presence of organic chemicals that adversely affect the PCR step.
- The concentration of DNA is determined by measuring absorbance at 260 nm. 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 ($A_{260} = 1 = 50 \mu\text{g/ml}$).

Total amount of DNA purified (ng) = concentration of DNA (ng/ μl) x volume of sample (μl).

- If the A_{260}/A_{280} ratio is below 1.7, and/or if the gDNA concentration is below 10 ng/ μl , the sample must not be further processed.

Genomic DNA sample normalization

Dilute the DNA to 10 ng/ μl in TE buffer provided in the CALR RGQ PCR Kit.

The Rotor-Gene Q PCR reaction is optimized for 50 ng of purified gDNA diluted in a final sample volume of 5 μl .

qPCR on Rotor-Gene Q 5plex HRM Instrument

The CALR RGQ PCR Kit must be run on the Rotor-Gene Q 5plex HRM instrument. Take time to familiarize yourself with the Rotor-Gene Q instrument before starting the protocol. See the instrument user manual for details.

Sample processing on Rotor-Gene Q instrument with 72-tube rotor

We recommend testing 6 gDNA samples in the same experiment to optimize use of the controls and reaction mixes.

Each reaction mix (CALR TYPE 1, CALR TYPE 2, CALR CLAMP 1, CALR CLAMP 2, CALR CLAMP 3, CALR CLAMP 4 and CALR CLAMP 5) is used for 9 reactions: 6 samples of gDNA and 3 external controls [1 CALR Mutant Control (MTC), 1 CALR Wild-Type Control (WTC) and 1 No Template Control (NTC = TE buffer provided in the CALR RGQ PCR Kit)].

The scheme shown in Figure 4 and Figure 5 provides an example of the loading block and rotor setup for an optimized experiment with the CALR RGQ PCR Kit.

Numbers in Figure 4 denote positions in the loading block and indicate final rotor position.

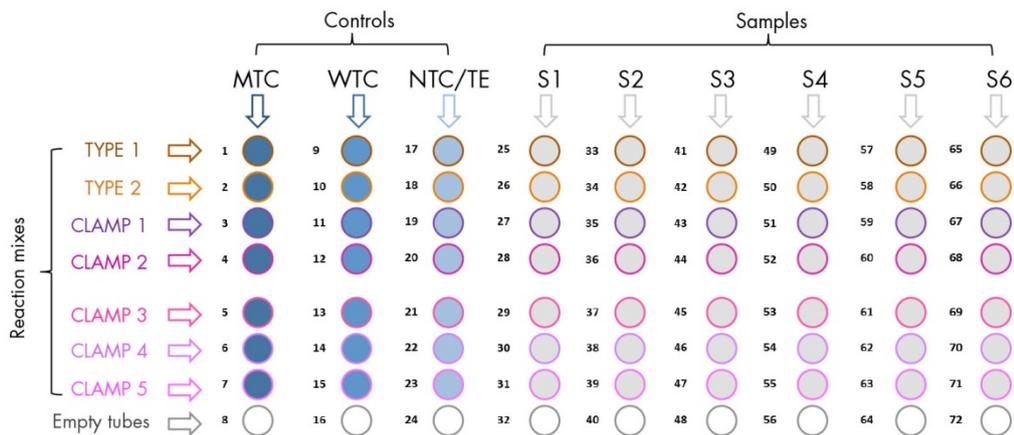


Figure 4. Suggested loading block setup for an experiment with the CALR RGQ PCR Kit. TYPE 1: CALR Reaction mix TYPE 1; TYPE 2: CALR Reaction mix TYPE 2; CLAMP 1: CALR Reaction mix CLAMP 1; CLAMP 2: CALR Reaction mix CLAMP 2; CLAMP 3: CALR Reaction mix CLAMP 3; CLAMP 4: CALR Reaction mix CLAMP 4; CLAMP 5: CALR Reaction mix CLAMP 5; MTC: CALR Mutant Control; WTC: CALR Wild-Type Control; NTC/TE: No Template Control (TE); S1–S6: gDNA samples.

