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ipsogen[®] BCR-ABL1 MbcR RGQ RT-PCR Kit Handbook



Version 1

IVD

Quantitative in vitro diagnostics

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



REF

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QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden,
GERMANY

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Intended Use

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is a quantitative in vitro diagnostic test for the measure of the BCR-ABL1 fusion gene b3a2 (e14a2) and b2a2 (e13a2) transcripts in total RNA extracted from whole blood.

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is intended to monitor deep molecular response in patients diagnosed with chronic phase Philadelphia chromosome positive (Ph+) p210 chronic myeloid leukemia (CML).

It is calibrated against the World Health Organization (WHO) International Genetic Reference Panel.

Summary and Explanation

Background on chronic myeloid leukemia

CML belongs to the group of myeloproliferative neoplasms and is seen in >90% of cases characterized by the presence of the Philadelphia chromosome (Ph CHR5). This chromosome is the product of a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22), with the breakpoint cluster region (BCR) located on chromosome 22 and the c-ABL oncogene situated on chromosome 9. The corresponding fusion gene, BCR-ABL1, is transcribed into an 8.5 kb mRNA, with 2 junction variants, b2a2 (seen in 40% of cases) and b3a2 (present in 55% of cases). This fusion gene encodes a chimeric protein, p210, with elevated tyrosine kinase activity. The b2a3 and b3a3 transcripts represent less than 5% of cases. A Ph chromosome can also be detected in 35% of adult patients with acute lymphoblastic leukemia (ALL).

The annual incidence of CML is approximately 1–2 per 100,000, and CML accounts for 20% of all adult leukemias. It is characterized clinically by an excess of myeloid cells that differentiate and function normally. In 90–95% of cases of CML, patients will be diagnosed in the chronic or stable phase of the disease. In the past, patients would progress to blastic crisis and acute leukemia with fatal consequences within an average of 4 to 6 years. The advent of imatinib and, more recently, second generation tyrosine kinase inhibitors (TKI), however, has dramatically changed the natural course of the disease. Most patients will now remain in remission and as such require long-term follow-up and disease monitoring.

Disease monitoring

The current goal of CML therapy is to achieve 100% survival and Ph chromosome negativity. Disease monitoring is therefore an essential tool to assess treatment response and detect relapse in each patient as early as possible. When treated with TKIs, patients typically progress from hematologic to cytogenetic then molecular remission, with a corresponding decrease in the number of leukemic cells and BCR-ABL1 transcripts, as shown in Figure 1.

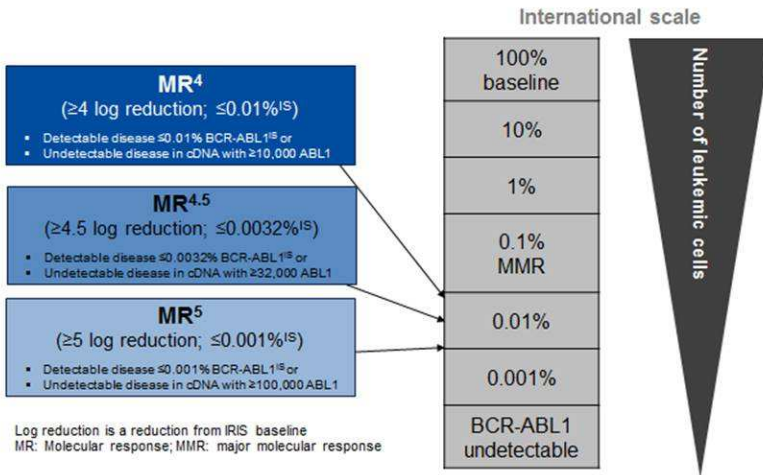


Figure 1. Definition of molecular response. Adapted from references 1, 2 and 9. **MR:** molecular response. **MMR:** major molecular response.

The reference method to estimate tumor burden in CML patients is conventional cytogenetic analysis (G-banding) on bone marrow (BM) metaphases. Cytogenetic response is assessed on at least 20 marrow metaphases. The level of cytogenetic response is estimated based on the percentage of Ph chromosome-positive metaphases (3). However, this assessment is influenced by laboratory performances and expertise and has a low sensitivity, at 5% when 20 metaphases are analyzed.

Real-time quantitative polymerase chain reaction (qPCR) quantification of BCR-ABL1 Mbc mRNA on peripheral blood (PB) specimens defines the molecular response, and this is now part of the disease monitoring techniques used in CML. It is less invasive than conventional bone marrow metaphase cytogenetics and more sensitive.

Recommendations for CML disease monitoring have also been recently updated to incorporate new clinical evidence from drug trials, higher clinical efficacy of second generation TKIs, and BCR-ABL1 quantification technical improvements, all leading to enhanced disease monitoring

objectives. Specifically, second generation TKIs lead to a more significant molecular response in a large number of CML patients, achieving what is defined as a deep molecular response, and corresponds to a BCR-ABL1 load below 0.01% (MR4.0) or 0.0032% (MR4.5). Being able to accurately quantify these very low levels of BCR-ABL1 load could be of clinical relevance, as it has been shown in observational trials that TKIs may be safely discontinued in patients with a sustained MR4.5 molecular response (4). Additional clinical trials, however, are under way to confirm these findings.

The latest recommendations on response definition and monitoring of CML patients on TKIs come from the ELN experts (3).

From a technical standpoint, efforts have been made by international experts to harmonize BCR-ABL1 MbcR testing and reporting (5–7). Additionally, a reference panel has been validated recently under auspices of the WHO, to allow a simple standardization of BCR-ABL1 quantification (8).

Principle of the Procedure

qPCR permits the accurate quantification of PCR products during the exponential phase of the PCR amplification process. qPCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during and/or subsequent to PCR cycling, thereby drastically reducing the risk of PCR product contamination. 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 double-dye oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. A double-dye oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream 3' quencher dye, hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes exploits the 5'→3' exonuclease activity

of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer.

During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'→3' exonuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR (Figure 2). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and hence amplified during PCR. Because of these requirements, nonspecific amplification is not detected. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

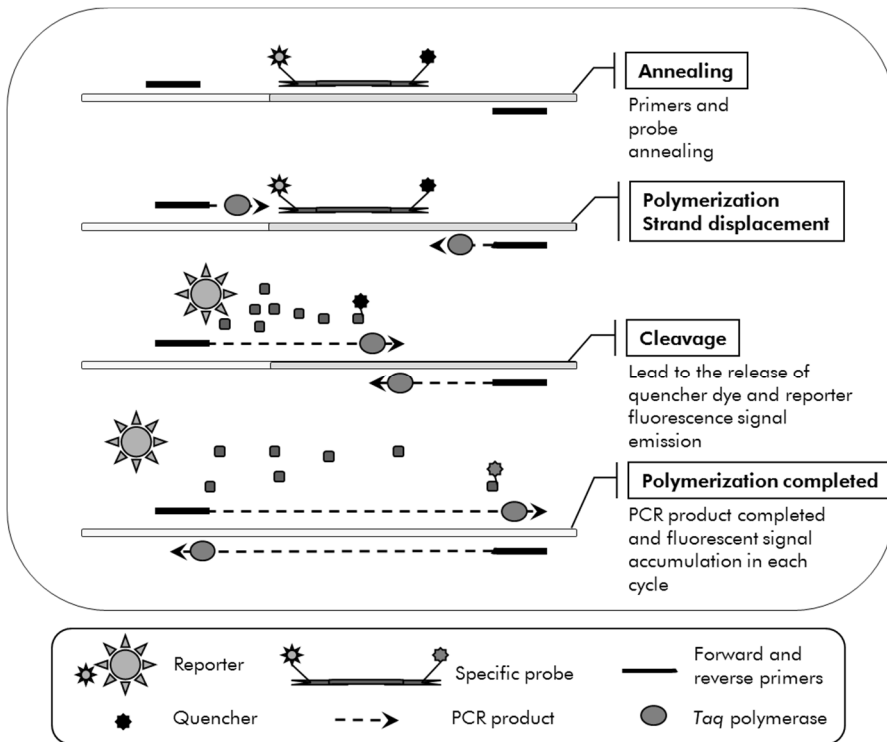


Figure 2. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™-BHQ®-1). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'→3' exonuclease activity of the *Taq* DNA polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them and leading to an increase in fluorescence from the FAM and a decrease in fluorescence from the BHQ-1.

Materials Provided

Kit contents

<i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit		(24)
Catalog no.		670923
Number of reactions		24
Reagents for reverse transcription (RT)		24 μ l
Reverse transcriptase	Purple	100 μ l
RT Mix	Purple	300 μ l
Reagents for calibration		
High Positive RNA Control	White	15 μ l x 3
Low Positive RNA Control	White	15 μ l x 3
IS-MMR Calibrator	White	15 μ l x 3
SP1-BCR-ABL MbcR and ABL: MbcR and ABL1 Single Plasmid Standard Dilution (10^1 copies/5 μ l)	Yellow	35 μ l
SP2-BCR-ABL MbcR and ABL: MbcR and ABL1 Single Plasmid Standard Dilution (10^2 copies/5 μ l)	Yellow	35 μ l
SP3-BCR-ABL MbcR and ABL: MbcR and ABL1 Single Plasmid Standard Dilution (10^3 copies/5 μ l)	Yellow	70 μ l
SP4-BCR-ABL MbcR and ABL: MbcR and ABL1 Single Plasmid Standard Dilution (10^4 copies/5 μ l)	Yellow	35 μ l
SP5-BCR-ABL MbcR and ABL: MbcR and ABL1 Single Plasmid Standard Dilution (10^5 copies/5 μ l)	Yellow	70 μ l
SP6-BCR-ABL MbcR and ABL: MbcR and ABL1 Single Plasmid Standard Dilution (10^6 copies/5 μ l)	Yellow	70 μ l

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Reagents for qPCR			
Taq DNA polymerase	Mint		85 µl
qPCR Mix ABL1 *	Green		720 µl x 3
qPCR Mix Mbcrt	Red		720 µl x 3
ipsogen <i>BCR-ABL1 Mbcrt RGQ RT-PCR Handbook</i>			1

* Contains a mix of specific reverse and forward primers for the ABL1 control gene plus a specific FAM-BHQ-1 probe.

† Contains a mix of specific reverse and forward primers for the BCR-ABL1 Mbcrt fusion gene plus a specific FAM-BHQ-1 probe.

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.

Reagents for erythrocyte lysis

- Erythrocyte Lysis (EL) Buffer (cat. no. 79217)
- 14.3 M β-mercaptoethanol*
- RNeasy® Midi Kit (cat. no. 75144)

* Recommended chemicals and equipment for erythrocyte lysis and RNA isolation can be potentially hazardous. Ensure that the appropriate personal protective equipment and protective measures are set before use.

