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ipsogen® CALR 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

CE

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Sample to Insight

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Contents

Intended Use

The *ipsogen* CALR RGQ PCR kit is an in vitro real-time PCR test intended for the detection of *CALR* mutations in genomic DNA from whole blood of subjects suspected of myeloproliferative neoplasms (MPN). The *ipsogen* CALR RGQ PCR Kit also enables the identification of the two major CALR mutations (Type 1 and Type 2), and is for use with the QIAGEN Rotor-Gene Q MDx 5Plex HRM Platform. The product is intended to be used by professionals, such as technicians and physicians, who are trained in molecular biology techniques.

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.

Summary and Explanation

Myeloproliferative neoplasms are a group of diseases representing 39% of hematological malignancies, characterized by chronic accumulation of different mature blood cell types in blood, which are either Philadelphia chromosome positive (Ph+) or negative (Ph–).

A recurrent somatic mutation, V617F, affecting the Janus tyrosine kinase 2 gene (*JAK2*) was identified in 2005 (1–4), leading to a major breakthrough in the understanding, classification and diagnosis of MPN. Among the total of patients with MPN Ph–, the *JAK2* V617F mutation is detected in >95% of patients with polycythemia vera (PV), 50–60% of patients with essential thrombocythemia (ET) and in 50% of patients with primary myelofibrosis (PMF). In addition, 5–10% of ET and PMF have activating mutations in the thrombopoietin receptor gene (*MPL*). No specific molecular marker had been identified in the remaining 30 to 45% of patients.

Discovery of somatically acquired mutations in the *CALR* gene (encoding the calreticulin protein) in a substantial proportion of patients with MPN Ph– has provided a new marker of clonal disease (5, 6), advancing both diagnosis and prognosis in these previously molecularly uncharacterized cases. Somatic insertions or deletions in *CALR* exon 9 were found in a majority of patients with MPN Ph– without *JAK2* mutation. A total of 36 "types" (Table 1), consisting of insertions, deletions, substitutions, or a combination of these, were initially identified for *CALR*. Most of them lead to a frameshift with the same alternative reading frame causing the generation of mutant CALR proteins sharing the same novel amino acid sequence at the C-terminal. This frameshift was suggested to alter the cellular localization of the different mutant proteins and to affect the $Ca2^{+}$ binding function of their C-terminal domains.

The exact pathological mechanism has not yet been fully elucidated, but in vitro studies showed that over-expression of the most frequent *CALR* deletion (Type 1 mutation, see Table 1) caused cytokine-independent cell growth (5).

Table 1. List of *CALR* mutations from Type 1 to Type 36

Type	COSMIC ID*	Frequency (%)†	Mutant *CALR* cDNA notation
1	COSM1738055	53	c.1092_1143del
2	COSM1738056	31.7	c.1154_1155insTTGTC
3	COSM1738150	1.7	c.1095_1140del
4	COSM1738151	1	c.1102_1135del
5	COSM1738057	0.7	c.1091_1142del
6	COSM1738152	0.7	c.1094_1139del
7	COSM1738343	0.7	c.1102_1153del
8	COSM1738153	0.7	c.1104_1137del
9	COSM1738154	0.7	c.1140del
10	COSM1738155	0.7	c.1154delinsTGTGTC

Type	COSMIC ID*	Frequency (%)†	Mutant *CALR* cDNA notation
11	NI;‡ COSM1738150	0.3	[c.1092G>C;1095_1140del]
12	COSM1738359	0.3	c.1098_1131del
13	COSM1738339	0.3	c.1100_1134delinsA
14	COSM1738368	0.3	c.1101_1134del
15	COSM1738151; NI‡	0.3	[c.1102_1135del;1145C>G]
16	COSM1738157	0.3	c.1102_1137delinsCA
17	COSM1738153; NI‡	0.3	[c.1104_1137del;1148A>T]
18	COSM1738158	0.3	c.1104_1155del
19	COSM1738349	0.3	c.1110_1140del
20	COSM1738159	0.3	c.1118_1136del
21	COSM1738160	0.3	c.1118_1145delinsCGTTTA
22	COSM1738328	0.3	c.1120_1123del
23	COSM1738344	0.3	c.1120_1131delinsTGCGT
24	COSM1738345	0.3	c.1120_1138del
25	COSM1738346	0.3	c.1122_1141delinsA
26	COSM1738350	0.3	c.1122del
27	COSM1738351	0.3	c.1123_1125delinsTGTTT
28	COSM1738329	0.3	c.1131_1152del
29	COSM1738352	0.3	c.1135_1152delins CCTCCTCTTTGTCT
30	COSM1738353	0.3	c.1137_1154delins CCATCCTTGTC
31	NI;‡ COSM1738056	0.3	[c.1151A>G;1154_1155insTTGTC]
32	COSM1738330	0.3	c.1153_1154delinsTGTC

Type	COSMIC ID*	Frequency (%)†	Mutant *CALR* cDNA notation
33	COSM1738355	0.3	c.1154_1155insATGTC
34	COSM1738331	0.3	c.1154_delinsCTTGTC
35	COSM1738356	0.3	c.1154delinsTTTGTC
36	COSM1738332	0.3	c.1155_1156insTGTCG

* IDs from COSMIC v72 (**cancer.sanger.ac.uk/cosmic/**).
† Frequencies from Klampfl et al (2013) (5).
‡ NI: Mutation event not identified in COSMIC.

Traditionally, the diagnosis of MPNs was based on clinical, bone marrow histology and cytogenetic criteria. The discovery of a disease-specific molecular marker resulted in both simplification of the process and increased diagnostic accuracy. Understanding the molecular basis for ET and PMF in patients without *JAK2* and *MPL* mutations has been a major goal in the field of MPN. In this way, the discovery of *CALR* mutations provides an additional molecular marker for the purposes of both diagnosis and prognosis of patients with MPN Ph–. Detection of *CALR* mutation is now part of the reference World Health Organization (WHO) 2016 criteria for the diagnosis of MPN (Table 2), and presence of this mutation is a major criterion for diagnostic confirmation.

Table 2. WHO criteria for the diagnosis of MPN (adapted from reference 7)

WHO criteria for a diagnosis of essential thrombocythemia

Major criteria:

1. Platelet count ≥ 450 x 109/L.
2. Bone marrow biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left-shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor increase in reticulin fibers.
3. Not meeting WHO criteria for *BCR-ABL1+* CML*, PV, PMF, myelodysplastic syndromes (MDS), or other myeloid neoplasms.
4. Presence of *JAK2*, *CALR* or *MPL* mutation.

Minor criterion:

Presence of a clonal marker or absence of evidence for reactive thrombocytosis

WHO criteria for a diagnosis of primary myelofibrosis

Major criteria:

1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis.
2. Not meeting WHO criteria for ET, PV, *BCR-ABL1+* CML, MDS, or other myeloid neoplasms.
3. Presence of *JAK2*, *CALR* or *MPL* mutation or in the absence of these mutations, presence of another clonal marker or absence of reactive myelofibrosis.