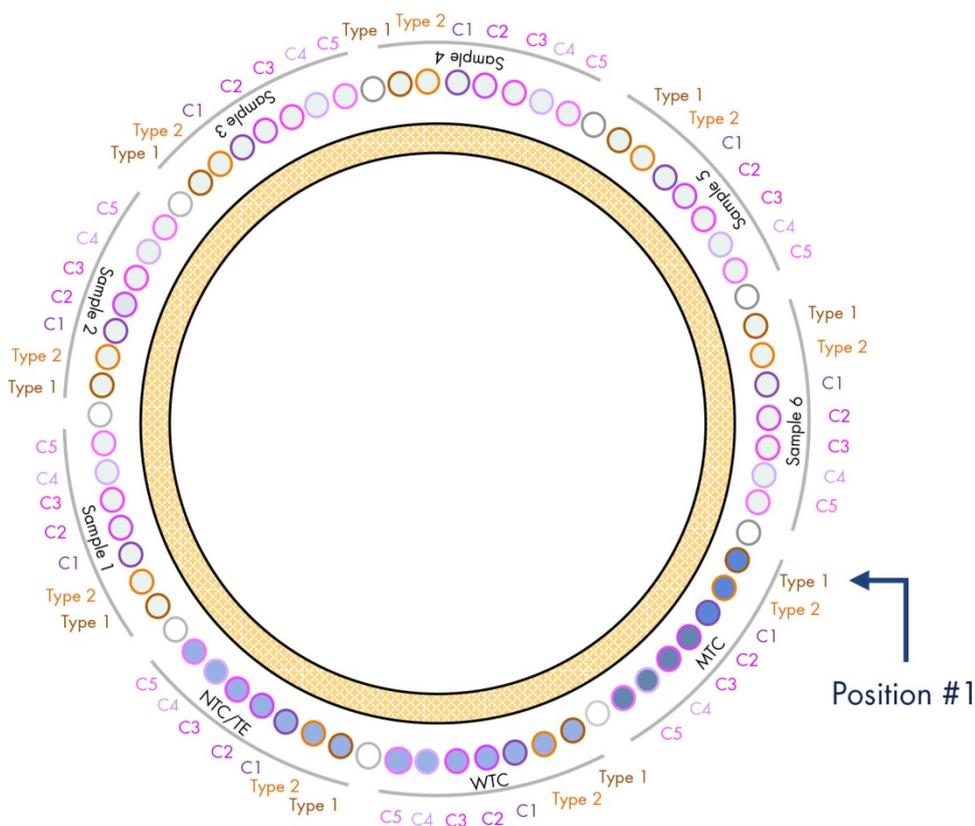


Figure 5. Suggested rotor setup for an experiment with the CALR RGQ PCR Kit.

From position 1 MTC: CALR Mutant Control; WTC: CALR Wild-Type Control; NTC/TE: No Template Control (TE); Type 1: CALR Reaction mix TYPE 1; Type 2: CALR Reaction mix TYPE 2; C1: CALR Reaction mix CLAMP 1; C2: CALR Reaction mix CLAMP 2; C3: CALR Reaction mix CLAMP 3; C4: CALR Reaction mix CLAMP 4; C5: CALR Reaction mix CLAMP 5; Sample 1 to Sample 6: gDNA samples. **Note:** All of the remaining positions ○ should be filled with empty tubes.

Things to do before starting

- Thaw all necessary components except the Taq DNA polymerase; the enzyme must be kept in the freezer when it is not being used. Place the tubes containing the components to be thawed on ice (or on a cooling block).
- Clean the bench area that is dedicated for the PCR mix preparation to reduce the risk of template or nuclease contamination.
- Vortex (10–12 seconds) and then centrifuge briefly the tubes containing controls and reaction mixes before use.

Procedure

1. Prepare qPCR master mixes for each reaction mix (CALR TYPE 1, CALR TYPE 2, CALR CLAMP 1, CALR CLAMP 2, CALR CLAMP 3, CALR CLAMP 4 and CALR CLAMP 5) on ice (or using a cooling block) according to the number of samples to be processed.

The pipetting scheme for the preparation of all CALR reagent master mixes shown in the table below is calculated to achieve final reaction volumes of 25 μ l after addition of 5 μ l of gDNA or control. Extra volume is included to compensate for pipetting error and to allow preparation of sufficient reaction master mix for 6 samples plus 3 external controls.

Component	1 reaction (μ l)	9 + 1 reactions (μ l)*
CALR Reaction Mix	19.83	198.3
Taq DNA polymerase	0.17	1.7
Total volume of qPCR master mix (μl)	20	200
qPCR Master mix distribution		20 μ l per tube
Sample distribution		5 μ l per tube
Total volume of qPCR reaction		25 μl

* An extra reaction volume is included to compensate for pipetting error.