Reagents for total RNA isolation

- RNeasy Midi Kit (cat. no. 75144)
- Ethanol (70%, 80% and 96–100%)
- RNA cleanup and concentration step: RNeasy MinElute® Cleanup Kit (cat. no. 74204)
- Nuclease-free PCR-grade water

Consumables

- Nuclease-free, aerosol-resistant, sterile PCR pipet tips with hydrophobic filters
- 18–20-gauge needle* fitted to RNase-free syringe
- 0.5 ml or 0.2 ml nuclease-free tubes
- 1.5 ml or 2 ml nuclease-free tubes
- 50 ml centrifuge tubes
- Strip Tubes and Caps 0.1 ml for the Rotor-Gene Q (cat. nos. 981103 or 981106)
- Ice

Equipment

- Pipets* dedicated for PCR (1–10 µl; 10–100 µl; 100–1000 µl)
- Benchtop centrifuge* with rotor for 0.2 ml and 2 ml reaction tubes (capable of attaining 8000 x *g* or 10,000 rpm)
- Spectrophotometer*
- Laboratory centrifuge* with rotor for 15 and 50 ml centrifuge tubes (capable of 3000–5000 x *g*) that allows refrigerated centrifugation (4°C)

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

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- Thermomixer, heated orbital incubator, heating block or water bath (for the reverse transcription step)*
 - Rotor-Gene Q MDx 5plex HRM* (cat. no. 9002032) and associated specific material
Note: Rotor-Gene Q MDx 5plex HRM cannot be used for the reverse transcription step.

Equipment for qPCR with manual analysis

Rotor-Gene Q software version 2.1.0 or higher

Equipment for qPCR with automated analysis

- Rotor-Gene AssayManager® software version 2.1.x (x≥0)
- Rotor-Gene AssayManager Gamma Plug-in v1.0.x (x≥0)
- Assay Profile ipsogen_BCR-ABL1Mbc(ABL)_blood_CE_V1_0_x.iap (x≥1)

Warnings and Precautions

For in vitro diagnostic use

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view and print the SDS for each QIAGEN® kit and kit component.

All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazard materials. Blood is considered potentially infectious. All necessary precautions recommended by the appropriate regulatory authorities in the country of use should be taken when working with whole blood.

Recommended chemicals and equipment for erythrocyte lysis and RNA isolation can be potentially hazardous. Ensure that the appropriate personal protective equipment and protective measures are set before use.

General precautions

Use of qPCR tests requires good laboratory practices, including maintenance of equipment dedicated to molecular biology and compliant with applicable regulations and relevant standards. The components of this product are sufficient to perform 24 reactions for each assay.

- Discard sample and assay waste according to your local safety procedures.
- Reagents provided by the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit are optimally diluted. Do not further dilute reagents, as this may result in a loss of performance.

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- All reagents supplied in the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit are intended to be used solely with the other reagents supplied in the same kit. Do not substitute any reagent between *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kits, as this may affect performance.
 - Refer to the Rotor-Gene Q MDx 5plex HRM instrument, Rotor-Gene AssayManager v2.1 and Gamma plug-in user manuals for additional warnings, precautions and procedures.
 - Alteration of incubation times and/or temperatures may result in erroneous or discordant data.
 - Do not use expired or incorrectly stored components.
 - Use extreme caution to prevent cross-contamination using Positive Controls.
 - Use extreme caution to prevent cDNA or PCR product carryover contamination resulting in a false positive signal.
 - Use extreme caution to prevent contamination by RNase or DNase, which may cause degradation of RNA or cDNA templates.
 - Do not open the Rotor-Gene Q MDx 5plex HRM instrument until the run is finished.
 - 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 at all times so maintaining traceability.

We therefore recommend the following:

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials)
- Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Prepare pre-PCR master mixes with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrices (cDNA, plasmid, or PCR products) are introduced.
- Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).

Refer to the corresponding handbooks for safety information specific to the reagents and kits used for sample preparation. Safety information for the RNeasy Midi Kit (cat. no. 75144) in association with Buffer EL (cat. no. 79217) is provided in the *RNeasy Midi/Maxi Handbook* and safety information for the RNeasy MinElute Cleanup Kit (cat. no. 74204) is provided in the *RNeasy MinElute Cleanup Handbook*.

Reagent Storage and Handling

Shipping conditions

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is shipped on dry ice. If any component of the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is not frozen on arrival, if the outer packaging has been opened during transit, or if the shipment does not contain a packing note or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Storage conditions

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit should be stored immediately upon receipt at -30°C to -15°C in a constant-temperature freezer. Care must be taken to protect the qPCR mixes from light.

For storage information relative to the reagents and kits used for sample preparation: RNeasy Midi Kit (cat. no. 75144), Buffer EL (cat. no. 79217), RNeasy MinElute Cleanup Kit (cat. no. 74204), refer to the corresponding handbooks.

Stability

When stored under the specified storage conditions, the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is stable until the stated expiration date.

Once opened, reagents can be stored in their original packaging at -30°C to -15°C until the stated expiration date shown on the packaging. Do not exceed a maximum of five freeze-thaw cycles.

For stability information relative to the reagent and kits used for sample preparation: RNeasy Midi Kit (cat. no. 75144), Buffer EL (cat. no. 79217), RNeasy MinElute Cleanup Kit (cat. no. 74204), refer to the corresponding handbooks.

Specimen Handling and Storage

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is for use with RNA samples extracted from whole blood. All samples should be treated as potentially hazardous.

Whole blood samples

- Whole blood samples should be anti-coagulated with potassium EDTA ($\text{K}_2\text{-EDTA}$) and stored at $2\text{--}8^{\circ}\text{C}$ for no more than 4 days before RNA extraction.
- Do not use frozen blood.
- Label, handle and store blood samples in a controlled manner, according to local procedures.

Note: Whole blood samples must be shipped under the same conditions as storage to avoid temperature changes.

RNA samples

- Following isolation, purified RNA may be stored at -30°C to -15°C or lower (-90°C to -65°C) if long-term storage is required.
- Label, handle and store RNA samples in a controlled manner, according to local procedures.

Note: RNA samples must be shipped under the same conditions as storage to avoid temperature changes during storage and shipment.

Procedure

Total RNA should be purified from 10 ml of peripheral whole blood collected in EDTA tubes.

- Ensure reagents to be used for erythrocyte lysis, RNA isolation and RNA concentration are not expired and have been transported and stored under appropriate conditions.
- Use the RNeasy Midi Kit (cat. no. 75144) and Buffer EL for erythrocyte lysis (cat. no. 79217) for RNA purification from peripheral whole blood.

Erythrocyte lysis protocol for isolation of total leukocytes from whole blood

This protocol is designed for isolation of total leukocytes from 10 ml of human whole blood using Buffer EL (cat. no. 79217).

Note: This protocol is not designed for the use of frozen whole blood samples.

Important notes before starting

- Blood and body fluids of all human subjects are considered potentially infectious. All necessary precautions recommended by the appropriate regulatory authorities in the country of use should be taken when working with whole blood.
- Buffer RLT may form a precipitate upon storage. If necessary, dissolve by warming, and then place at room temperature.
- Erythrocyte lysis step should be performed on ice.
- Centrifugation steps 3 and 5 of this protocol should be performed at 4°C in a standard laboratory centrifuge.

Things to do before starting

- Prepare Buffer RLT (supplied with the RNeasy Midi Kit) by adding β -mercaptoethanol (β -ME): add 10 μ l β -ME per 1 ml Buffer RLT.
- Buffer RLT is stable for 1 month after addition of β -ME.
Note: β -ME is toxic; dispense in a fume hood and wear appropriate protective clothing.
Note: Buffer RLT contains guanidine isothiocyanate, which can form highly reactive compounds when combined with bleach. Do not add bleach or acidic solutions directly to the sample-preparation waste.

Procedure

1. Add 40 ml of Buffer EL to 10 ml whole blood in a single 50 ml centrifuge tube. Mix by inverting briefly.
2. Incubate for 15 minutes on ice. Mix by inverting briefly twice during the incubation.
Note: The cloudy suspension becomes translucent during incubation, indicating lysis of red blood cells.
3. Centrifuge at 400 x g for 10 minutes at 4°C. Completely discard the supernatant. Save the leukocyte pellet.
Note: The leukocytes will form a pellet after centrifugation. Ensure complete removal of the supernatant. Trace amounts of red blood cells that might remain are eliminated in the following steps.
Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, affecting the RNA binding conditions to the RNeasy membrane. Both effects may reduce RNA yield.
4. Add 20 ml of Buffer EL to the leukocyte pellet and resuspend by pipetting up and down.
5. Centrifuge at 400 x g for 10 minutes at 4°C. Completely discard the supernatant. Save the leukocyte pellet.
Note: The following centrifugation steps (e.g., RNA isolation) must be performed at 20–25°C.

6. Loosen the leukocyte pellet by flicking the tube in 4 ml of Buffer RLT supplemented with β -ME. Vortex or pipet to mix.

Note: Ensure that β -ME is added to Buffer RLT before use.

7. Ensure disruption using a conventional rotor–stator homogenizer for at least 45 seconds at full speed until the sample is uniformly homogeneous. Alternatively, vortex the sample for 10 seconds, and pass the lysate at least 10 times through an 18–20-gauge needle fitted to an RNase-free syringe.

Note: Incomplete disruption will lead to significantly reduced yields by clogging of the RNeasy column. Disruption with rotor–stator homogenizers generally results in higher total RNA yields compared to other homogenization methods.

Note: Samples can be stored at -90°C to -65°C in lysis buffer after disruption. Frozen samples are stable for months.

Isolation of total RNA

This protocol is designed for isolation of total cellular RNA from homogenized leukocyte lysate resuspended in 4 ml of RLT/ β -ME.

Important notes before starting

- DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- The RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20 – 25°C . Ensure that the centrifuge does not cool below 20°C .

- At each centrifugation step, the entire volume must pass through the column. It may be necessary to repeat the centrifugation.

Things to do before starting

- If needed, thaw leukocyte lysate at room temperature before starting the RNA isolation protocol.
- Prepare 4 ml of 70% ethanol per sample.
- RPE Buffer is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.

Procedure

1. Add 4 ml 70% ethanol to the lysate, and mix thoroughly by shaking vigorously. Do not centrifuge.

Note: Visible precipitates may form after the addition of ethanol. Dissolve precipitates completely by vigorous shaking, and proceed immediately to step 2. Insufficient dissolution of precipitates may cause DNA contamination leading to an impure total RNA sample.

2. Apply the sample, including any precipitate that may have formed, to an RNeasy Midi Column placed in a 15 ml centrifuge tube (supplied). Close the tube gently, and centrifuge for 5 minutes at 4000 x g. Discard the flow-through.

Note: The maximum loading volume is 4 ml. If the volume exceeds 4.0 ml, load aliquots successively onto the RNeasy Column, and centrifuge as above. Discard the flow-through after each centrifugation step.

Reuse the centrifuge tube in step 3.

-
3. Add 4 ml Buffer RW1 to the RNeasy Column. Close the centrifuge tube gently, and centrifuge for 5 minutes at 4000 x *g* to wash the column. Discard the flow-through.

Note: Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.

Reuse the centrifuge tube in step 4.