Minor criteria:

Presence of at least one of the following, confirmed in two consecutive determinations:

a) Anemia not attributed to a comorbid condition
b) Leukocytosis ≥11 x 10^9/L
c) Palpable splenomegaly
d) LDH* increased to above upper normal limit of institutional reference range
e) Leukoerythroblastosis

WHO criteria for polycythemia vera

Major criteria:

1. Hemoglobin (Hgb) >16.5 g/dL in men, Hgb >16.0 g/dL in women; or, Hematocrit (Hct) >49% in men, Hct >48% in women; or, increased red cell mass.
2. Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size).
3. Presence of *JAK2* V617F or *JAK2* exon 12 mutation

Minor criterion:

Subnormal serum erythropoietin level

* CML: chronic myeloid leukemia; LDH: lactate dehydrogenase.

Detection of *CALR* mutations in gDNA extracted from peripheral blood cells is now used as a diagnostic tool in the same way as detection of *JAK2* mutations, which have simplified and improved the accuracy of diagnosis of patients with MPN. The CALR and JAK2 tests (*ipsogen* CALR RGQ PCR Kit and *ipsogen* JAK2 RGQ PCR Kit) have been validated with the same gDNA extraction methods, therefore the same sample can be tested with the two different qPCR kits.

Principle of the Procedure

The *ipsogen* CALR RGQ PCR Kit is a real-time PCR test. The kit uses quantitative real-time PCR (qPCR) techniques for the qualitative detection of somatic mutations in the region c.1091_1162 (cDNA annotation) of exon 9 in the *CALR* gene (GenBank® Accession Number CR457070) (5, 6), and also allows the identification of the two major *CALR* mutations (Type 1 and Type 2).

The kit provides reagents to perform seven separate PCR amplification reactions in the same run for the identification of *CALR* mutations Type 1 and Type 2 and the detection of additional minor variants (listed in "Performance Characteristics/Specificity", page 68) in genomic DNA extracted from human peripheral whole blood. The turnaround time to execute all tasks, from gDNA extraction (using manual or automated extraction) to data analysis, is less than one working day.

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. Analysis by qPCR 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.

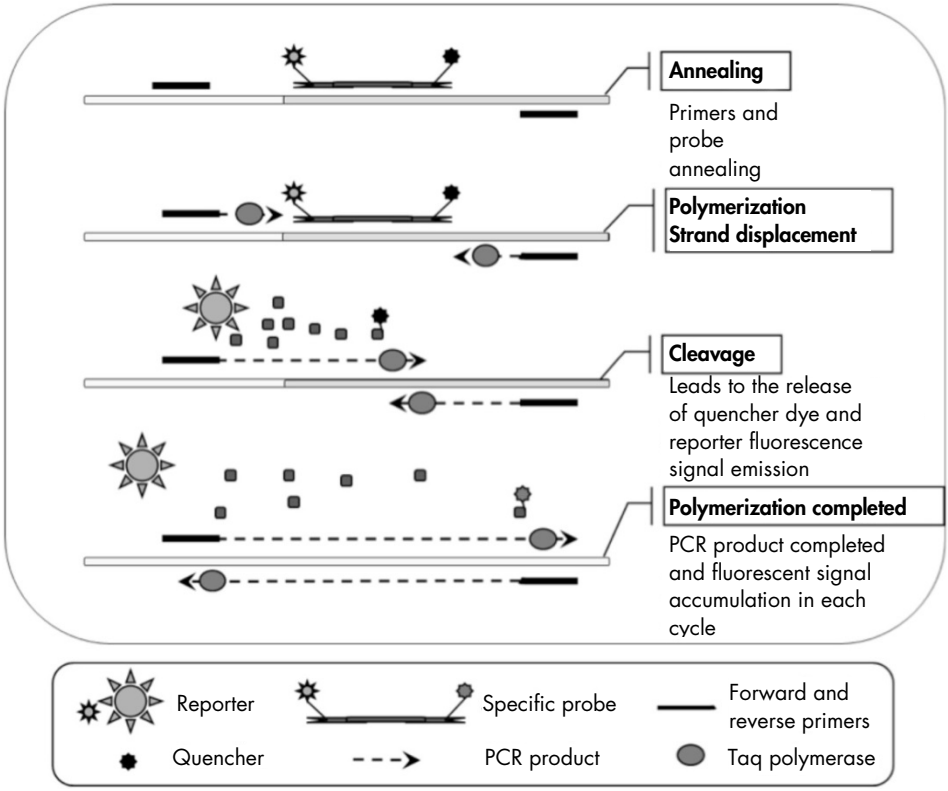


Figure 1. Principle of real-time PCR reactions.

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).

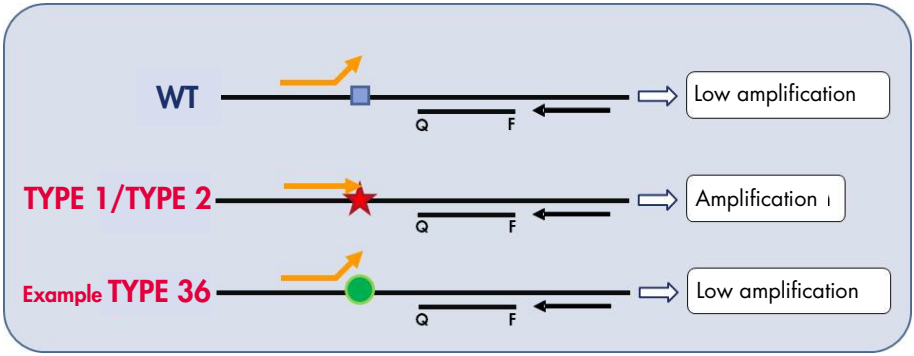


Figure 2. Identification of Type 1 and Type 2 *CALR* mutations by ARMS PCR. WT: wild-type; Q — F: BHQ® — FAM™ double-dye probe; ⇆ forward primer (orange) and reverse primer (black).

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).

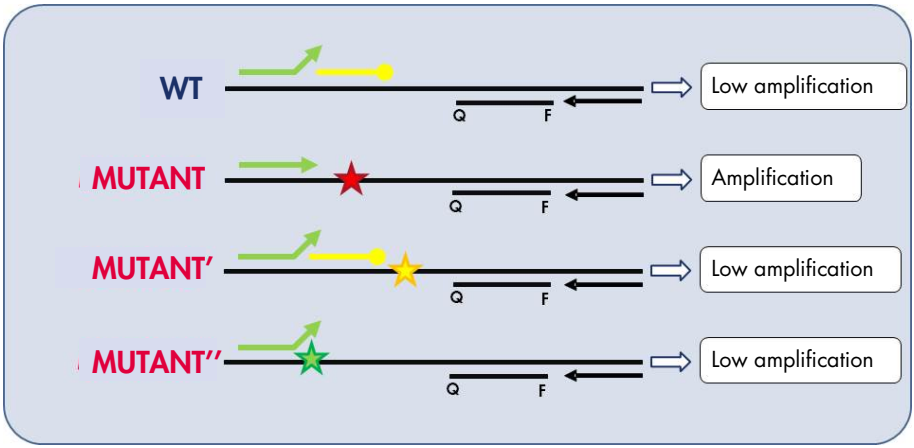


Figure 3. Detection of *CALR* minor mutations. WT: wild-type; Q — F: BHQ — FAM double-dye probe; ⇆ forward primer (green) and reverse primer (black); —o : 3'-phosphate oligonucleotide (CLAMP oligonucleotide; yellow).