Note: We recommend that you do not pipet volumes less than 1 μ l

2. Vortex and briefly centrifuge all qPCR master mixes.
3. Place the qPCR strip tubes on a cooled Loading Block 72 x 0.1 ml Tubes and dispense 20 μ l of the CALR qPCR master mixes per strip tube following the loading block setup shown in Figure 4.
4. Vortex and briefly centrifuge gDNA samples, CALR Wild-Type Control (WTC), CALR Mutant Control (MTC) and TE buffer (NTC). Then, add 5 μ l of sample or control material into its corresponding tube according to the setup in Figure 4 to give a total volume of 25 μ l. Mix gently by pipetting up and down.
Note: Be careful to change tips between each tube to avoid false-positive results from contamination by any non-specific template or reaction mix. Close all tubes and check that no bubbles are present at the bottom of the tubes.
5. Return all the CALR RGQ PCR Kit components to the appropriate storage conditions to avoid any material degradation.
6. Place a 72-well rotor on the Rotor-Gene Q rotor holder.

- Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Figure 5 with empty capped strip tubes placed into all unused positions.

Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions as shown in Figure 4 and Figure 5.

Note: Always keep the TYPE 1 reaction mix and the three controls (MTC, WTC, NTC) in positions 1, 9 and 17 so that gain optimization (performed on tube position 1) is always performed on the same amplification (see Figure 4 and Figure 5).

- Attach the locking ring.
- Load the Rotor-Gene Q instrument with the rotor and locking ring. Close the instrument lid.
- Open the Rotor-Gene Software and select **New run**.
- In the **New Run Wizard** dialog, select **72-Well Rotor** from the drop-down list. Check the **Locking Ring Attached** box. Click **Next**.
- Enter the PCR reaction volume (**25**). Click **Next**.
Optional: Enter details in the **Operator:** and **Notes:** fields.
- Click **Gain Optimisation** in the **New Run Wizard** dialog box to open the **Auto-Gain Optimisation Setup** dialog.
- Set parameters for the FAM and HEX channels according to the table below.

Optimize before first acquisition	Yes	
Optimize at beginning of run	No	
Channel	Green (FAM)	Yellow (HEX)
Tube position	1	1
Min Reading	5	5
Max Reading	10	10
Gain minimum	-10	-10
Gain maximum	10	10

- Click **Edit Profile** in the **New Run Wizard** dialog box to open the **Edit Profile** dialog.

16. Program the Rotor-Gene Q instrument with the thermal cycling program as indicated in the table below.

Mode of analysis	Parameters
Hold	Temperature: 95°C Time: 10 minutes
Cycling	45 times 95°C for 15 seconds 60°C for 1 minute with: a) Acquisition of FAM fluorescence in channel Green: single b) Acquisition of HEX fluorescence in channel Yellow: single

17. Start the thermal cycling program.

18. Fill the **Edit samples** window by creating two separate pages: one for the TYPE 1 assay (which is analyzed with a 0.06 threshold), and one for the TYPE 2, CLAMP 1, CLAMP 2, CLAMP 3, CLAMP 4 and CLAMP 5 assays (which are analyzed with a 0.05 threshold).

19. When thermal cycling has ended, analyze each qPCR assay.

20. Target analysis of all CALR assays — FAM fluorescence (see table below):

20a. Select **Analysis** and double-click on **Cycling A. Green**.

20b. Click on the name of the CALR assay to be analyzed.

20c. Select **Dynamic Tube**.

20d. Select **Slope Correct**.

20e. Click on **Take Off Adjustment** and adjust the cycle to be used as take off point:

If take off point was calculated before cycle: **15**

Then use the following cycle as take off point: **25**

20f. Click on **Outlier Removal** and set the NTC threshold to **10%**.

20g. Check that **Reaction Efficiency Threshold** is disabled.

20h. Define the threshold at **0.06** for the TYPE 1 assay and **0.05** for all other assays.

20i. Set the graph to **Linear Scale** and **Auto Scale**.

21. Analysis of internal amplification control in all CALR assays — HEX fluorescence (see table below):

21a. Select **Analysis** and double-click on **Cycling A. Yellow**.

21b. Click on the name of the CALR assay to be analyzed.

21c. Select **Dynamic Tube**.

- 21d. Check that **Slope Correct** is **not selected**.
- 21e. Check that **Outlier Removal** is set to **0%** (corresponding to the NTC threshold).
- 21f. Check that **Reaction Efficiency Threshold** is disabled.
- 21g. Define the threshold at **0.05**.
- 21h. Set the graph to **Linear Scale** and **Auto Scale**.

The summary of parameters for target analysis is shown in the table below.