4. Add 2.5 ml Buffer RPE to the RNeasy Column. Close the centrifuge tube gently, and centrifuge for 2 minutes at 4000 x *g* to wash the column.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

Reuse the centrifuge tube in step 5. The flow-through need not be discarded.

5. Add another 2.5 ml Buffer RPE to the RNeasy Column. Close the centrifuge tube gently, and centrifuge for 5 minutes at 4000 x *g* to dry the RNeasy silica-gel membrane.

Note: It is important to dry the RNeasy membrane since residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

Note: Following the centrifugation, remove the RNeasy column from the centrifuge tube carefully so the column does not contact the flow-through as this will result in the carryover of ethanol.

6. To elute, transfer the RNeasy Column to a new 15 ml collection tube (supplied). Pipet 200 µl of RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 minute, and then centrifuge for 3 minutes at 4000 x *g*.
7. Repeat the elution step (step 6) using the eluate from step 6 and then centrifuge for 5 minutes at 4000 x *g*.

Note: For long term storage, RNA can be stored at –90°C to –65°C.

Qualification and quantification of RNA

Assay quality is largely dependent on the quality of input RNA. We recommend analyzing purified RNA by agarose gel electrophoresis or spectrophotometry prior to analysis.

- A blank of nuclease-free PCR-grade water should be used to calibrate the spectrophotometer.
- OD of 1.0 at 260 nm is equivalent to approximately 40 µg/ml single-stranded RNA.
- An OD_{260}/OD_{280} ratio between 1.8 and 2.1 is indicative of highly purified RNA.

To perform the RT step, the required RNA concentration is 200 ng/µl. If the RNA concentration in the eluate is less than 200 ng/µl, the RNA concentration in the eluate should be increased using the RNeasy MinElute Cleanup Kit (QIAGEN, cat. no. 74204).

If RNA concentration in the eluate is above the upper range limit, the concentration should be adjusted to 200 ng/µl with RNase-free water.

Note: Check RNA concentration after normalization.

RNA concentration

This protocol is optimized for concentrating RNA.

Important notes before starting

- DNase digestion is not required since RNeasy MinElute silica-membrane technology efficiently removes most of the DNA.
- Buffer RLT contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.

- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20° C.
- Buffer RLT may form a precipitate during storage. If necessary, dissolve by warming, and then place at room temperature (15–25°C).

Things to do before starting

- Prepare 500 µl of 80% ethanol for each RNA sample to be concentrated.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to provide a working solution.
- Set up the columns at room temperature before starting.
- Measure the volume of the samples to be treated and adjust so that the final sample volume is 200 µl.

Procedure

1. After adjusting the sample to a volume of 200 µl with RNase-free water, add 700 µl Buffer RLT, and mix well.
2. Add 500 µl of 96–100% ethanol to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer a maximum of 700 µl of the sample to a RNeasy MinElute Spin Column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through. Transfer any remaining sample (up to 700 µl) and repeat the centrifugation. Discard the flow-through.

Note: Flow-through contains Buffer RLT and is therefore not compatible with bleach. See “Warnings and Precautions”, page 15, for safety information.

4. Place the RNeasy MinElute Spin Column in a new 2 ml collection tube (supplied).

5. Add 500 μ l Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

Reuse the collection tube in step 6.

6. Add 500 μ l of 80% ethanol to the RNeasy MinElute Spin Column. Close the lid gently, and centrifuge for 2 minutes at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through and collection tube.

Note: Flow-through contains Buffer RLT and is therefore not compatible with bleach.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

7. Place the RNeasy MinElute Spin Column in a new 2 ml collection tube (supplied).
8. Open the lid of the spin column, and centrifuge at full speed for 5 minutes. Discard the flow-through and collection tube.

To avoid damaging the lids of the spin columns, place the spin columns into the centrifuge with at least one empty position between columns. Orientate the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

It is important to dry the spin column membrane since residual ethanol may interfere with downstream reactions. Centrifuging the spin columns with their lids open ensures no ethanol is carried over during RNA elution.

9. Place the RNeasy MinElute Spin Column in a new 1.5 ml collection tube (supplied).
10. Add 20 μ l RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 minute at full speed to elute the RNA.
11. When the elution step is complete, place samples on ice.

12. Measure the volume of the samples to be treated and adjust so that the final concentration is 200 ng/μl.

See “Qualification and quantification of RNA”, page 25, for further details if needed.

Reverse transcription

Things to do before starting

- Thaw all necessary components except the reverse transcriptase, which must be kept in the freezer when it is not being used. Place the tubes containing the components to be thawed on ice.
Note: Do not exceed 30 minutes for the thawing step to avoid any material degradation.
- Clean the bench area dedicated for reverse transcription (RT) mix preparation to ensure no template or nuclease contamination.
- Mix well by pipetting up and down 10 times the tubes containing the reverse transcription reagents, RNA samples, positive controls and IS-MMR Calibrator, and briefly centrifuge before use. Subsequently, keep on ice.
- The RT-negative control is generated during the reverse transcription step using nuclease-free PCR-grade water.
- The required input is 3 μg of RNA per sample.
- The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit contains enough reagents to perform three runs of eight samples.

Procedure

1. Incubate 15 μl of each sample, positive controls (high and low positive controls), water (used to generate the RT-negative control) and IS-MMR calibrator for 5 minutes at 65°C. Then, immediately cool on ice for at least 5 minutes.

2. Centrifuge briefly (approximately 5 seconds), to collect the liquid in the bottom of the tube. Subsequently, keep on ice.
3. Prepare the following RT mix according to the number of samples, controls, and calibrators being processed (Table 1).

Note: the final volume per reaction must be 25 μ l.

Table 1. Preparation of RT mix

Component	Volume per sample (μ l)	RT mix: 12 + 1 reactions (μ l)	Final concentration
RT Mix, 3.33x	7.5	97.5	1x
Reverse transcriptase, 10x	2.5	32.5	1x
Final volume of RT mix (to be added in step 4)	10	130	–
Sample, positive controls, IS-MMR Calibrator or water (from step 1)	15	15 each	–
Total volume	25	25 each	–

4. Pipet 10 μ l RT mix into each labeled tube containing RNA sample, positive controls, water or calibrator (from step 3).
5. Mix well by pipetting up and down 10 times and centrifuge briefly (approximately 5 seconds), to collect the liquid in the bottom of the tube.

Note: Return all the reverse transcription reagents in the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit to the freezer after setting up the reactions to avoid any material degradation.

6. Place the tubes in the thermal cycler and set the reverse transcription program (Table 2).

Table 2. Temperature profile for reverse transcription

Step	Parameters
Reverse transcription 1	Temperature: 25°C Time: 10 minutes
Reverse transcription 2	Temperature: 46°C Time: 45 minutes
Inactivation	Temperature: 85°C Time: 5 minutes
Cooling	Temperature: 4°C Time: 5 minutes

7. Once the program has finished, centrifuge the tubes briefly (approximately 5 seconds) to collect the liquid in the bottom of the tube. Keep the tubes on ice or at -20°C until performing the qPCR experiment.

Manual analysis: qPCR on the Rotor-Gene Q MDx 5plex HRM instrument with 72-tube rotor with RGQ software

We recommend performing all measurements in duplicate, as indicated in Table 3. The kit allows testing eight cDNA samples in the same experiment in duplicate. Three experiments can be performed with the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit.

Table 3. Number of reactions for Rotor-Gene Q instrument with 72-tube rotor

Sample	Reactions
With the qPCR Mix ABL1 (34 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA High Positive Control	2 reactions
1 cDNA Low Positive Control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	4 x 2 reactions (SP3, SP4, SP5, and SP6)
RT negative control	2 reactions
Water control	2 reactions
With the qPCR Mix MbcR (34 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA High Positive Control	2 reactions
1 cDNA Low Positive Control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	5 x 2 reactions (SP1, SP2, SP3, SP5, and SP6)
Water control	2 reactions

Setup of loading block and rotor

We recommend testing at least eight cDNA samples in the same experiment to optimize the use of the standards, primers and probe mixes. The rotor scheme in Figure 3 shows an example experiment.

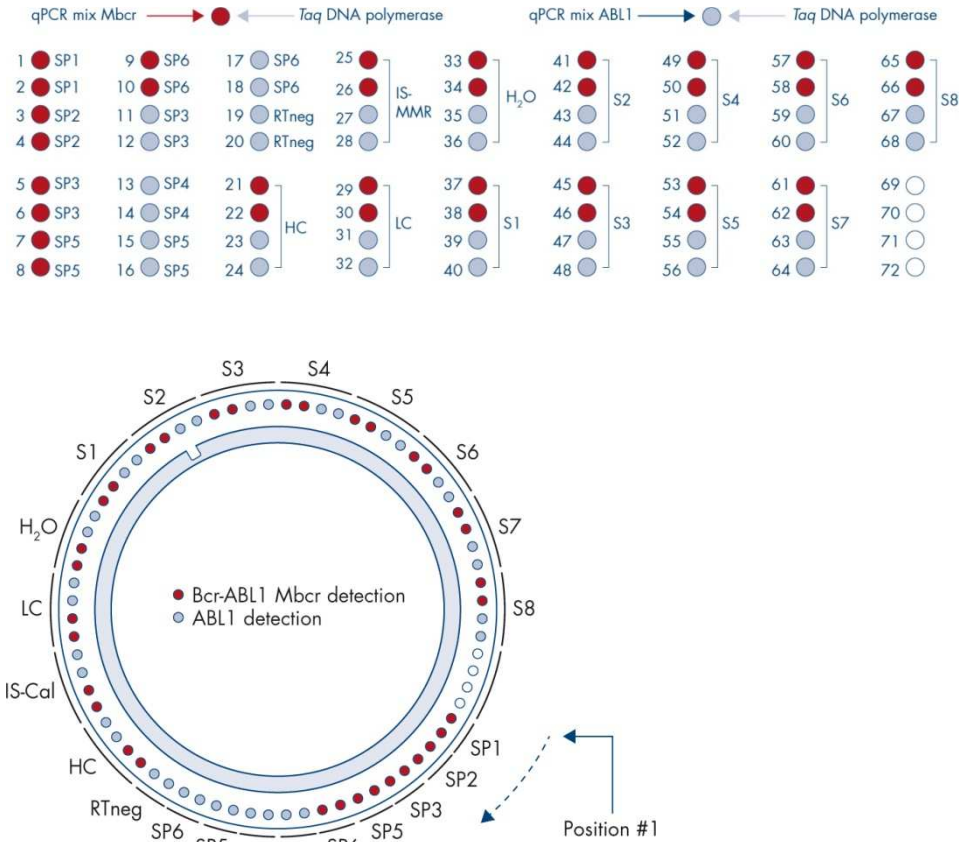


Figure 3. Rotor setup for each experiment. SP1–SP6: BCR-ABL1 Mbcr and ABL1 standards; RTneg: RT negative control; IS-Cal: IS-MMR Calibrator; HC: High Positive Control; LC: Low Positive Control; H₂O: water control; S1–S8: cDNA samples.

Note: Fill all empty positions with empty tubes. Numbers denote positions in the loading block and indicate final rotor position.