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

***ipsogen* CALR RGQ PCR Kit**		**(24)**
Catalog number		**674023**
Number of reactions		**24**
Color	**Identity**	**Volume**
Yellow	CALR Reaction Mix Type 1	850 µl
Yellow	CALR Reaction Mix Type 2	850 µl
Purple	CALR Reaction Mix CLAMP 1	850 µl
Purple	CALR Reaction Mix CLAMP 2	850 µl
Purple	CALR Reaction Mix CLAMP 3	850 µl
Purple	CALR Reaction Mix CLAMP 4	850 µl
Purple	CALR Reaction Mix CLAMP 5	850 µl
Green	CALR Wild-Type Control	145 µl
Red	CALR Mutant Control	145 µl
Mint	Taq DNA Polymerase	85 µl
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

- QIAsymphony® 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)
- QIAsymphony 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 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)
- Rotor-Gene AssayManager v2.1 Gamma Plug-in version 1.0.x (where x = 0 or higher)
- CALR Assay Profile ipsogen_CALR_blood_CE version 1.0.x (where x = 2 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

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. For safety information regarding instruments, see the relevant instrument user manual.

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 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 *ipsogen* 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 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 performances.
- Make sure that the assay profile files and required Rotor-Gene AssayManager v2.1 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.
- 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.
- 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 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.

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 *ipsogen* CALR RGQ PCR Kit is shipped on dry ice. If any component of the *ipsogen* 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 *ipsogen* 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 *ipsogen* 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. Do not exceed a maximum of 5 freeze-thaw cycles.

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 *ipsogen* 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 for no more than 24 months, either directly after extraction or after being diluted in TE buffer.

Procedure

Genomic DNA extraction and preparation

The *ipsogen* 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 QIAsymphony SP instrument in combination with the QIAsymphony 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 x *g* (8000 rpm), centrifuge again at full speed (up to 20,800 x *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 µl 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 x *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 µl 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 x *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 x *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 µl 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 x *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 whole blood for analysis with the *ipsogen* 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 QIAsymphony SP 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.

- 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 into the drawer.

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 QIAsymphony 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 QIAsymphony SP after the run is completed may experience condensation or evaporation.

14. Export the QIAsymphony 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 QIAsymphony 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 QIAsymphony 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 QIAsymphony SP.

    Follow the maintenance instructions in the QIAsymphony SP 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 QIAsymphony 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.

Quantitation 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 µg/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 *ipsogen* CALR RGQ PCR Kit.

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

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

The *ipsogen* CALR 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. The cycling parameters are locked for the run.

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.

Installation of 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 at **www.qiagen.com/Products/Rotor-GeneAssayManager_v2_1.aspx**.

For details about 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 *ipsogen* CALR RGQ PCR Kit with Rotor-Gene AssayManager v2.1, the latest Gamma Plug-in must be installed to your Rotor-Gene AssayManager v2.1. Refer to **Product Resources** at the Rotor-Gene AssayManager v2.1 product page at **www.qiagen.com/Products/Rotor-GeneAssayManager_v2_1.aspx** to access the latest version of the plug-in.

* 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.

For details about installation of the plug-in, refer to the section "Installing Plug-ins" in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

The *ipsogen* CALR RGQ PCR Kit also requires an assay profile. The assay profile contains all parameters needed for cycling and analyzing the qPCR assay. The CALR assay profile (ipsogen_CALR_blood_CE) corresponds to an .iap file that can be downloaded from the *ipsogen* CALR RGQ PCR Kit product page on the **Product Resources** tab under **Protocol Files**. The assay profile must be imported in Rotor-Gene AssayManager v2.1 software.

For detailed information on the installation of the Gamma plug-in and assay profile, refer to *Rotor-Gene AssayManager v2.1 Core Application User Manual* and *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

1. Download both the Gamma Plug-in and the latest version of the CALR assay profile from **www.qiagen.com**.
2. Start the installation process by double-clicking on the file **RGAM_V2_1_Gamma_Plug-in.Installation.V1_0_0.msi**, and follow the installation instructions.
   For a detailed description refer to the section "Installing Plug-ins" in *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

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

   - Select the **Settings** tab in the **Configuration** environment.
   - In the **Work list** panel under **Closed mode**, check the boxes for **Material number required**, **Valid expiry date required** and **Lot number required**.

   This can only be done by a user with "Administrator" rights.
3. After the Gamma Plug-in is installed, import the CALR assay profile (.iap file).
   Log in to Rotor-Gene AssayManager v2.1 software as a user with "Administrator" rights for Rotor-Gene AssayManager v2.1.
4. Select the **Configuration** environment.

5. Select the tab **Assay Profiles**.
6. Click the **Import** button.
7. Select the CALR assay profile ipsogen_CALR_blood_CE file in the open file dialog.
8. Click **Open**. The assay profile is loaded and added to the list of available assay profiles and can be used in the **Setup** environment.

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

Setup of loading block and 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 *ipsogen* CALR RGQ PCR Kit)].

The schemes shown in Figure 4 and Figure 5 provide an illustration of the loading block and rotor setup for an optimized experiment with the *ipsogen* CALR RGQ PCR Kit.

The position of CALR reaction mixes and controls is set in the CALR assay profile and cannot be changed. If reaction mixes/controls are not placed as instructed below, the automated result analysis cannot be performed.

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

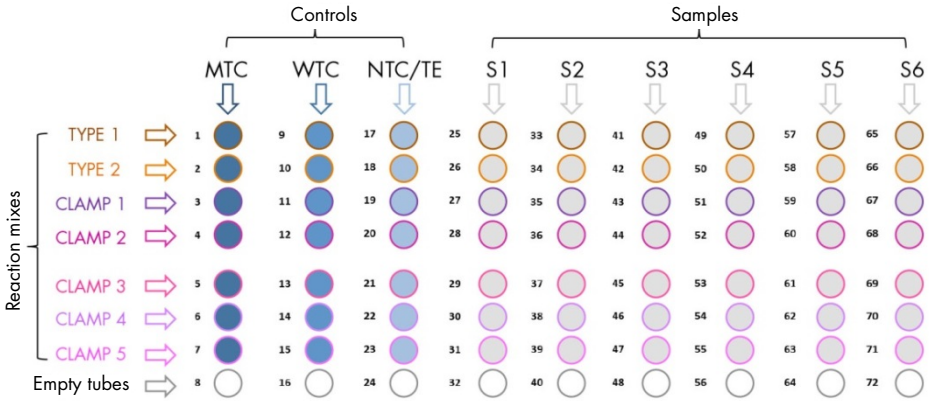


Figure 4. Loading block setup for an experiment with the *ipsogen* CALR RGQ PCR Kit. TYPE 1: CALR Reaction mix TYPE 1; TYPE 2: CALR Reaction mix TYPE 2; CLAMP 1: CALR Reaction mix CLAMP 1; CLAMP 2: CALR Reaction mix CLAMP 2; CLAMP 3: CALR Reaction mix CLAMP 3; CLAMP 4: CALR Reaction mix CLAMP 4; CLAMP 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.