Parameter	Channel	
	Green (FAM)	Yellow (HEX)
Digital Filter	Light	Light
Eliminate Cycles before	1	1
Threshold for the TYPE 1 assay	0.06	0.05
Threshold for the TYPE 2 and CLAMP assays	0.05	0.05
Ignore first xx cycles	0	0
NTC threshold	10%	0%
Reaction efficiency threshold	Disabled	Disabled
Take off adjustment	If take off point was calculated before cycle: 15 Then use the following cycle as take off point: 25	No
Slope correction	Yes	No
Dynamic tube	Yes	Yes
Auto scale	Yes	Yes
Linear scale	Yes	Yes

- 22. To interpret the qPCR results, click on **File, Save as** and **Excel Analysis sheet** to export the run report as .csv data.
- 23. To record all qPCR results, including the amplification curves, click on **Report**, select the report to record, and finally click on **Quantitation (FullReport)** to export the .mht files.
- 24. Make sure that the raw data (.rex file) have been recorded and proceed to "Interpretation of Results", page 28.
- 25. Unload the Rotor-Gene Q instrument and discard the strip tubes according to your local safety regulations.

Interpretation of Results

Data analysis

The PCR cycle at which the fluorescence from a particular reaction crosses a threshold value is defined as the C_T value.

The Rotor-Gene Q software interpolates fluorescence signals between any two recorded values. The C_T values can therefore be any real number (not limited to integers) within the range of 0 to 45.

The C_T values obtained with the CALR RGQ PCR Kit assay reaction mixes (CALR TYPE 1, CALR TYPE 2, CALR CLAMP 1, CALR CLAMP 2, CALR CLAMP 3, CALR CLAMP 4 and CALR CLAMP 5) for all controls and samples are collected and checked for validity and/or positivity.

The data for all controls and samples from the CALR assays in Green (FAM) and Yellow (HEX) channels are extracted from the run report (.csv file) or from the single assay reports (.mht file). The .mht files also contain amplification curves of each single assay. The Rotor-Gene Q .rex file is kept as a record of the run raw data.

The following steps are required to determine the *CALR* status of a sample:

- The assay (CALR TYPE 1, CALR TYPE 2, CALR CLAMP 1, CALR CLAMP 2, CALR CLAMP 3, CALR CLAMP 4 and CALR CLAMP 5) must be checked for validity
- The internal amplification controls of test samples must be checked for validity
- *CALR* positive or negative status for each valid assay is assigned to valid samples

Assay validity

For each CALR RGQ PCR Kit qPCR assay to be valid, the C_T values in the FAM and HEX channels for the external controls (MTC, WTC and NTC) must comply with the assay validity criteria. These criteria are presented in Table 1 for FAM and Table 2 for HEX.

Important: Each CALR assay is analyzed independently.

Table 1. Criteria for assay validity: CALR targets for external controls in the Green channel (FAM)

CALR assay	FAM CALR targets (C_T)		
	Mutant control (MTC)	Wild-type control (WTC)	No template control (NTC/TE)
TYPE 1	[27.50 ; 31.50]	≥ 32.25 or NA*	> 40.00 or NA
TYPE 2	[26.50 ; 33.00]	≥ 35.00 or NA	> 40.00 or NA
CLAMP 1	[23.50 ; 29.50]	≥ 30.50 or NA	> 40.00 or NA
CLAMP 2	[22.00 ; 29.00]	≥ 31.50 or NA	> 40.00 or NA
CLAMP 3	[22.00 ; 29.00]	≥ 30.50 or NA	> 40.00 or NA
CLAMP 4	[24.00 ; 30.00]	≥ 35.00 or NA	> 40.00 or NA
CLAMP 5	[23.00 ; 30.00]	≥ 32.00 or NA	> 40.00 or NA

* NA: No amplification.

Table 2. Criteria for assay validity: Internal amplification control targets for external controls in the Yellow channel (HEX)

CALR assay	HEX internal amplification control targets (C_T)		
	Mutant control (MTC)	Wild-type control (WTC)	No template control (NTC/TE)
TYPE 1	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA*
TYPE 2	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA
CLAMP 1	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA
CLAMP 2	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA
CLAMP 3	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA
CLAMP 4	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA
CLAMP 5	[19.00 ; 26.00]	[19.00 ; 26.00]	> 40.00 or NA

* NA: No amplification.

If the internal amplification control in a given tube is invalid according to the criteria defined above, the external control in the same tube is deemed invalid.

If at least one external control is invalid for a given CALR assay, the results obtained with that reaction mix for all test samples are considered invalid. In this case, only the given CALR assay is invalid, not the entire qPCR run.

Validity of test sample amplification

The internal amplification controls of the test samples are valid, if the C_T values in the HEX channel obtained for test samples are compliant with the validity criteria presented in Table 3.