Setting up the qPCR

Things to do before starting

- Thaw all necessary components except the *Taq* DNA polymerase, which must be kept in the freezer when it is not being used. Place the tubes containing the components to be thawed on ice.

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

- Clean the bench area dedicated for PCR mix preparation to ensure no template or nuclease contamination
- Mix well by pipetting up and down 10 times the tubes containing the qPCR Mix ABL1 and qPCR Mix Mbcr and briefly centrifuge before use. Subsequently, keep on ice.

Procedure

1. Prepare the PCR master mix according to the number of samples being processed.

Table 4 describes the pipetting scheme for the preparation of a reagent mix, calculated to achieve a final reaction volume of 25 μ l. A pre-mix is prepared, according to the number of reactions, using the same primers and probe mix (either qPCR Mix ABL1 or qPCR Mix Mbcr). Extra volumes are included to compensate for pipetting error.

Note: Do not use reaction volumes (reaction mix plus sample) of less than 25 μ l.

Table 4. Preparation of PCR master mix

Component	1 reaction (µl)	Pre-mix ABL1 or Mbcr: 34 +2 reactions (µl)	Final concentration
qPCR Mix (either qPCR Mix ABL1 or qPCR Mix Mbcr)	19.75	711	1x
<i>Taq</i> DNA polymerase	0.25	9	1x
Sample, standard, control or IS-MMR Calibrator (to be added at step 3)	5	5 each	–
Total volume	25	25 each	–

2. Dispense 20 µl qPCR pre-mix into each 0.1 ml Rotor-Gene Q tube.
3. Add 5 µl of RT product (cDNA) obtained after the reverse transcription step (see “Reverse transcription”, page 28), 5 µl of standards, 5 µl of controls or IS-MMR Calibrator, according to the sample layout as shown in Figure 4 (total volume 25 µl).
4. Mix gently by pipetting up and down.

Preparing the Rotor-Gene MDx and starting the qPCR run

1. Place the tubes in the adapter provided with the instrument.
Note: Unused positions must be filled with empty tubes.
2. Place the locking ring above tubes and press to lock.
3. Load the full adapter into the Rotor-Gene Q MDx 5plex HRM instrument.
4. Program the Rotor-Gene Q MDx 5plex HRM instrument with the thermal cycling program as indicated in Table 5.

Note: Return all the *ipsogen* BCR-ABL1 Mbcr RGQ RT-PCR Kit components to the freezer to avoid any degradation of material.

Table 5. Temperature profile for qPCR

Step	Parameters
Mode of analysis	Quantitation
Hold 1	Temperature: 95°C Time: 15 minutes
Cycling	50 cycles 94°C; 15 seconds 60°C; 60 seconds with acquisition of FAM fluorescence in the Green channel.

5. Click "Gain Optimisation" in the "New Run Wizard" dialog box to open the "Auto-Gain Optimisation Setup" dialog. Check the range for the Green channel from "5 FI" for "Min Reading" to "10 FI" for "Max Reading" and the acceptable Gain range from -10 to 10.
6. Check that "Perform Optimisation Before 1st Acquisition" option is ticked, and close the "Auto-Gain Optimisation Setup" dialog box.
7. Start the thermal cycling program.
8. Create both ABL1 and MbcR subsets, filling "Edit samples" window.
9. Once the thermal cycling has finished, select "Options" and "Crop Start Cycles". Remove data before cycle 10. Then, select "Analysis" and "Cycling A. Green from 10", indicated on the report as "left threshold = 10.00".

10. For both ABL1 and Mbcr, proceed as follows:

- If the "Calculate Automatic Threshold" window opens, select "Cancel".
- Define the threshold at 0.03 (at the right of the window at the bottom).
- Select "Dynamic Tube" as a normalization method in the report and "Slope Correct" to correct the noise slope.
- Check that "Outlier Removal" is set to 0% (corresponding to the NTC threshold) and "Reaction Efficiency Threshold" is disabled.
- Set the graph to linear scale and "Auto-Scale".
- Right-click on the window displaying amplification curves and check that "Digital filter" is set to "Light".
- Select the option "named on" (to the right of the window) to be sure that all samples are displayed.

Once all steps are complete, ensure raw data recording and proceed to results analysis (see "Data analysis principle", page 47).

Automated analysis: qPCR on the Rotor-Gene Q MDx 5plex HRM instrument with 72-tube rotor with RGAM software

We recommend performing all measurements in duplicate, as indicated in Table 6. The kit allows testing eight cDNA samples in the same experiment in duplicate. Three experiments can be performed with the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit.

Table 6. Number of reactions for Rotor-Gene Q instrument with 72-tube rotor

Sample	Reactions
With the qPCR Mix ABL1 (34 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA High Positive Control	2 reactions
1 cDNA Low Positive Control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	4 x 2 reactions (SP3, SP4, SP5, and SP6)
RT negative control	2 reactions
Water control	2 reactions
With the qPCR Mix MbcR (34 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA High Positive Control	2 reactions
1 cDNA Low Positive Control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	5 x 2 reactions (SP1, SP2, SP3, SP5, and SP6)
Water control	2 reactions

Important notes before starting

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit must be run on the Rotor-Gene Q MDx 5plex HRM instrument using Rotor-Gene AssayManager v2.1. Take time to familiarize yourself with

the Rotor-Gene Q MDx instrument before starting the protocol. See the user manuals for the instrument, the Rotor-Gene AssayManager software version 2.1, and the Gamma Plug-in for details.

Rotor-Gene AssayManager v2.1 enables automated interpretation of the PCR results. The cycling parameters are locked for the run.

Things to do before starting

Rotor-Gene AssayManager software version 2.1 must be installed on the computer connected to the Rotor-Gene Q and can be downloaded from the QIAGEN website: http://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 *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

- The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit requires the specific Gamma Plug-in. This plug-in can be downloaded from the QIAGEN website page: <https://www.qiagen.com/resources/resourcedetail?id=bf8c9a8-245b-4ab4-99ea-1b39e2c243a0&lang=en>. This plug-in must be installed on a computer that already has Rotor-Gene AssayManager version 2.1 installed.
- The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit also requires an assay profile. This assay profile (*.iap file) contains all parameters needed for cycling and analyzing the qPCR assay. It can be downloaded from the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit dedicated webpage on the QIAGEN website <https://www.qiagen.com/shop/detection-solutions/personalized-healthcare/ipsogen-bcr-abl1-mbcR-rgq-rt-pcr-kit-ce/#resources>. The assay profile needs to be imported in Rotor-Gene AssayManager v2.1 software.

Note: The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit can only run if certain configuration settings in the Rotor-Gene AssayManager software v2.1 are programmed.

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

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

Installation of Gamma plug-in and importing the assay profile

The installation and importing of the Gamma Plug-in and the assay profile are detailed in the Rotor-Gene AssayManager v2.1 and Gamma Plug-in handbooks, respectively, *Rotor-Gene AssayManager v2.1 Core Application User Manual* and the *Gamma Plug-in User Manual*.

- Download both the Gamma Plug-in and the latest version of the ipsogen_BCR-ABL1 Mbc(ABL)_blood_CE assay profile from the QIAGEN website.
- Start the installation process by double-clicking on the RGAM_V2_1_Gamma_Plug-in.Installation.V1_0_0.msi file, and following the installation instructions. For a detailed description of this process, please refer to the section on “Installing Plug-ins” in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

Note: For system-wide process safety, select the “Settings” tab and check the boxes for “Material number required”, “Valid expiry date required” and “Lot number required” for the closed mode (section Work list). If these are not enabled (checked), click to enable.

-
- After successful installation of the plug-in, a person with administrator rights for the Rotor-Gene AssayManager software will need to import the ipsogen_BCR-ABL1Mbc(ABL)_blood_CE assay profile as follows:
 1. Log-in to Rotor-Gene AssayManager v2.1 software as user with administrator rights.
 2. Select the configuration environment.
 3. Select the tab "Assay Profiles".
 4. Click the "Import" button.
 5. Select the assay profile ipsogen_BCR-ABL1Mbc(ABL)_blood_CE to be imported in the dialog and click "Open".
 6. Once the assay profile has been successfully imported, it 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 at least eight cDNA samples in the same experiment to optimize the use of the standards, primers and probe mixes. The rotor scheme in Figure 4 shows an example experiment.

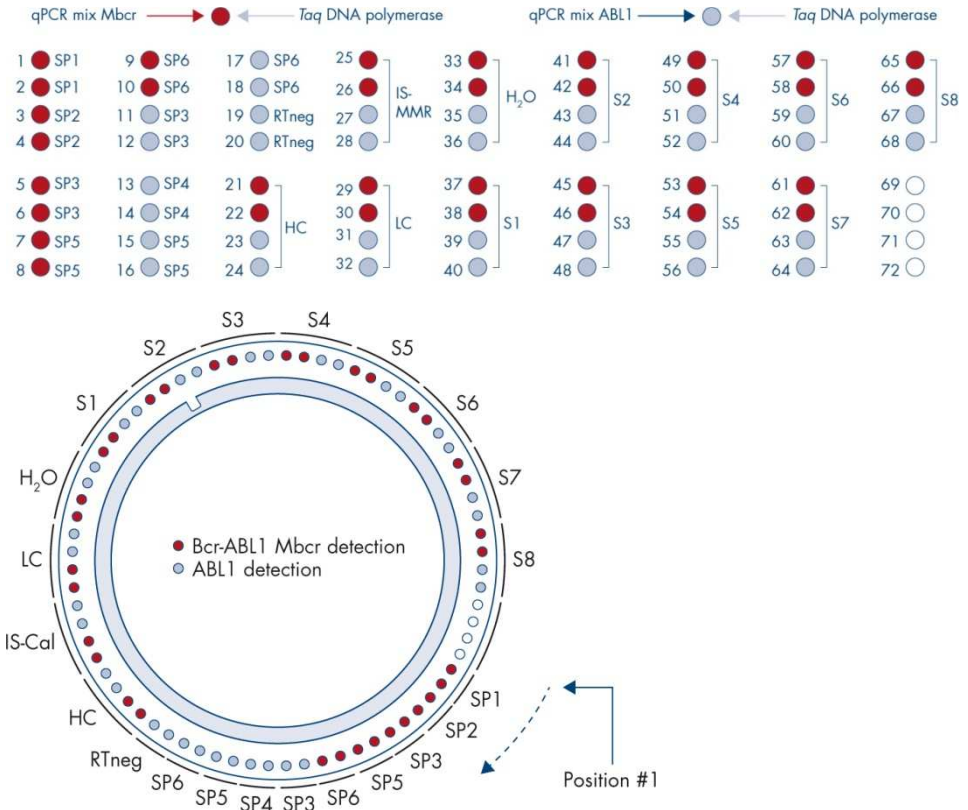


Figure 4. Rotor setup for each experiment. SP1–SP6: BCR-ABL1 Mbcr and ABL1 standards; RTneg: RT negative control; IS-Cal: IS-MMR Calibrator; HC: High Positive Control; LC: Low Positive Control; H₂O: water control; S1–S8: cDNA samples. **Note:** Fill all empty positions with empty tubes. Numbers denote positions in the loading block and indicate final rotor position.