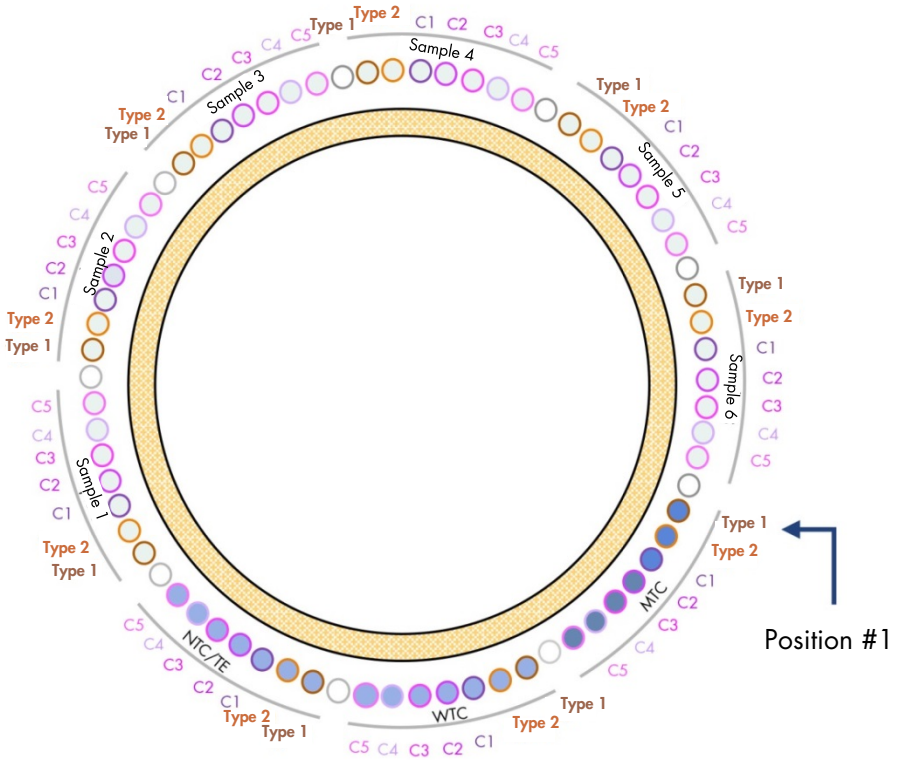


Figure 5. Rotor setup for an experiment with the *ipsogen* CALR RGQ PCR Kit.
From position 1 MTC: CALR Mutant Control; WTC: CALR Wild-Type Control; NTC/TE: No Template Control (TE); Type 1: CALR Reaction mix TYPE 1; Type 2: CALR Reaction mix TYPE 2; C1: CALR Reaction mix CLAMP 1; C2: CALR Reaction mix 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.

Creating a work list

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*.

Note: The work list file can be saved. The work list can be created before loading the samples into the instrument, or when the experiment is set up in the instrument.

1. Switch on the Rotor-Gene Q MDx instrument.
2. Open Rotor-Gene AssayManager v2.1 software and log in as a user with the "Operator" role in the closed mode.
3. Select the **Setup** environment.
4. Click the button **New manual work list** in the work list manager.
5. Select the CALR assay profile from the list of available assay profiles.
6. Click **Move** to transfer the selected assay profile to the list of **Selected assay profiles**. The assay profile should now be displayed in the **Selected assay profiles** list.
7. Enter the number of samples (up to 6) in the corresponding field.
8. Select the **Kit Information** step. Use the kit bar code, or enter manually the following kit information found on the lid of the *ipsogen* CALR RGQ PCR Kit box:
   - Material number 1100703
   - Valid expiry date
   - Lot number
9. Select the **Samples** step. A list with the sample details is shown. This list represents the expected layout of the rotor.
10. Enter the sample identification number(s) into this list as well as any optional sample information as a comment for each sample.
11. Select **Properties** and enter a work list name.
12. Enable the check box **worklist is complete (can be applied)**.

13. **Save** the work list.
14. Press **Print work list** to print the work list.
    Printing the work list may help with the preparation and setup of the qPCR. The sample details are included as part of the work list.

Setting up the qPCR

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 using 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 standards, controls and reaction mixes before use.

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 µl after addition of 5 µl 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 (µl)	9 + 1 reactions (µl)*
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 µl per tube	
Sample distribution	5 µl 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 µl.

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 µl 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 µl of sample or control material into its corresponding tube according to the setup in Figure 4 to give a total volume of 25 µl. 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 *ipsogen* CALR RGQ PCR Kit components to the appropriate storage conditions to avoid any material degradation.

Preparing the Rotor-Gene MDx and starting the qPCR run

1. Place a 72-well rotor on the Rotor-Gene Q MDx rotor holder.
2. 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).
3. Attach the locking ring.
4. Load the Rotor-Gene Q MDx instrument with the rotor and locking ring. Close the instrument lid.
5. Within the Rotor-Gene AssayManager v2.1 software select the corresponding work list from the work list manager and click **Apply**.
   Alternatively, if the work list is still open, click the **Apply** button.

   Note: If a work list dedicated to the experiment has not been created, log in to Rotor-Gene AssayManager v2.1 and follow the steps in "Creating a work list," page 37, before proceeding.
6. Enter the experiment name.
7. Select the cycler to be used in **Cycler Selection**. A Rotor-Gene Q MDx 5plex HRM cycler must be used.
8. Check that the locking ring is correctly attached and confirm on the screen that the locking ring is attached.
9. Click **Start run**.

   The qPCR run should start.
10. When the run has finished, click **Finish run**.

    Note: Until this step is completed, the experiment is not saved in the internal database.

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 depending on the plug-in corresponding to the assay profile and the rules and parameter values defined by the assay profile.

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

The first step in the approval process is to filter the assay to be approved. This is done by using filter criteria in the **Approval** environment.

1. Release and approve 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.
2. Select the filter options for the assay to be approved and click **Apply**.
3. Review results and click the **Release/Report data** button.
4. Click **OK**.
   The report will be generated in .pdf format and automatically stored in the pre-defined folder.

   The default folder path is **QIAGEN > Rotor-Gene AssayManager > Export > Reports**.

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

5. 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*.

Interpretation of Results

Data analysis

The analysis of the qPCR results for each individual assay and sample is entirely automated. Rotor-Gene AssayManager v2.1 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.