Table 3. Criteria for test sample validity: Internal amplification control targets for test samples in the Yellow channel (HEX)

HEX internal amplification control targets (C _T)	
CALR assay	Test sample
TYPE 1	[19.00 ; 26.00]*
TYPE 2	[19.00 ; 26.00]
CLAMP 1	[19.00 ; 26.00]
CLAMP 2	[19.00 ; 26.00]
CLAMP 3	[19.00 ; 26.00]
CLAMP 4	[19.00 ; 26.00]
CLAMP 5	[19.00 ; 26.00]

* This criterion is the same as the assay validity criterion for the internal amplification control targets for external controls in the Yellow channel (HEX) (see Table 2).

If the internal amplification control in a given tube is invalid according to the criteria defined above, the test sample is deemed invalid.

CALR mutation positive or negative status of test samples

CALR mutation positive or negative status of a test sample is assigned by comparing the C_T value of the test sample in the FAM channel with the test sample positivity/negativity criteria presented in Table 4.

Table 4. Criteria for positive or negative status of a test sample: CALR targets for test samples in the Green channel (FAM)

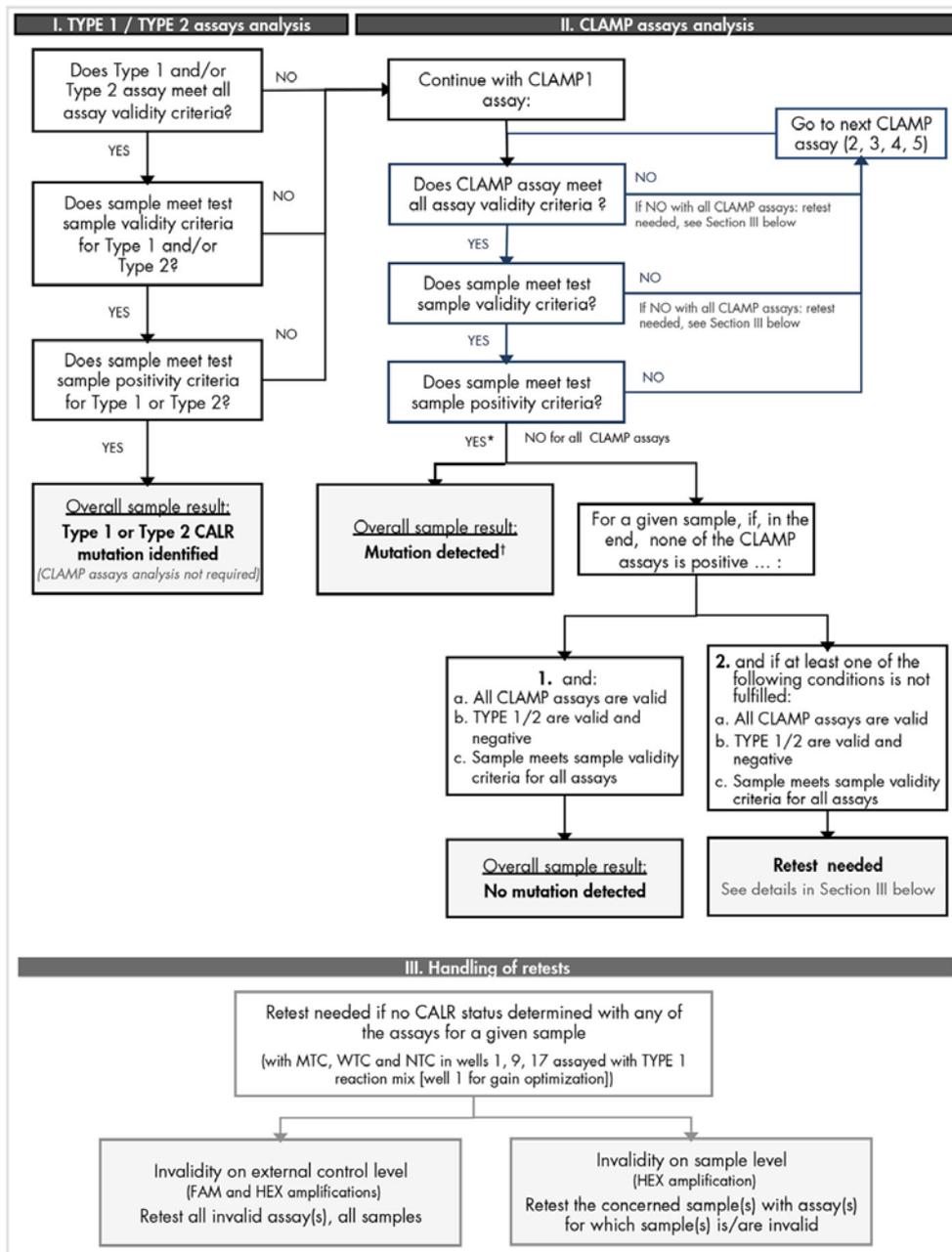
CALR assay	FAM CALR targets (C_T)	
	Positive test sample (mutation detected)	Negative test sample (no mutation detected)
TYPE 1	≤ 31.75	> 31.75 or NA*
TYPE 2	≤ 34.50	> 34.50 or NA
CLAMP 1	≤ 30.00	> 30.00 or NA
CLAMP 2	≤ 31.00	> 31.00 or NA
CLAMP 3	≤ 30.00	> 30.00 or NA
CLAMP 4	≤ 34.00	> 34.00 or NA
CLAMP 5	≤ 31.50	> 31.50 or NA