Tubes must be inserted into the rotor as indicated in Figure 4, as the automated analysis set in the assay profile is based on this organization. If a different layout is used, aberrant results will be obtained.

Note: Fill all resting positions with empty tubes.

Creating a work list

Create a work list for the samples to be processed as follows.

1. Switch on the Rotor-Gene Q MDx instrument.
2. Open the Rotor-Gene AssayManager v2.1 software and log-in as user with the operator role in the closed mode.
3. Click the button “New manual work list” in the work list manager (“Setup” environment).
4. Select the “ipsogen_BCR-ABL1Mbc(ABL)_blood_CE” assay profile from the list of available assay profiles in the “Assay” step.
5. Click the “Add assay to work list” button 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.
6. Enter the number of samples in the corresponding field.
7. Select the “Kit information” set and enter the following *ipsogen* BCR-ABL1 Mbc RGQ RT-PCR kit information, which is printed on the lid of the box.
 - Material number: 0670923
 - Valid expiry date
 - Lot number.
8. Select the “Samples” step. A list with the sample details is shown. This list represents the expected layout of the rotor.

-
9. Enter the sample identification number(s) into this list as well as any optional sample information as a comment for each sample.
 10. Select the "Properties" step and enter a work list name.
 11. Enable the check box "is applicable".
 12. Save the work list.
 13. The work list can be printed and this may help with the preparation and setup of the qPCR. To print the work list, press the "Print work list" button. The sample details are included as part of this work list.

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

Setting up the qPCR

Things to do before starting

- Thaw all necessary components except the *Taq* DNA polymerase, which must be kept in the freezer when it is not being used. Place the tubes containing the components to be thawed on ice.
Note: Do not exceed 30 minutes for the thawing step to avoid any material degradation.
- Clean the bench area dedicated for PCR mix preparation to ensure no template or nuclease contamination
- Mix well by pipetting up and down 10 times the tubes containing the qPCR Mix ABL1 and qPCR Mix Mbcr and briefly centrifuge before use. Subsequently, keep on ice.

Procedure

1. Prepare the PCR master mix according to the number of samples being processed.

Table 7 describes the pipetting scheme for the preparation of a reagent mix, calculated to achieve a final reaction volume of 25 μ l. A pre-mix is prepared according to the number of reactions, using the same primers and probe mix (either qPCR Mix ABL1 or qPCR Mix Mbcr). Extra volumes are included to compensate for pipetting error.

Note: Do not use reaction volumes (reaction mix plus sample) of less than 25 μ l.

Table 7. Preparation of PCR master mix

Component	1 reaction (μ l)	Pre-mix ABL1 or Mbcr: 34 +2 reactions (μ l)	Final concentration
qPCR Mix (either qPCR Mix ABL1 or qPCR Mix Mbcr)	19.75	711	1x
<i>Taq</i> DNA polymerase	0.25	9	1x
Sample, standard, control or IS-MMR Calibrator (to be added at step 3)	5	5 each	–
Total volume	25	25 each	–

2. Dispense 20 μ l qPCR pre-mix into each 0.1 ml Rotor-Gene Q tube.
3. Add 5 μ l of RT product (cDNA) obtained after the reverse transcription step (see “Reverse transcription”, page 28), 5 μ l of standards, 5 μ l of controls or IS-MMR Calibrator according to the sample layout as shown in Figure 4 (total volume 25 μ l).
4. Mix gently, by pipetting up and down.

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 4, 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.
3. Attach the locking ring.
4. Load the Rotor-Gene Q MDx instrument with the rotor and locking ring, and close the instrument lid.
5. Within the Rotor-Gene AssayManager software v2.1, either select the corresponding work list from the work list manager and click the “Apply” button, or if the work list is still open, click the “Apply” button.
Note: If the experiment dedicated work list has not been created, log in to Rotor-Gene AssayManager v2.1 and follow step “Creating a work list”, page 42, before proceeding as follows.
6. Enter the experiment name.
7. Select the cyclers to be used in the “Cycler selection”.
8. Check correct attachment of locking ring and confirm on the screen that the locking ring is attached.
9. Click the “Start run” button. The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR run should start.

Release and report qPCR results

1. When the run has finished, click “Finish run”.
2. 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”.

3. If “Release and go to approval” was clicked, the results for the experiment are displayed.
4. If “Release” was clicked by a user with user role, someone with an “Approver” role will need to log in and select the “Approval” environment.
 - a. Filter for the assay to be approved by selecting the filter options and clicking the “Apply” button.
 - b. Check the check box next to the Experiment that is to be approved.
 - c. Click the “Start approval” button.

As the experiment contains one calibrator, mandatory information about the calibrator needs to be entered in the “Calibrator” tab before samples can be finally approved.

5. Select the “Use calibrator” button and enter the corresponding value (found on the IS-MMR Calibrator tube or on the Certificate of Analysis).

Note: You must enter this value twice in the fields “Enter calibrator value” and “Reenter calibrator value”.

Confirm the entered values by pressing the “Apply” button: results are updated.

Note: Once at least one sample is released, the calibrator cannot be changed any more.

6. Review results and click the “Release/Report data” button.

Click “OK”. The report will be generated in *.pdf format and automatically stored in the pre-defined folder.

By default, this folder path is: **QIAGEN > Rotor-Gene AssayManager > Export > Reports**

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

Note: For troubleshooting, a support package from the run is required. Support packages can be generated from the approval or archive environment (*Rotor-Gene AssayManager v2.1 Core Application User Manual*, Section 1.8, “Troubleshooting” > “Creating a support package”). In addition, the audit trail from the time of incident ± 1 day might be helpful. The audit trail can be retrieved in the Service environment (*Rotor-Gene AssayManager v2.1 Core Application User Manual*, Section 1.5.5.5).

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

Interpretation of Results on RGQ Software

Data analysis principle

Using TaqMan® technology, the number of PCR cycles necessary to detect a signal above the threshold is called the threshold cycle (C_T) and is directly proportional to the amount of target present at the beginning of the reaction.

Using standards with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample. The standard curves are plasmid-based. To ensure accurate standard curves, four standard dilutions for ABL1 and five standard dilutions for MbcR are used. The kit also includes an IS calibrator allowing conversion of results to the international scale. Figure 5 and Figure 6 show examples of TaqMan amplification curves similar to those obtained for standards, the IS-MMR Calibrator, the High Positive RNA Control and the Low Positive RNA Control with the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit.

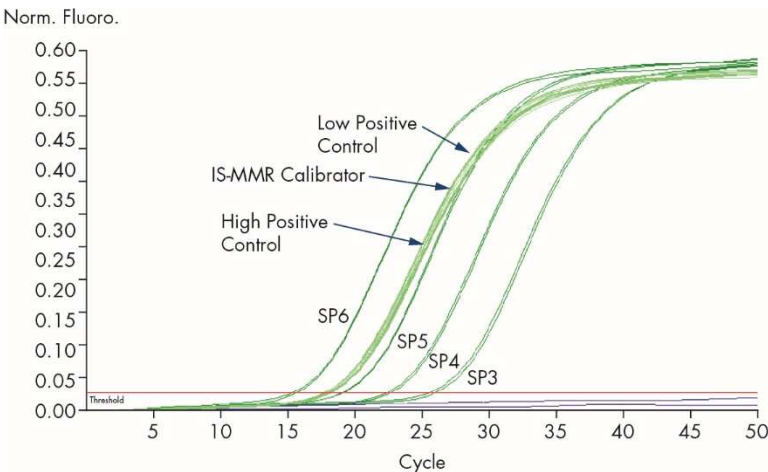


Figure 5. Detection of ABL1 with controls and standards SP3, SP4, SP5 and SP6. 10^3 , 10^4 , 10^5 and 10^6 copies/reaction.

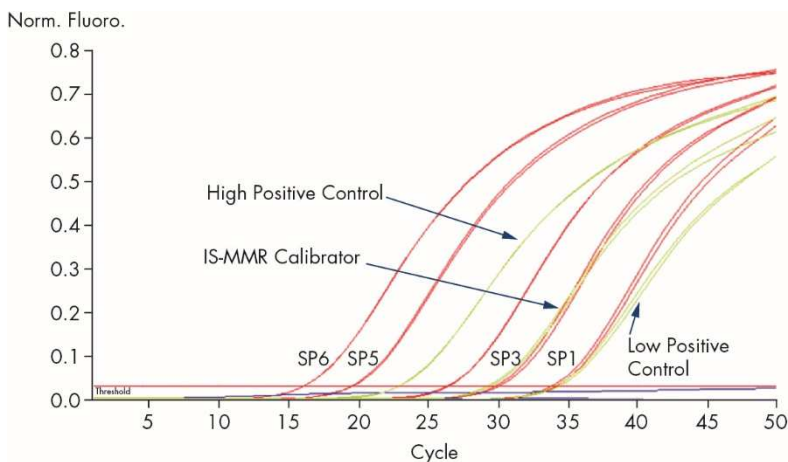


Figure 6. Detection of BCR-ABL1 Mbc with controls and standards SP1, SP2, SP3, SP5 and SP6. 10^1 , 10^2 , 10^3 , 10^5 and 10^6 copies/reaction.

Standard curves and quality criteria applicable to raw data

Reproducibility between replicates

The variation in C_T values between replicates should be ≤ 2 or the duplicate should be invalidated except in the following cases:

If mean $C_T \geq 36$ or if $C_{T_a} \geq 36$ and C_{T_b} is “not detected” then ΔC_T criteria do not apply; the duplicate conforms. If this is the case, the copy number (CN) calculated for C_{T_a} should be divided by 2.

Note: Users should measure reproducibility in their laboratory.

Standard curves

Raw data can be pasted into an Excel® file for analysis.

For each gene (ABL1 and BCR-ABL1 Mbcrr), C_T values obtained from plasmid standard dilutions are plotted according to the log copy number (3, 4, 5 and 6 for SP3, SP4, SP5 and SP6; 1, 2, 3, 5 and 6 for SP1, SP2, SP3, SP5 and SP6). Figure 7 shows an example of an ABL1 curve calculated with four standard dilutions. Figure 8 shows an example of a BCR-ABL1 Mbcrr curve calculated with five standard dilutions.

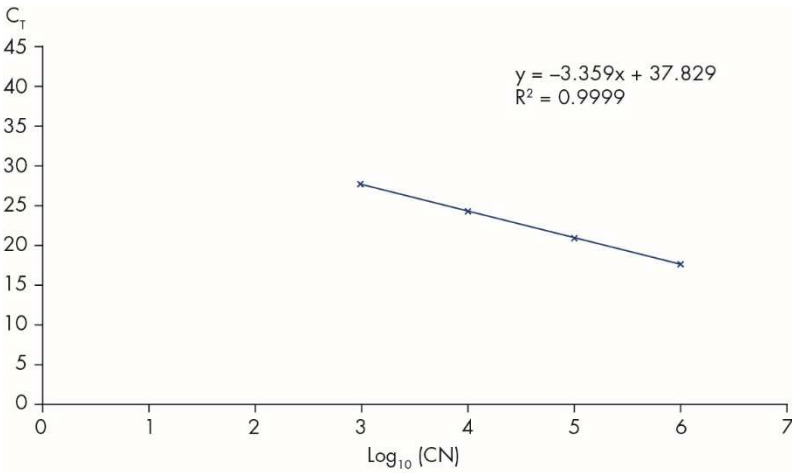


Figure 7. Standard curve for ABL1 calculated from four standard dilutions. A linear regression curve ($y = ax + b$) is calculated, where “a” is the slope of the line and “b” is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. The equation and coefficient of determination (R^2) are shown on the graph.