To determine assay validity, Rotor-Gene AssayManager v2.1 also analyzes the run controls, i.e., CALR Wild-Type Control (WTC), CALR Mutant Control (MTC) and TE buffer (NTC) in the Green (FAM) and Yellow (HEX) channels for the *ipsogen* CALR RGQ PCR Kit reaction mixes (CALR TYPE 1, CALR TYPE 2, CALR CLAMP 1, CALR CLAMP 2, CALR CLAMP 3, CALR CLAMP 4 and CALR CLAMP 5). Validity for each control is based on compliance of C_T values with pre-defined specifications.

Note: If the internal amplification control in a given tube is invalid (Yellow channel), the CALR-specific target in the same tube (Green channel) is deemed invalid.

Note: If at least one external control is invalid for a given CALR assay (e.g. CLAMP 1 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.

Rotor-Gene AssayManager v2.1 also analyzes the unknown samples by checking the validity of the ABL1 internal control.

Finally, a *CALR* status is assigned to the unknown samples. In the first instance, the software considers the results obtained for the TYPE 1 and TYPE 2 assays. If either Type 1 or Type 2 positive mutation status is assigned to a sample, the *CALR* status is determined. The results obtained for the CLAMP assays are then displayed 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 (i.e. mutation detected or no mutation detected) is determined.

To conclude that a sample is positive, detection by at least one of the seven CALR assays is required. All controls associated with the concerned assay(s) and control in the tested sample must be valid, i.e., MTC, WTC, NTC and ABL1 internal control.

To conclude that a sample is negative, the sample must be negative with all assays, and all controls (MTC, WTC, and NTC) from all seven CALR assays, as well as the ABL1 internal control in the sample, must be valid.

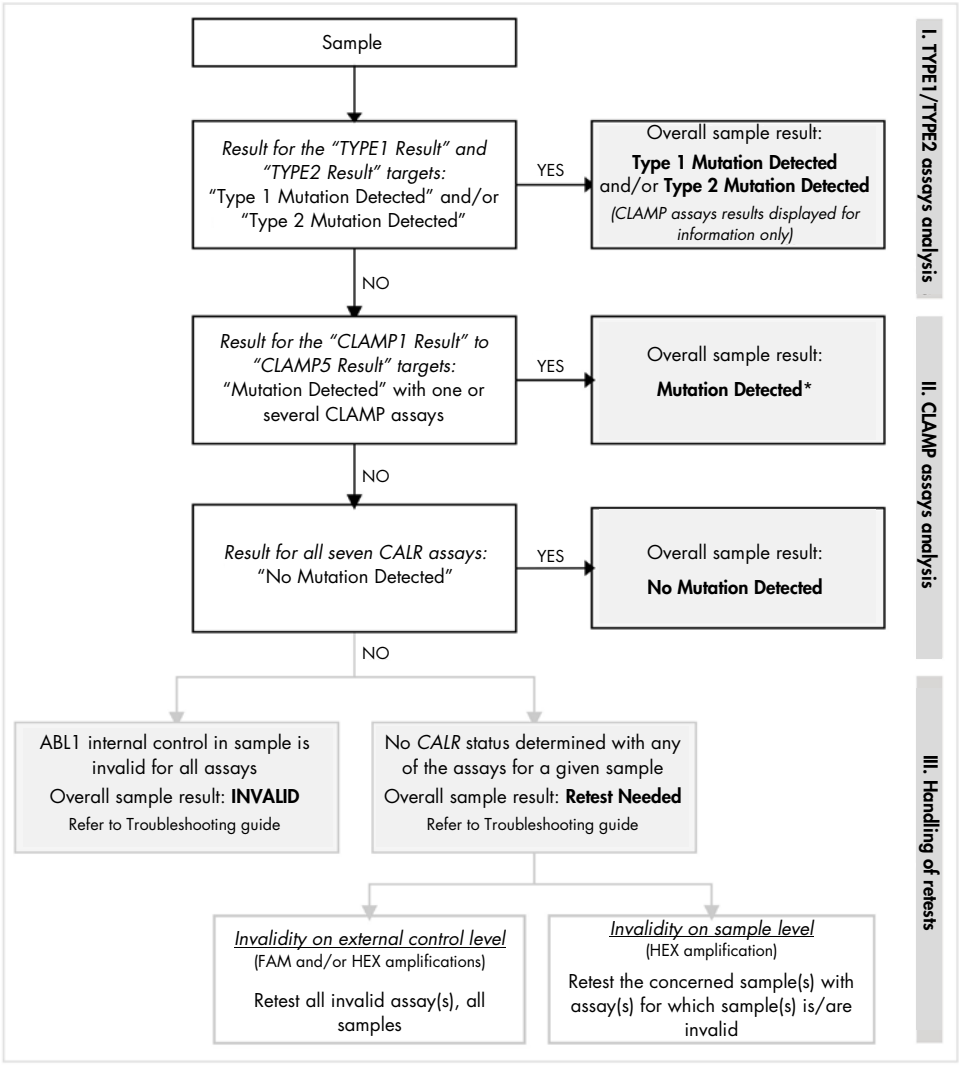
The results of the test samples, automatically analyzed and set by the Rotor-Gene AssayManager v2.1 software, have to be approved and released by a user logged in with the role of "Approver". Sample results to be approved have three additional approval buttons at the end of the dedicated row. These buttons 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*.

In case of invalid results, refer to the "Troubleshooting guide", page 54, to investigate the cause of failure and potentially identify any error that needs to be corrected.

Retests

In case of invalid results, follow the decision flowchart in Figure 6 to assess the need for retests.

Retests should not be needed if a *CALR* status could be assigned to the concerned sample(s) with any of the seven CALR assays.



* In cases where Type 1/Type 2 identification is mandatory, and the TYPE 1 and/or TYPE 2 assay is invalid, a retest may be needed — despite a positive CLAMP assay — 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.

Note: In cases where Type 1/Type 2 identification is mandatory, and the TYPE 1 and/or TYPE 2 assay is invalid, a retest may be needed — despite a positive CLAMP assay — to obtain a conclusive result for TYPE 1 and/or TYPE 2 assay.

Retests may be needed in other cases. When performing retests, 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 the tube in position 1) is always performed on the same amplification. Make sure to place each retested assay in its dedicated position (Figure 4) even if not all assays are present on the plate.

Note: If some of the seven CALR assays are missing when samples are retested, all empty positions that are usually filled will trigger an "INVALID" response from the software. For better traceability, empty positions and the expected nature of the associated response should be documented in the comments section of the report.

Results display

Targets

The results for each assay of the *ipsogen* CALR RGQ PCR Kit are displayed under the following target names:

- "ABL_AssayName" (e.g., ABL_TYPE_1) for the ABL1 internal amplification control (Yellow channel results)
- "AssayName" for a CALR Reaction Mix (e.g., TYPE 1 for the CALR Reaction Mix TYPE 1) (Green channel results)
- "AssayName Result" (e.g., TYPE 1 Result). These targets are combined targets; the corresponding result takes into account validity of controls (MTC, WTC, NTC and ABL1).

Results

Results for the above targets are displayed in the **Result** column of the report.