* NA: No amplification.

Determination of CALR status

The CALR status of a given sample can be either "Type 1 mutation detected" (specific to the TYPE 1 assay), "Type 2 mutation detected" (specific to the TYPE 2 assay), "Mutation detected" (specific to the CLAMP assays), "No mutation detected" or "Invalid" (i.e., validity criteria not fulfilled).

The final determination of the CALR status of a given sample, as well as the potential requirement for retests, can be made using the decision flowchart in Figure 6.



* For one or several valid assays.

† In case Type 1/Type 2 identification is mandatory, a retest may be needed if TYPE 1 and/or TYPE 2 is invalid, despite a positive CLAMP assay, in order to obtain a conclusive result for TYPE 1 and/or TYPE 2 assay.

Figure 6. Decision flowchart to determine CALR mutation status of test samples.

In the first instance, the operator should consider the results obtained for the TYPE 1 and TYPE 2 assays and follow the flow chart. If either Type 1 or Type 2 positive mutation status is assigned to a sample then the *CALR* status is determined. The results obtained for the CLAMP assays for these samples can be analyzed for informational purposes.

If neither Type 1 nor Type 2 mutation is identified, the analysis is continued with the results obtained for the CLAMP assays until the *CALR* status is determined.

When invalid results are reported, either on an assay or a sample level, retests may be needed. When performing retests, always keep the TYPE 1 reaction mix and the three controls (MTC, WTC, NTC) in position 1, 9 and 17 so that gain optimization (performed on the tube in position 1) is always performed on the same amplification. Follow the decision flowchart in Figure 6 to assess the need for retests.

Troubleshooting guide

This troubleshooting guide may be helpful in solving any problems that may arise in the assessment of *CALR* mutation status using the CALR RGQ PCR Kit. For contact information, see back cover or visit www.qiagen.com.

For troubleshooting information relating to the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) or QIASymphony DNA DSP Mini Kit (cat. no. 937236), please refer to the respective kit handbooks.

For troubleshooting information relating to the Rotor-Gene Q instrument and software, please refer to the respective handbooks.

Comments and suggestions

A sample is detected as positive with multiple assays

A given mutation can be detected by several assays	For example, it is common for a sample bearing a Type 1 mutation to be amplified by the CLAMP 1 and CLAMP 2 assays in addition to the TYPE 1 assay. For a sample bearing a Type 2 mutation, it is common to obtain amplification with the CLAMP 5 assay in addition to the TYPE 2 assay.
--	---

No or low amplification of the internal amplification control in external controls and/or samples ($C_T > 26.00$ or no amplification)

- | | |
|---|---|
| a) Reaction mix and/or Taq DNA polymerase and/or template not added | Check the pipetting scheme and setup of the reaction.
Check that all template DNA and all components of the qPCR master mix have been added. Repeat the PCR run. |
| b) 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, if necessary, to repeat the PCR run. |

Comments and suggestions

- | | |
|--|--|
| c) The pipetting volume may be incorrect | Check the pipetting scheme and setup of the reaction.
Check that a 5 µl volume of control/sample and a 20 µl volume of qPCR master mix have been added. Perform a visual inspection of all pipetted volumes.
Check and recalibrate the pipets, if necessary, before repeating the qPCR step. |
| d) DNA concentration is too low | Check the DNA concentration of the sample. The CALR RGQ PCR kit is optimized for a working DNA concentration of 10 ng/µl. If the DNA concentration is less than 10 ng/µl, concentrate or re-extract DNA from whole blood, decreasing the elution volume, before repeating the qPCR step. |
| e) Protein contamination of the DNA or the presence of organic chemicals | Check the A_{260}/A_{280} ratio. The A_{260}/A_{280} ratio must be ≥ 1.7 . If the ratio is < 1.7 , perform a new DNA extraction and repeat the PCR run. |