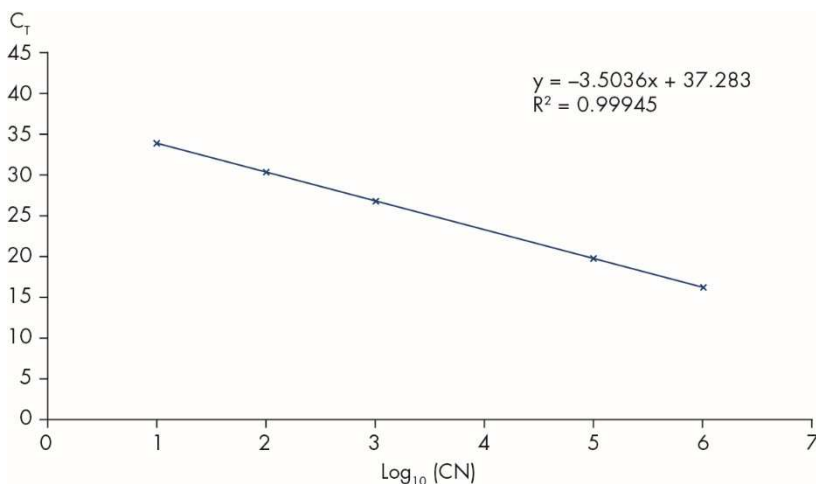


Figure 8. Standard curve for BCR-ABL1 MbcR calculated from five standard dilutions. A linear regression curve ($y = ax + b$) is calculated, where “a” is the slope of the line and “b” is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. The equation and coefficient of determination (R^2) are shown on the graph.

As standards are tenfold dilutions, the theoretical slope of the curve is -3.3 . A slope between -3.1 and -3.6 is acceptable as long as R^2 is >0.95 . However, a value for $R^2 >0.98$ is desirable for precise results.

Note: The SP1 standard dilution (BCR-ABL1 plasmid, 10 copies) must be detected to establish the BCR-ABL MbcR standard curve.

Copy number (CN)

The ABL1 or BCR-ABL1 MbcR standard curve equation should be used to transform raw C_T values (obtained with the qPCR Mix ABL1 or the qPCR Mix MbcR for the unknown samples into ABL1 or BCR-ABL1 copy numbers ($ABL1_{CN}$ or $BCR-ABL1 MbcR_{CN}$).

$$\text{Log}_{10} \text{ sample ABL1}_{\text{CN}} = \frac{\text{Mean ABL } C_T - \text{ABL1 standard curve intercept}}{\text{ABL standard curve slope}}$$

$$\text{Log}_{10} \text{ sample BCR-ABL1 M}_{\text{bcrCN}} = \frac{\text{Mean BCR-ABL1 M}_{\text{bcr}} C_T - \text{BCR-ABL1 M}_{\text{bcr}} \text{ standard curve intercept}}{\text{BCR-ABL1 M}_{\text{bcr}} \text{ standard curve slope}}$$

Quality control on all ABL1_{CN} values

Poor RNA quality or problems during the RT-qPCR can result in low ABL1 copy numbers.

To reach optimal sensitivity of the test ABL1_{CN} should be equal or greater than 100,000 for the High Positive RNA Control, the Low Positive RNA Control, and the IS-MMR Calibrator.

RT negative and water controls

The no template control (NTC) for the PCR step (water control) and the reverse transcription step (RT negative control) should give zero CN for both ABL1 and BCR-ABL1 M_{bcr}. Consequently, no C_T should be obtained or the C_T value is above the intercept of standard curves, respectively. A positive result for these NTCs indicates cross-contamination during reverse transcription and/or qPCR.

Normalized copy number (NCN)

The ratio of these CN values gives the normalized copy number (NCN):

$$\text{NCN} = \frac{\text{BCR-ABL1 Mbcrc}_{\text{CN}}}{\text{ABL1}_{\text{CN}}} \times 100$$

Calculate the NCN result for the High Positive RNA Control (NCN_{HC}), the Low Positive RNA Control (NCN_{LC}), the IS MMR calibrator (NCN_{cal}), and each sample (NCN_{sample}).

Quality control on normalized copy number values

High Positive RNA Control, Low Positive RNA Control, and IS-MMR Calibrator allow the monitoring of the reverse transcription and amplification steps of ABL1 and BCR-ABL1 Mbcrc during transcript quantification.

- The NCN result obtained for the IS-MMR-Calibrator, tested with the *ipsogen* BCR-ABL1 Mbcrc RGQ RT-PCR Kit, must be within the interval 0.05–0.3 as otherwise, NCN values cannot be converted to the International Scale.
- The sensitivity of the experiment can be assessed only if the Low Positive RNA Control is detected.

International scale conversion

Note: Before interpretation, refer to the value indicated on the IS-MMR Calibrator tube label or on the certificate of analysis provided with the kit. (Check that the same value is indicated on both the label and the certificate).

Use the experimental IS-MMR Calibrator NCN result (NCN_{cal}), and its assigned value (IS-Cal value) indicated in the certificate of analysis, to calculate the normalized copy number on the international scale (IS-NCN_{sample}).

$$\text{IS-NCN}_{\text{sample}} = \frac{\text{NCN}_{\text{sample}} \times \text{IS-Cal value}}{\text{NCN}_{\text{cal}}}$$

Quality control on IS-NCN values

- The IS-NCN_{HC} result (NCN on the international scale for the High Positive RNA Control) should give no major molecular response (“No MMR”, see “Molecular response reporting” below).
- The IS-NCN_{LC} result (NCN on the international scale for the Low Positive RNA Control) should be <0.01 (MR4) to ensure that MR4.5 status can be established with confidence.

Molecular response reporting

Determine the molecular response status of each sample according to the interpretation in Table 8.

Table 8. Molecular response reporting

Case	ABL CN	BCR-ABL1 Mbcn CN	IS-NCN%	Status
1	<10,000	<10	–	Poor quality sample
2	<10,000	≥10	>0.1	No MMR
		≥10	≤0.1	Inconclusive
3	10,000 ≤CN _{ABL} <32,000	≥LOD	>0.1	No MMR
			≤0.1	MMR
		LOB<CN<LOD Replace CN by LOD	>0.1	No MMR
			≤0.1	MMR
		≤LOB	–	Not detected/MR4
4	32 000 ≤CN _{ABL} <100,000	≥LOD	>0.1	No MMR
			0.01<IS≤0.1	MMR
			≤0.01	MR4
		LOB<CN<LOD Replace CN by LOD	>0.1	No MMR
			0.01<IS≤0.1	MMR
			≤0.01	MR4
5	100,000 ≤CN _{ABL}	≥LOD	>0.1	No MMR
			0.01<IS≤0.1	MMR
			0.0032<IS≤0.01	MR4
			≤0.0032	MR4.5
		LOB<CN<LOD Replace CN by LOD	>0.1	No MMR
			0.01<IS≤0.1	MMR
			0.0032<IS≤0.01	MR4
	≤0.0032	MR4.5		
	≤LOB	–	Not detected/MR5	

LOB: limit of blank; **LOD:** limit of detection; **MR:** molecular response; **MMR:** major molecular response.

Summary of quality criteria

Fehler! Ungültiger Eigenverweis auf Textmarke. summarizes the various quality criteria and associated values or results.

Table 9. Quality criteria summary

Criteria	Acceptable values/results
Variations in C_T values between replicates	$\leq 2 C_T$ Except if mean $C_T \geq 36$ or if $C_{T_a} \geq 36$ and C_{T_b} is "not detected": the duplicate conforms. CN calculated for C_{T_a} should be divided by 2.
Slope for standard curves	Between -3.1 and -3.6
R^2 for standard curves	At least >0.95 (and ideally >0.98)
SP1 standard dilution (BCR-ABL1 10 copies plasmid)	Must be detected to establish the standard curve
Quality control on ABL_{CN} value for biological samples	Refer to Table 8
High Positive RNA Control, Low Positive RNA Control, and the IS-MMR-Calibrator	$ABL_{CN} \geq 100,000$
NTC (water) and RTneg controls	For each $ABL_{CN} = 0$ and $Mb_{CTCN} = 0$ (no C_T value or $C_T >$ standard curve intercept)
NCN obtained for IS-MMR Calibrator (NCN_{cal})	Must be within the range $0.05-0.3$
High Positive RNA Control	Must be detected
Low Positive RNA Control	Must be detected
IS- NCN_{HC}	Status: No major molecular response
IS- NCN_{LC}	$IS-NCN_{LC} \leq 0.01$ (MR4) Must be detected to ensure that MR4.5 status can be established with confidence.

C_T : threshold cycle; **HC**: high control; **IS**: International Standard; **LC**: low control; **MR**: molecular response; **MMR**: major molecular response; **NCN**: normalized copy number; **NTC**: No Template Control; **RTneg**: reverse transcription negative.

Interpretation of Results on RGAM Software

The analysis is entirely automated.

Rotor-Gene AssayManager v2.1 first 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.

The results of the test samples are automatically analyzed and set by Rotor-Gene AssayManager v2.1 but have to be approved and released by the 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 *Gamma Plug-in User Manual*.

Rotor-Gene AssayManager v2.1 will then analyze the run controls:

- NTC (RT-neg and H2O) are checked for the absence of specific amplification (ABL1 and BCR-ABL1 Mbcf).
- ABL1 and BCR-ABL1 Mbcf SP: The validation is based on the R^2 and the slope values of each.
- HC: The ABL1 copy number must be high enough for this control to be interpreted. If this is the case, the IS-NCN percentage will be calculated. This run control is validated if its status is No MMR according to the test.
- LC: The ABL1 copy number must be high enough for this control to be interpreted. If this is the case, the IS-NCN percentage will be calculated. This run control is validated if its status is MR4 according to the test.
- IS-MMR Calibrator: The ABL1 copy number must be high enough for this control to be interpreted. If this is the case, the NCN will be calculated. This run control is validated if its NCN is within the acceptable range according to the test.

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

If all the controls in the run conform, then Rotor-Gene AssayManager v2.1 will analyze the unknown samples.

In the sample, the variation in C_T values between replicates must be low enough for the results to be interpreted. The IS-NCN percentage will then be calculated and the sample status will be given.

Note: If both the run controls and the sample results are valid, the report will show the ABL1 and BCR-ABL1 Mbc copy numbers, the NCN (%), the IS-NCN (%) and the molecular response status for each sample.