Table 3. Displayed results for each target

Target	Samples	Displayed results
ABL_AssayName (e.g., ABL_TYPE_1)	MTC, WTC, NTC, Test samples	Internal Control Valid, INVALID
AssayName (e.g., TYPE 1)	MTC, WTC, NTC	Signal, No Signal, INVALID
AssayName (e.g., TYPE 1)	Test samples	Significant Amplification Detected, No Significant Amplification Detected, No Amplification Detected, INVALID
TYPE 1 Result	Test samples	Type 1 Mutation Detected, No Mutation Detected, INVALID
TYPE 2 Result	Test samples	Type 2 Mutation Detected, No Mutation Detected, INVALID
CLAMP X Result (e.g., CLAMP 1 Result)	Test samples	Mutation Detected, No Mutation Detected, INVALID

If one of the controls (MTC, WTC, NTC) linked to a given sample is invalid for a given assay, or if the ABL1 internal control is invalid, the displayed result for the combined target result will be “INVALID”.

The conclusion of the analysis for each sample is displayed in the **Overall Sample Result** column of the report.

Table 4. Overall sample results

Sample result	Description
Type 1 Mutation Detected	The assayed sample carries the Type 1 *CALR* mutation.
Type 2 Mutation Detected	The assayed sample carries the Type 2 *CALR* mutation.
Type 1 and Type 2 Mutation Detected	The assayed sample carries the Type 1 and Type 2 *CALR* mutations. This event is rare but has been observed once during the *ipsogen* CALR RGQ PCR kit clinical validation.
Mutation Detected	The assayed sample carries a *CALR* mutation other than Type 1 or Type 2.
No Mutation Detected	No *CALR* mutation is detected in the assayed sample.
Retest Needed	The result is inconclusive due to invalidity of one or several controls for one or several CALR reaction mixes. A retest is needed to obtain a conclusive answer (see Figure 6). Example: A sample is positive (i.e., "Significant Amplification Detected") with the CLAMP1 assay only, but the NTC for the CLAMP1 assay is invalid due to contamination in the well. The sample result for CLAMP1 cannot be taken into account and the CLAMP1 Result will be displayed as INVALID. A retest must be performed for the CLAMP1 assay (MTC, WTC, NTC and concerned sample) to confirm a positive result for the sample.
INVALID	The ABL1 internal amplification control is invalid for the assayed sample with all seven CALR reaction mixes, while all external controls (MTC, WTC, NTC) are valid. This is most likely due to sample quality, or to incorrect sample normalization. See "Troubleshooting guide," page 54, for more details.

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 *ipsogen* CALR RGQ PCR Kit assay may provide the following assay-specific and universal flags:

Flag	Description
Assay-specific flags	
CONSECUTIVE_FAULT	A target that was used for calculation of this target is invalid.
IC_INVALID	The internal control is invalid. Target and internal control share the same tube.
INVALID_SIGNAL	Flag specific to the NTC. The C_T value is too low for the internal control or the CALR-specific amplification.
MC_HIGH_CT (CLAMP X)	The C_T value is too high for the Mutant Control.
MC_HIGH_CT (TYPE X)	The C_T value is too high for the Mutant Control.
MC_IC_ HIGH _CT (CLAMP X)	The C_T value is higher than expected for the internal control in the tube containing the mutant control.

Flag	Description
MC_IC_HIGH_CT (TYPE X)	The C_T value is higher than expected for the internal control in the tube containing the mutant control.
MC_IC_LOW_CT (CLAMP X)	The C_T value is lower than expected for the internal control in the tube containing the mutant control.
MC_IC_LOW_CT (TYPE X)	The C_T value is lower than expected for the internal control in the tube containing the mutant control.
MC_LOW_CT (CLAMP X)	The C_T value is too low for the Mutant Control.
MC_LOW_CT (TYPE X)	The C_T value is too low for the Mutant Control.
MC_NO_CT (CLAMP X)	No detectable C_T for the mutant control with the CLAMP X reaction mix.
MC_NO_CT (TYPE X)	No detectable C_T for the mutant control with the TYPE X reaction mix.
NO_SIGNAL_IC_INVALID	No internal control signal detected. Target and internal control share the same tube.
NTC_IC_LOW_CT (CLAMP X)	The C_T value is too low for the internal control in the tube containing the no template control.
NTC_IC_LOW_CT (TYPE X)	The C_T value is too low for the internal control in the tube containing the no template control.

Flag	Description
NTC_LOW_CT (CLAMP X)	The C_T value is too low for the No Template Control.
NTC_LOW_CT (TYPE X)	The C_T value is too low for the No Template Control.
SAMPLE_CLAMP X_IC_HIGH_CT	The C_T value is higher than expected for the internal control in a tube containing a sample.
SAMPLE_CLAMP X_IC_LOW_CT	The C_T value is lower than expected for the internal control in a tube containing a sample.
SAMPLE_TYPE X_IC_HIGH_CT	The C_T value is higher than expected for the internal control in a tube containing a sample.
SAMPLE_TYPE X_IC_LOW_CT	The C_T value is lower than expected for the internal control in a tube containing a sample.
WTC_IC_HIGH_CT (CLAMP X)	The C_T value is higher than expected for the internal control in the tube containing the WT control.
WTC_IC_HIGH_CT (TYPE X)	The C_T value is higher than expected for the internal control in the tube containing the WT control.
WTC_IC_LOW_CT (CLAMP X)	The C_T value is lower than expected for the internal control in the tube containing the WT control.

Flag	Description
WTC_IC_LOW_CT (TYPE X)	The C_T value is lower than expected for the internal control in the tube containing the WT control.
WTC_LOW_CT (CLAMP X)	The C_T value is too low for the WT Control.
WTC_LOW_CT (TYPE X)	The C_T value is too low for the WT Control.
Other flags	
ANALYSIS_FAILED	Assay is set to invalid because the analysis has failed due to various reasons. Contact QIAGEN Technical Services.
CURVE_SHAPE_ANOMALY	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.
FLAT_BUMP	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).
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.
NO_BASELINE	No initial baseline has been found. The subsequent analysis cannot be performed.

Flag	Description
RUN_FAILED	Assay is set to invalid due to a problem with the cycler or the cycler connection.
RUN_STOPPED	Assay is set to invalid because the run has been stopped manually.
SATURATION	The raw data fluorescence is saturating strongly before the inflection point of the amplification curve.
SPIKE	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the C_T is determined.
SPIKE_CLOSE_TO_CT	A spike is detected in the amplification curve close to the C_T.
STEEP_BASELINE	A steeply rising baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_BASELINE_DIP	A strong drop in the baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_NOISE	Strong noise is detected outside the growth phase of the amplification curve.
STRONG_NOISE_IN_GROWTH_PHASE	Strong noise is detected in the growth (exponential) phase of the amplification curve.
WAVY_BASE_FLUORESCENCE	Wavy baseline for the raw data fluorescence detected in the amplification curve.