Early amplification of the internal amplification control in external controls and/or samples ($C_T < 19.00$)

- | | |
|--|--|
| a) DNA concentration is too high | Check the DNA concentration of the sample. The CALR RGQ PCR kit is optimized for a working concentration of 10 ng/µl. If the DNA concentration is greater than 10 ng/µl, dilute the DNA in TE buffer and repeat the PCR run. |
| b) The pipetting volume may be incorrect | Check the pipetting scheme and setup of the reaction.
Check that a 5 µl volume of control/sample and a 20 µl volume of qPCR master mix have been added. Perform a visual inspection of all pipetted volumes.
Check and recalibrate the pipets, if necessary, before repeating the qPCR step. |
| c) The amplification curve may be incorrect. | Check the corresponding amplification for unusual curves.
Repeat the PCR run. |

Comments and suggestions

No or low signals for the internal amplification control in samples ($C_T > 26.00$ or no amplification), but the external controls are valid

- | | |
|--|--|
| a) DNA concentration is too low | Check the DNA concentration of the sample. The CALR RGQ PCR kit is optimized for a working DNA concentration of 10 ng/ μ l. If the DNA concentration is less than 10 ng/ μ l, concentrate or re-extract DNA from whole blood decreasing the elution volume before repeating the qPCR step. |
| b) Protein contamination of the DNA or the presence of organic chemicals | Check the A_{260}/A_{280} ratio. The A_{260}/A_{280} ratio must be ≥ 1.7 . If the ratio is < 1.7 , perform a new DNA extraction and repeat the PCR run. |
| c) The pipetting volume may be incorrect | Check the pipetting scheme and setup of the reaction. Check that a 5 μ l volume of control/sample and a 20 μ l volume of qPCR master mix have been added. Perform a visual inspection of all pipetted volumes. Check and recalibrate the pipets, if necessary, before repeating the qPCR step. |

No template control (NTC/TE buffer) is positive (FAM and/or HEX) ($C_T \leq 40.00$)

- | | |
|---|---|
| a) Cross-contamination or contamination of reagents | Replace all critical reagents and repeat the PCR run.
Always handle samples, kit components and consumables in accordance with recommended practices to prevent carryover contamination.
Make sure tips are changed in between pipetting different reagents or when loading different tubes.
Prepare the pre-PCR master mix with dedicated material (pipets, tips, etc.)
Prepare the pre-PCR master mix and NTC reaction in a dedicated area where no DNA matrices (DNA, plasmid or PCR products) are introduced.
If possible, close the PCR tubes directly after addition of the sample to be tested. |
|---|---|

Comments and suggestions

- | | |
|---|--|
| b) Strip tube and/or sample ID inversion | Check the pipetting scheme and setup of the reaction.
Repeat the PCR run. |
| c) The reaction mix or the probe 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, if necessary to repeat the PCR run. |
| d) The amplification curve may be incorrect | Check the corresponding amplification for unusual curves.
Repeat the PCR run. |

No or low amplification of the mutant control (MTC) (FAM amplification) (see Table 1)

- | | |
|---|--|
| a) Reaction mix and/or Taq DNA polymerase not added | Check the pipetting scheme and setup of the reaction.
Check that all components of the qPCR master mix have been added. Repeat the PCR run. |
| b) 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, if necessary, to repeat the PCR run. |
| c) Strip tube and/or sample ID inversion | Check the pipetting scheme and setup of the reaction.
Repeat the PCR run. |
| d) The pipetting volume may be incorrect | Check the pipetting scheme and setup of the reaction.
Check that a 5 μl volume of control/sample and a 20 μl volume of qPCR master mix have been added. Perform a visual inspection of all pipetted volumes.
Check and recalibrate the pipets, if necessary, before repeating the qPCR step. |

Comments and suggestions

Early amplification of the mutant control (MTC) (FAM amplification) (see Table 1)

- | | |
|---|--|
| a) Pipetting volume may be incorrect | Check the pipetting scheme and setup of the reaction.
Check that a 5 µl volume of control/sample and a 20 µl volume of qPCR master mix have been added. Perform a visual inspection of all pipetted volumes.
Check and recalibrate the pipets, if necessary, before repeating the qPCR step. |
| b) The amplification curve may be incorrect | Check the corresponding amplification for unusual curves.
Repeat the PCR run. |
| c) Strip tube and/or sample ID inversion | Check the pipetting scheme and setup of the reaction.
Repeat the PCR run. |

Early amplification of the wild-type control (WTC) (FAM amplification) (see Table 1)