Table 10 and Table 11 show the invalidating and warning sample flags respectively that may be assigned to an individual tube during the analysis by Rotor-Gene AssayManager v2.1, along with an explanation of what this flag means.

Table 10. Invalidating sample flags and description of terms

Flag	Description
ANALYSIS_FAILED	Assay is set to invalid because the analysis has failed. Contact QIAGEN Technical Services.
ASSAY_INVALID	The assay is invalid because at least one external control is invalid.
CONSECUTIVE_FAULT	Target that was used for calculation of this target is invalid.
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).
HIGH_PC_HIGH_DELTA_CT (ABL or Mbcr)	Variation in C _T values between High Positive Control replicates in the control gene mix is too high .
HIGH_PC_HIGH_DELTA_CT (Mbcr)	Variation in C _T values between High Positive Control replicates in the fusion gene mix is too high .
HIGH_PC_LOW_ABL_CN	Control gene copy number for the High Positive Control is too low.
HIGH_PC_LOW_IS-NCN	The Normalized Copy Number (International Scale) for the High Positive Control is too low.
HIGH_PC_NO_CT (ABL)	No detectable C _T for the High Positive Control in the control gene mix.
HIGH_PC_NO_CT (Mbcr)	No detectable C _T for the High Positive Control in the fusion gene mix.
HIGH_PC_REPLICATE_NO_CT (ABL)	One of High Positive Control replicates undetected in the control gene mix.
HIGH_PC_REPLICATE_NO_CT (Mbcr)	One of High Positive Control replicates undetected in the fusion gene mix.
INVALID_CALCULATION	Calculation for this target failed.
IS-CAL_HIGH_DELTA_CT (ABL)	Variation in C _T values between IS-MMR-Calibrator Positive Control replicates in the control gene mix is too high.

Flag	Description
IS-CAL_HIGH_DELTA_CT (Mbcrr)	Variation in C _T values between IS-MMR-Calibrator replicates in the fusion gene mix is too high .
IS-CAL_HIGH_NCN	The Normalized Copy Number for the IS-MMR-Calibrator is too high.
IS-CAL_LOW_ABL_CN	Control gene copy number for the IS-MMR-Calibrator is too low.
IS-CAL_LOW_NCN	The Normalized Copy Number for the IS-MMR-Calibrator is too low.
IS-CAL_REPLICATE_NO_CT (ABL)	One of IS-MMR-Calibrator replicates undetected in the control gene mix.
IS-CAL_REPLICATE_NO_CT (Mbcrr)	One of IS-MMR-Calibrator replicates undetected in the fusion gene mix.
LOW_PC_HIGH_DELTA_CT (ABL)	Variation in C _T values between Low Positive Control replicates in the control gene mix is too high.
LOW_PC_HIGH_DELTA_CT (Mbcrr)	Variation in C _T values between Low Positive Control replicates in the fusion gene mix is too high.
LOW_PC_HIGH_IS-NCN	The Normalized Copy Number (International Scale) for the Low Positive Control is too high.
LOW_PC_LOW_ABL_CN	Control gene copy number for the Low Positive Control is too low.
LOW_PC_NO_CT (ABL)	No detectable C _T for the Low Positive Control in the control gene mix.
LOW_PC_NO_CT (Mbcrr)	No detectable C _T for the Low Positive Control in the fusion gene mix.
LOW_PC_REPLICATE_NO_CT (ABL)	One of Low Positive Control replicates undetected in the control gene mix.
LOW_PC_REPLICATE_NO_CT (Mbcrr)	One of Low Positive Control replicates undetected in the fusion gene mix.
MULTIPLE_THRESHOLD_CROSSING	The amplification curve crosses the threshold more than once. An unambiguous C _T cannot be determined.
NO_BASELINE	No initial baseline has been found. The subsequent analysis cannot be performed.
NTC_UNEXPECTED_VALUE	C _T have been detected in the no template control.
OTHER_TARGET_INVALID	Another target for the same sample is invalid.
OUT_OF_COMPUTATION_RANGE	The calculated concentration for this sample exceeds the technical limit.

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.
SAMPLE_HIGH_DELTA_CT (ABL)	Variation in C_T values between test sample replicate in the control gene mix is too high.
SAMPLE_HIGH_DELTA_CT (Mbcf)	Variation in C_T values between test sample replicate in the fusion gene mix is too high.
SAMPLE_REPLICATE_NO_CT (ABL)	One of test sample replicates undetected in the control gene mix.
SAMPLE_REPLICATE_NO_CT (Mbcf)	One of test sample replicates undetected in the fusion gene mix.
SATURATION	The raw data fluorescence is saturating strongly before the inflection point of the amplification curve.
SPIKE_CLOSE_TO_CT	A spike is detected in the amplification curve close to the C_T .
SP_HIGH_SLOPE (ABL)	The upper limit of the control gene slope is exceeded.
SP_HIGH_SLOPE (Mbcf)	The upper limit of the fusion gene slope is exceeded.
SP_LOW_RSQUARED (ABL)	The lower limit of the control gene R^2 is not met.
SP_LOW_RSQUARED (Mbcf)	The lower limit of the fusion gene R^2 is not met.
SP_LOW_SLOPE (ABL)	The lower limit of the control gene slope is not met.
SP_LOW_SLOPE (Mbcf)	The lower limit of the fusion gene slope is not met.
SPI_NO_CT (Mbcf)	No detectable C_T for the standard plasmid 1 in the fusion gene mix.
STANDARD_HIGH_DELTA_CT (ABL)	Variation in C_T values between standard replicates in the control gene mix is too high.
STANDARD_HIGH_DELTA_CT (Mbcf)	Variation in C_T values between standard replicates in the fusion gene mix is too high.
STANDARD_REPLICATE_NO_CT (ABL)	One of standard replicates undetected in the control gene mix.
STANDARD_REPLICATE_NO_CT (Mbcf)	One of standard replicates undetected in the fusion gene mix.
STEEP_BASELINE	A steeply rising baseline for the raw data fluorescence is detected in the amplification curve.

Flag	Description
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.

Table 11. Warning sample flags and description of terms

Flag	Description
CN MbcR Between LOB and LOD	The fusion gene copy number for test sample is between the LOB and the LOD values and is replaced by the LOD value.
CN MbcR Single Replicate	The fusion gene copy number for test sample is calculated from only one replicate and is divided by 2.
CN MbcR Single Replicate Between LOB and LOD	The fusion gene copy number for test sample (calculated from only one replicate and divided by 2) is between the LOB and the LOD values and is replaced by the LOD value.
IS-NCN (%) Between LOB and LOD	The normalized copy number (international scale) for test sample is obtained based on a fusion gene copy number value between LOB and LOD.
IS-NCN (%) Single Replicate	The normalized copy number (international scale) for test sample is obtained based on a fusion gene copy number calculated from only one replicate.
IS-NCN (%) Single Replicate Between LOB and LOD	The normalized copy number (international scale) for test is obtained based on a fusion gene copy number value (calculated from only one replicate) between the LOB and the LOD values.
LOW_FLUORESCENCE_CHANGE	The percentage fluorescence change for this sample relative to the sample tube with the largest fluorescence change is lower than a defined limit.
LOW_REACTION_EFFICIENCY	The reaction efficiency for this sample has not reached a defined limit.
NCN (%) Between LOB and LOD	The normalized copy number for test sample is obtained based on a fusion gene copy number value between LOB and LOD.
NCN (%) Single Replicate	The normalized copy number for test sample is obtained based on a fusion gene copy number calculated from only one replicate.
NCN (%) Single Replicate Between LOB and LOD	The normalized copy number for test sample is obtained based on a fusion gene copy number value (calculated from only one replicate) between the LOB and the LOD values.
SPIKE	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the C_T is determined.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

Comments and suggestions

RNA isolation

For troubleshooting for RNA purification from whole blood using the RNeasy Midi Kit and Buffer EL, refer to the relevant kit handbooks.

Insufficient RNA in the eluate

Insufficient blood used

Repeat RNA isolation using more samples. Consider pooling both eluates and concentrating RNA using the RNeasy MinElute Cleanup Kit (cat. no. 74204).

Insufficient RNA in the eluate

RNA concentration must be 200 ng/ μ l for optimal sensitivity

Use the RNeasy MinElute Cleanup Kit, (cat. no. 74204) to concentrate the sample, then adjust concentration to 200 ng/ μ l.

Standard, Control or IS-Cal not detected

- | | |
|--|--|
| a) Pipetting errors or omitted reagents; tube or well inversions | Check pipetting scheme and setup of the reaction. Repeat the PCR run. |
| b) Inappropriate storage of kit components | Store the <i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit at -30°C to -15°C and keep qPCR Mix ABL1 and qPCR Mix MbcR protected from light.
Do not exceed a maximum of three freeze-thaw cycles. |
| c) The <i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit has expired | Check the storage conditions and the expiration date (see the kit label) of the reagents and, if necessary, use a new kit. |

Comments and suggestions

No signal, including no signal for controls

- | | |
|--|--|
| a) No reaction tube in position 1 of the Rotor-Gene Q MDx 5plex HRM instrument | Make sure to always place a sample in position 1 of the rotor. Otherwise the instrument will not perform calibration, and incorrect fluorescence data will be acquired. |
| b) Pipetting errors or omitted reagents; tube or well inversions | Check the pipetting scheme and reaction setup.
Repeat the PCR run. |
| c) Inappropriate storage of kit components | Store the <i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit at -30°C to -15°C and keep primers and probe mixes protected from light.
Do not exceed a maximum of three freeze-thaw cycles. |
| d) The <i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit has expired | Check the storage conditions and the expiration date (see the kit label) of the reagents and, if necessary, use a new kit. |
| e) Incorrect detection channel chosen | Set the detection channel to Cycling Green or 470 nm/510 nm. |
| f) No data acquisition program | Check the cycling program. See Table 5, page 35.
Select acquisition mode "Single" at the end of each annealing segment of the PCR program. |

Fluorescence intensity varies

- | | |
|---|---|
| Pipetting errors or omitted reagents; tube or well inversions | Check the pipetting scheme and reaction setup.
Repeat the PCR run. |
|---|---|

Fluorescence intensity too low

- | | |
|--|--|
| a) Inappropriate storage of kit components | Store the <i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit at -30°C to -15°C and keep qPCR Mix ABL1 and qPCR Mix MbcR protected from light.
Do not exceed a maximum of three freeze-thaw cycles. |
| b) The <i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit has expired | Check the storage conditions and the expiration date (see the kit label) of the reagents and, if necessary, use a new kit. |
| c) Very low amount of target RNA | Always check the RNA concentration before starting. |

Comments and suggestions

Negative control (H₂O) gives a positive result

Cross-contamination, reagent contamination, instrument error, well or capillary inversion, or probe degradation

Replace all critical reagents, or use a new kit.

Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent cross contamination.

Keep qPCR Mix ABL1 and qPCR Mix Mbcr protected from light.

Check for false positive readings on fluorescence curves.

Check the reaction setup.