Troubleshooting guide

This troubleshooting guide may be helpful in solving any problems that may arise in the assessment of *CALR* mutation status using the *ipsogen* 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 MDx instrument and Rotor-Gene AssayManager v2.1 software, please refer to the respective user manuals.

		Comments and suggestions
A sample is detected as positive with multiple assays		
	A given mutation can be detected by several assays	For instance, 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		
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.

		Comments and suggestions
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)	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 *ipsogen* 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.

	Comments and suggestions	
Early amplification of the internal amplification control in external controls and/or samples		
a)	DNA concentration is too high	Check the DNA concentration of the sample. The *ipsogen* 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.
No or low signals for the internal amplification control in samples, but the external controls are valid		
a)	DNA concentration is too low	Check the DNA concentration of the sample. The *ipsogen* 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.

		Comments and suggestions
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 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.
No template control (NTC/TE buffer) is positive (FAM and/or HEX)		
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)

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.

		Comments and suggestions
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.

Early amplification of the mutant control (MTC) (FAM amplification)

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.

		Comments and suggestions
Early amplification of the wild-type control (WTC) (FAM amplification)		
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)

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.

	Comments and suggestions
Frequent wild-type control (WTC) failure due to high background amplification below the assay validity target (C_T)	
Fault 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.
Run failure due to inconsistent fluorescence signal in controls and/or samples (across all tubes)	
Fault in Rotor-Gene Q MDx instrument accessories	Check instrument maintenance logs. The 72-Well Rotor 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 *ipsogen* CALR 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 product is to be used only by personnel specially instructed, trained for molecular biology techniques and familiar with this technology.

This kit should be used following the instructions given in this manual, in combination with a validated instrument mentioned in "Materials Required but Not Provided" page 15.

All reagents supplied in the *ipsogen* CALR RGQ PCR Kit are intended to be use solely with the other reagents supplied in the same kit. This may affect performance.

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

The *ipsogen* CALR RGQ PCR Kit is validated only for whole blood anticoagulated in 2K EDTA.

The *ipsogen* CALR RGQ PCR Kit is validated only for use with the QIAsymphony DNA DSP Mini Kit (cat. no. 937236) or the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104).

Only the Rotor-Gene Q MDx 5plex HRM (for PCR) and the QIAsymphony SP (for sample preparation) have been validated.

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

Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings. If the *CALR* status of a sample is "No Mutation Detected", it concerns only the absence of one of the 36 mutations described in this manual (see Table 1) – within the limits of the kit's sensitivity – or the lack of detection of mutations Type 23 and Type 27 (see "Performance Characteristics/Specificity", page 68). This does not exclude the presence of other *CALR* mutations.

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

Limit of blank

The limit of blank (LOB) was determined following the CLSI/NCCLS EP-17-A2 standard (8) on healthy donor whole blood samples, with a wild-type *CALR* status (5 samples, 60 measurements per reagent lot, 2 *ipsogen* CALR RGQ PCR Kit lots used). The LOB was determined for each given assay, as the lowest LOB value obtained.

The LOB results are summarized in Table 5.

Table 5. Summary of the limit of blank results for the *ipsogen* CALR RGQ PCR Kit

CALR assay	Limit of blank (C_T FAM values)
TYPE 1	35.24
TYPE 2	45.00
CLAMP 1	40.01
CLAMP 2	45.00
CLAMP 3	45.00
CLAMP 4	45.00
CLAMP 5	38.90

Limit of detection

The limit of detection (LOD) was determined based on the "Probit approach" described in the CLSI/NCCLS EP-17-A2 standard (8). In this study, 5 low-mutation levels were analyzed for 3 independent samples (gDNA extracted from *CALR* mutation-positive patient mixed with

wild-type DNA). A total of 20 replicates per dilution, per positive sample, were performed for the TYPE 1 and TYPE 2 assays with 2 lots of *ipsogen* CALR RGQ PCR Kit.

The LOD was determined for a given assay as the highest LOD value obtained out of the two considered batches. The results indicated that the analytical sensitivity for the Type 1 *CALR* mutation is 0.60% and the analytical sensitivity for the Type 2 *CALR* mutation is 0.08% (Table 6).

Table 6. Summary of the limit of detection results for the *ipsogen* CALR RGQ PCR Kit

CALR assay	Limit of detection
TYPE 1	0.60%
TYPE 2	0.08%

DNA input

The optimized gDNA input to be used in combination with the *ipsogen* CALR RGQ PCR Kit was evaluated with one kit lot on 3 *CALR*-positive samples (plasmids mixed with wild-type gDNA) and one *CALR*-negative sample for 5 different gDNA inputs. In this study, 3 replicates were performed per input sample and per CALR assay. The results showed that the optimized input to be used is 50 ng (10 ng/µL).

Repeatability and reproducibility

The precision study was performed according to the CLSI/NCCLS EP5-A2 standard (9). For each CALR assay, the precision was assessed on a given *CALR* mutation, i.e., Type 1 for the TYPE 1, CLAMP 1 and CLAMP 2 assays, Type 2 for the TYPE 2 and CLAMP 5 assays, and Type 28 for the CLAMP 3 and CLAMP 4 assays. Testing was performed on 3 levels of mutation: 5%, 25% and 50% (plasmids mixed with wild-type gDNA). Each level was tested in duplicate on 49 runs performed over 20 days, with a minimum of 73 measurements per mutation level and per assay. The 3 samples showed a coefficient of variation for total precision (CV_{Total}) below 5% for most assays (Table 7).

Note: For the CLAMP assays, total precision may vary from one *CALR* mutant to the other.

Table 7. Repeatability and reproducibility results for the *ipsogen* CALR RGQ PCR Kit

CALR assay	Level of mutation	No. of measure-ments	Sr*	Srr†	Total‡	CV_{Total}§
TYPE 1	50%	88	0.10	0.07	0.21	0.80
	25%	88	0.10	0.07	0.20	0.76
	5%	88	0.15	0.05	0.30	1.04
TYPE 2	50%	80	0.11	0.08	0.21	0.85
	25%	80	0.11	0.00	0.19	0.73
	5%	80	0.12	0.08	0.27	0.95
CLAMP 1	50%	106	0.14	0.13	0.27	1.05
	25%	105	0.13	0.28	0.50	1.90
	5%	106	0.20	0.37	0.55	1.92
CLAMP 2	50%	84	0.13	0.31	0.59	2.24
	25%	85	0.19	0.36	0.90	3.28
	5%	82	0.37	0.59	1.27	4.16
CLAMP 3	50%	84	0.49	0.52	2.33	8.04
	25%	84	0.73	0.70	3.54	11.26
	5%	84	1.28	3.18	5.70	15.03
CLAMP 4	50%	73	0.22	0.33	1.32	4.46
	25%	76	0.24	0.33	1.37	4.46
	5%	73	0.26	0.37	1.59	4.66
CLAMP 5	50%	100	0.17	0.17	0.66	2.52
	25%	100	0.21	0.05	0.75	2.73
	5%	104	0.39	0.55	0.94	3.04

* Sr: Repeatability expressed as standard deviation.