- | | |
|---|--|
| 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, if necessary, to repeat the PCR run. |
| b) The pipetting volume may be incorrect | Check the pipetting scheme and setup of the reaction.
Check that a 5 µl volume of control/sample and a 20 µl volume of qPCR master mix have been added. Perform a visual inspection of all pipetted volumes.
Check and recalibrate the pipets, if necessary, 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 for unusual curves.
Repeat the PCR run. |

Comments and suggestions

- e) Carryover contamination Replace all critical reagents.
Repeat the experiment with new aliquots of all reagents.
Always handle samples, kit components and consumables in accordance with recommended practices to prevent carryover contamination.
Make sure tips are changed in between pipetting different reagents.

Early amplification of the wild-type control (WTC) (FAM amplification) and no or low amplification of the mutant control (MTC) (FAM amplification) (see Table 1)

- a) Cross-contamination Check the pipetting scheme and setup of the reaction and repeat the PCR run.
- b) Inversion of the reaction mixes in the tubes or premix Check the pipetting scheme and setup of the reaction and repeat the PCR run.
- c) Strip tube and/or sample ID inversion Check the pipetting scheme and setup of the reaction.
Repeat the PCR run.

Frequent wild-type control (WTC) failure due to high background amplification below the assay validity target (C_T) (see Table 1)

- Fault in Rotor-Gene Q 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.

Run failure due to inconsistent fluorescence signal in controls and/or samples (across all tubes)

- Fault in Rotor-Gene Q instrument accessories Check instrument maintenance logs.
The Rotor-Disc® may be faulty.

If a problem cannot be attributed to any of the causes listed in the “Troubleshooting guide”, or if the suggested corrective actions fail to resolve a problem, please contact QIAGEN Technical Services for advice.

Quality Control

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

References

1. Klampfl T., et al. (2013) Somatic mutations of calreticulin in myeloproliferative neoplasms. N. Engl. J. Med. **369**, 2379.
2. Nangalia J., et al. (2013) Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N. Engl. J. Med. **369**, 2391.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Catalog number
	Manufacturer
	Material number
	Lot number
	Global Trade Item Number
	Use by
	Contains reagents sufficient for N reactions
	Temperature limitation
	Consult instructions for use
	Keep away from sunlight

Ordering Information

Product	Contents	Cat. no.
CALR RGQ PCR Kit (24)	For 24 reactions: CALR Wild-type Control, CALR Mutant Control, CALR TYPE 1 Reaction Mix, CALR TYPE 2 Reaction Mix, CALR CLAMP 1 Reaction Mix, CALR CLAMP 2 Reaction Mix, CALR CLAMP 3 Reaction Mix, CALR CLAMP 4 Reaction Mix, CALR CLAMP 5 Reaction Mix, Taq DNA polymerase, TE buffer for dilution and NTC	674013
Rotor-Gene Q and accessories		
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt (HRM) analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9001580
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 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, 1-year warranty on parts and labor, installation and training	9001650

Product	Contents	Cat. no.
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cyclers 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
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
QIASymphony SP and accessories		
QIASymphony SP System	QIASymphony sample prep module: includes installation and training, 1 year warranty on parts and labor	9001751
QIASymphony SP	QIASymphony sample prep module: includes 1-year warranty on parts and labor	9001297
Sample Prep Cartridges, 8-well (336)	8-well sample prep cartridges for use with the QIASymphony SP	997002
8-Rod Covers (144)	8-Rod Covers for use with the QIASymphony SP	997004
Filter-Tips, 200 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube® and the QIASymphony SP/AS instruments	990332

Product	Contents	Cat. no.
Filter-Tips, 1500 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIASymphony SP/AS instruments	997024
Tube Insert 3b, 2 ml, v2, sample carrier, Qsym	Secondary tube adapter (for 2 ml screw-cap tubes) for use with the QIASymphony tube carrier	9242083
Elution Microtubes CL (24 x 96)	Nonsterile polypropylene tubes (0.85 ml maximum capacity, less than 0.7 ml storage capacity, 0.4 ml elution capacity); 2304 in racks of 96; includes cap strips	19588
Related products		
QIAamp DNA DSP Blood Mini Kit (50)	For 50 preps: QIAamp Mini Spin Columns, Buffers, Reagents, Tubes, VacConnectors	61104
QIASymphony DSP DNA Mini Kit (192)	For 192 preps of 200 µl each: Includes 2 reagent cartridges and enzyme racks and accessories.	937236
RNase A (17,500 U)	2.5 ml (100 mg/ml; 7000 units/ml, solution)	19101

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