Interpretation of results

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

Quality Control

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. Certificate 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 12.

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

All reagents supplied in the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit are intended to be used solely with the other reagents supplied in the same kit. Use of other reagents or reagents from other lots may affect performance.

The *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit is validated only for whole blood anticoagulated in potassium EDTA (K₂EDTA) collected from patients diagnosed with chronic phase Philadelphia chromosome positive (Ph+) p210 CML.

The performance of the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit was established using the RNeasy Midi Kit (cat. no. 75144), the Buffer EL (cat. no. 79217) and, for the RNA cleanup and concentration step, the RNeasy MinElute Cleanup Kit (cat. no. 74204).

Only the Rotor-Gene Q MDx 5plex HRM instrument has been validated for PCR with this kit.

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.

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

Limit of blank (LOB) was determined following the CLSI/NCCLS EP17-2A standard on healthy whole blood samples, (seven samples, 12 measurements/two lots).

The LOB was found to be equal to 1.02 copies of BCR-ABL1 Mbcr transcript.

Limit of detection

Limit of detection (LOD or analytical sensitivity) was determined based on the “Classical approach” described in the CLSI/NCCLS EP17-2A standard. In this study, known low positive samples (seven samples, 12 measurements/two lots) were analyzed.

The LOD was found to be equal to 3.21 copies of BCR-ABL1 Mbcr transcript or 0.0030% IS-NCN.

Linearity

Linearity was determined following CLSI/NCCLS EP6-A standard with one lot of the *ipsogen* BCR-ABL1 Mbcr RGQ RT-PCR Kit on nine different samples prepared by successive dilutions of positive RNA extracted from cell line into negative RNA extracted from healthy donors. The determination was performed for three different RNA inputs.

The quantification of the BCR-ABL1 Mbcr transcript is linear from the LOD value to 56% IS-NCN as long as the quantified sample RNA concentration is close to 200 ng/ μ l, the recommended input of the assay (total input 3 μ g).

At lower RNA input, the range of linearity may be reduced.

Repeatability and reproducibility

The precision study was performed according to the CLSI/NCCLS EP5-A2 standard. Testing was performed on nine different samples tested 45 times in duplicates on 45 runs performed over 23 days, giving rise to 90 measurements per sample.

The Precision results are summarized in Table 12.

Table 12. Precision results

Sample	Mean BCR-ABL1 MbcR IS-NCN	SDR+	SDRUN++	SDTOTAL+++	CV _{TOTAL}
S1	64.5243	4.3105	12.3610	13.0910	20.29%
S2	36.1684	1.7104	5.9078	6.8581	18.96%
S3	6.4876	0.4231	0.7857	1.0941	16.86%
S4	0.7305	0.0512	0.0779	0.1178	16.12%
S5	0.0754	0.0068	0.0073	0.0133	17.62%
S6	0.0075	0.0016	0.0009	0.0022	28.81%
S7	0.0036	0.0014	0.0002	0.0014	38.64%
S8	0.0020	0.0010	0.0000	0.0010	48.71%
S9	0.0011	0.0007	0.0000	0.0007	63.32%

CV_{TOTAL}: Coefficient of variation for the total precision (BCR-ABL1 MbcR IS-NCN); **SD**: standard deviation; **R+**: Repeatability; **RUN++**: Between run reproducibility; **S**: standard; **TOTAL+++**: Total precision (including inter-instrument, inter-operator and inter-lot).

Interfering substances

The study design was based on recommendations described in the NCCLS standard EP7-A2 "Interference Testing in clinical Chemistry". The following substances potentially present in blood samples or that may be introduced during the purification of RNA were chosen for their potential effect on PCR (unconjugated bilirubin, conjugated bilirubin, hemoglobin [human], serum albumin [human], excess of potassium EDTA [K2-EDTA], ethanol).

The obtained results showed no interfering effect for these substances.

Clinical validation and methods comparison

Two studies were performed to compare the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit with alternative methods.

Study 1: 76 RNA samples extracted from peripheral blood were analyzed with the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit and the *ipsogen* BCR-ABL1 MbcR IS-MMR DX kit.

Deming regression compared the measured IS-NCN from both methods. There was a strong correlation between the *ipsogen* BCR-ABL1 MbcR RGQ RT-PCR Kit and the *ipsogen* BCR-ABL1 MbcR IS-MMR DX ($R^2= 0.97$), as shown in Figure 9.

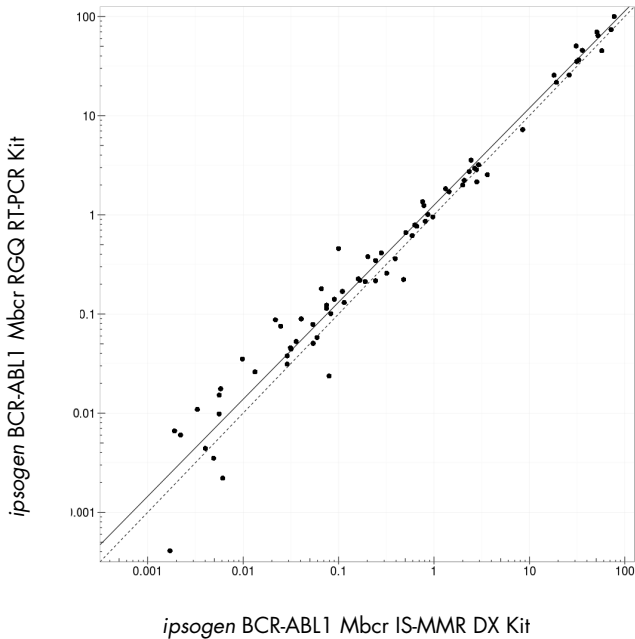


Figure 9. Plot of IS-NCN obtained with the *ipsogen* BCR-ABL1 Mbcr RGQ RT-PCR Kit and the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit.

Study 2: 39 RNA samples extracted from peripheral blood of patients previously diagnosed with Ph+ CML and under TKI treatment were analyzed in a French clinical center with the *ipsogen* BCR-ABL1 Mbcr RGQ RT-PCR Kit and a laboratory developed test (reference method). The reference method could report results standardized to the international scale using a conversion factor.

The following contingency table was built to compare the clinical status established with both methods. There was a strong agreement between the *ipsogen* BCR-ABL1 Mbcr RGQ RT-PCR Kit and the reference method (overall agreement = 97.4%), as shown in Figure 10.

		Reference method		n
		no MR 4	MR 4 or below	
ipsogen BCR-ABL1 Mbc RGQ RT-PCR Kit	no MR 4	15	1	16
	MR 4 or below	0	23	23
n		15	24	39

Figure 10. Contingency table comparing the *ipsogen* BCR-ABL1 Mbc RGQ RT-PCR Kit and a laboratory developed test standardized to the International Scale.

Concordance study: ERM-AD623 BCR-ABL1 single plasmid (IRMM) versus *ipsogen* single plasmid (QIAGEN) standards

The most recent working definitions of BCR-ABL1 Mbc molecular response in CML are given by the European LeukemiaNet/European Treatment Outcome Study (ELN/EUTOS) Molecular Monitoring Steering Group, recommending the use of the ERM-AD623 BCR-ABL1 plasmid, from the Institute for Reference Materials and Measurements (IRMM), Belgium (9).

To comply with this recommendation, QIAGEN conducted a concordance study to compare the *ipsogen* multi-target, single plasmid used in the *ipsogen* BCR-ABL1 Mbc RGQ RT-PCR Kit (24) CE (cat. no. 670923) with the ERM-AD623 BCR-ABL1 plasmid (IRMM).

The comparison was based on BCR-ABL1 Mbc/ABL1 normalized copy number ratio (NCN), assessed the two standards' dilutions (*ipsogen* or ERM-AD623 BCR-ABL1), on control samples included in *ipsogen* kits and on certified reference material from the National Institute for Biological Standards and Control (NIBSC) (8). Results show that the two standard curves are aligned (Figure 11) and NCN ratios comparable (Figure 12).

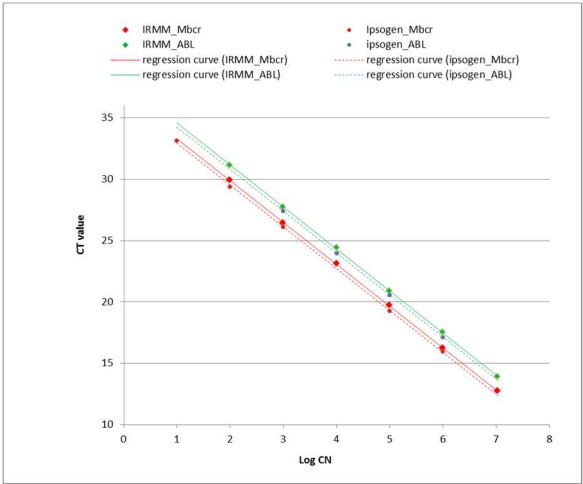


Figure 11. Comparison of *ipsogen* and ERM-AD623 BCR-ABL1 plasmids shows that standard curves are aligned.

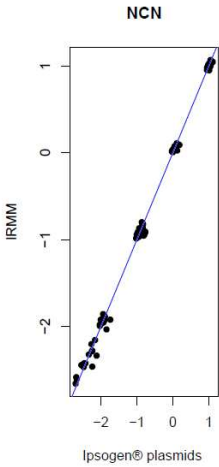


Figure 12. NCN values for the *ipsogen* and ERM-AD623 plasmids are comparable.

The QIAGEN study concluded that there is no statistically significant difference: ERM-AD623 BCR-ABL1 single plasmid and *ipsogen* single plasmid standards give equivalent results.

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Protect from light
	Consult instructions for use
	Caution

Ordering Information

Product	Contents	Cat. no.
<i>ipsogen</i> BCR-ABL1 MbcR RGQ RT-PCR Kit (24)	For 24 reactions: ABL1 and BCR-ABL1 MbcR quantitative Single Plasmid Standards, Low and High Positive Controls, IS-MMR Calibrator, qPCR mix ABL1, qPCR mix MbcR, Reverse Transcription and qPCR reagents.	670923
Rotor-Gene Q MDx 5plex HRM instrument – for IVD-validated real-time PCR analysis in clinical applications		
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cyclers and High-Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included.	9002032
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Rotor-Gene Assay Manager 2.1 Software	Software for routine testing in combination with the Rotor-Gene Q	9024203

Rotor-Gene Assay Manager 2.1 License	Single license software for installation on one computer	9025620
Loading Block 72 x 0.1ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes.	9018901
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RNA isolation		
RNeasy Midi Kit	50 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers. For purification of total RNA.	75144
Buffer EL	1000 ml Erythrocyte Lysis Buffer.	79217
RNeasy MinElute Cleanup Kit	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers. For RNA cleanup and concentration with small elution volumes.	74204

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Handbook Revision History

Document	Changes	Date
HB-1904_005	Inclusion of sections "Automated analysis: qPCR on the Rotor-Gene Q MDx 5plex HRM instrument with 72-tube rotor with RGAM software" and "Interpretation of Results on RGAM Software".	June 2018

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