† Srr: Between run reproducibility expressed as standard deviation.

‡ Total precision (inter-instrument, inter-operator and inter-lot; expressed as a standard deviation).

§ Coefficient of variation for the total precision.

Interfering substances

The study design was based on recommendations described in the NCCLS standard EP07-A2 (10). A total of 17 substances that may be present in blood samples were chosen for their potential effect on PCR: busulfan, citalopram hydrobromide, paroxetine hydrochloride hemihydrate, sertraline hydrochloride, fluoxetine hydrochloride, acetaminophen [paracetamol], bilirubin unconjugated, potassium EDTA, hemoglobin [human], triglycerides, lisinopril dehydrate, hydroxyurea, acetylsalicylic acid, salicylic acid, thiotepa, anagrelide, interferon alpha 2b. In addition, the potential effect of one substance used during the gDNA extraction process (proteinase K) was also assessed.

The results showed that none of these substances had an interfering effect.

Specificity

The specificity of the *ipsogen* CALR RGQ PCR Kit was evaluated by testing the ability of the kit to identify correctly the Type 1 and Type 2 mutations, and to detect mutations described in Table 1.

For the Type 1 and Type 2 mutations, the study was conducted on gDNA samples extracted from whole blood of MPN Ph– patients, at concentrations ≥16% mutation for Type 1 and ≥9% mutation for Type 2. Specificity for Type 1 and Type 2 was confirmed: all samples were detected and correctly identified.

Specificity for mutations Type 3 to Type 36 was tested using gDNA samples extracted from whole blood of MPN Ph– patients when available (i.e., for Types 3, 4, 5, 24, 25, 27, 29). For each rare mutation where no patient sample could be obtained, specificity was assessed using synthetic material, comprising wild-type human gDNA mixed with plasmid DNA carrying a known *CALR* mutation, at clinically relevant concentrations >10% mutation (average concentration is around 30% mutation).

The results showed that all *CALR* mutations from Type 3 to Type 10, which are the most frequently observed, are detected by at least one assay of the *ipsogen* CALR RGQ PCR Kit. Most *CALR* mutations from Type 11 to 36 (0.3% occurrence) are detected by at least one assay of the *ipsogen* CALR RGQ PCR Kit. Only Types 23 and 27 are not detected by the kit, while Types 22, 25, 26, 29 and 30 may only be detected in samples with high *CALR* allelic burden.

Important note: The specificity study showed that Type 5 and Type 17 mutations are detected by the TYPE 1 assay. The TYPE 2 assay allows amplification of Type 10, Type 31 and Type 33–36 mutations. This was expected based on high sequence similarity between these *CALR* mutation types (see Table 1), with the exception of the Type 17 mutation. Therefore, the *ipsogen* CALR RGQ PCR Kit is not able to distinguish between Type 1 and Type 5/17 mutations, and cannot distinguish between Type 2 and Type 10/31/33–36 mutations. There is currently no necessity to differentiate each *CALR* mutation in terms of diagnosis or treatments; most *CALR* mutations lead to the generation of similar mutant CALR proteins.

Clinical validation and method comparison

The purpose of this study was to validate the *ipsogen* CALR RGQ PCR Kit under conditions of normal use. The study assessed the ability of the kit to identify Type 1 and Type 2 *CALR* mutations in a sample cohort composed of patients suspected of having MPN. This validation study was performed on gDNA samples extracted from 227 patients suspected of having MPN (including *CALR*-positive and *CALR*-negative samples).

The *CALR* status of the gDNA samples obtained with the *ipsogen* CALR RGQ PCR Kit were compared with the *CALR* status obtained with an independent mutation detection method based on fragment size analysis coupled with Sanger bidirectional sequencing. In cases of discordant results, a third mutation detection method was employed, next-generation sequencing (NGS).

The *CALR* status of all samples used in this study as determined by the reference methods is listed in Table 8. The sample cohort is composed of 54.6% positive samples and 45.4% negative samples. Among the positive samples, 42.7% were characterized as Type 1 and 33.1% as Type 2 by the reference methods. These proportions are consistent with those described by Klampfl et al. (5), i.e., 53% for Type 1 and 31.7% for Type 2 (see Table 1).

Table 8. *CALR* mutation status of the overall cohort determined by reference methods: fragment size analysis, Sanger bidirectional sequencing and NGS analysis

CALR status	Number
Type 1 mutation	53
Type 2 mutation	41
Type 1 and Type 2	1
Other *CALR* mutations	29
CALR mutation positive	124 (54.6%)
CALR mutation negative	103 (45.4%)
Total samples	**227**

All samples of the cohort characterized with a Type 1 and/or a Type 2 *CALR* mutation status were correctly identified with the *ipsogen* CALR RGQ PCR Kit. A Type 1 mutation was incorrectly assigned by the *ipsogen* CALR RGQ PCR Kit to two samples: one sample characterized as a Type 5 mutation by reference methods, and one sample characterized as a mutation not described in Klampfl et al. (5). Similarly, a Type 2 mutation was incorrectly assigned to one sample characterized by reference methods as a mutation not described in Klampfl et al. (5). *In silico* analysis showed that these discordant samples are likely due to high sequence similarity between these mutations and Type 1 or Type 2 mutations.

Consequently, the overall concordance between results obtained for Type 1 and Type 2 mutations combined with the *ipsogen* CALR RGQ PCR Kit and with fragment size analysis/Sanger sequencing/NGS is 98.7% (confidence interval [96.2%; 99.5%]). The sensitivity and specificity of the *ipsogen* CALR RGQ PCR Kit for Type 1 and Type 2 *CALR* mutations combined are 100% (confidence interval [96.2%; 100%] and 97.7% [93.5%; 99.5%]) (Table 9).

Table 9. Summary of the performance outcome for Type 1 and Type 2 *CALR* mutations combined

Variable	Estimate	95% confidence interval
Overall concordance	98.7%	[96.2% ; 99.7%]
Sensitivity	100%	[96.2% ; 100%]
Specificity	97.7%	[93.5% ; 99.5%]

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8. Clinical and Laboratory Standards Institute (CLSI) (2012). *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures: Approved Guideline,* 2nd ed. CLSI Document EP17-A2. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

9. Clinical and Laboratory Standards Institute (CLSI) (2004). *Evaluation of Precision Performance of Quantitative Measurement Methods: Approved Guideline*, 2nd ed. CLSI Document EP5-A2. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
REF	Catalog number
	Manufacturer
MAT	Material number
Rn	R is for revision of the Handbook and n is the revision number
LOT	Lot number
GTIN	Global Trade Item Number
IVD	In vitro diagnostic medical device

Symbol	Symbol definition
	CE mark for European conformity
	Use by
 <N>	Contains reagents sufficient for N reactions
	Temperature limitation
	Consult instructions for use
	Keep away from sunlight

Ordering Information

Product	Contents	Cat. no.
ipsogen 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	674023
Rotor-Gene Q MDx 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.
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

Product	Contents	Cat. no.
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
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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