July 2016

BCR-ABL1 Mbcr RGQ RT-PCR Kit Handbook

For quantification of BCR-ABL1 p210 b2a2 or b3a2 transcripts

For research use only. Not for use in diagnostic procedures.

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

REF 670913

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Contents

Intended Use

The BCR-ABL1 Mbcr RGQ RT-PCR Kit is intended for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Principle of the Procedure

qPCR permits the accurate quantification of PCR products during the exponential phase of the PCR amplification process. Quantitative PCR 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 1). 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.

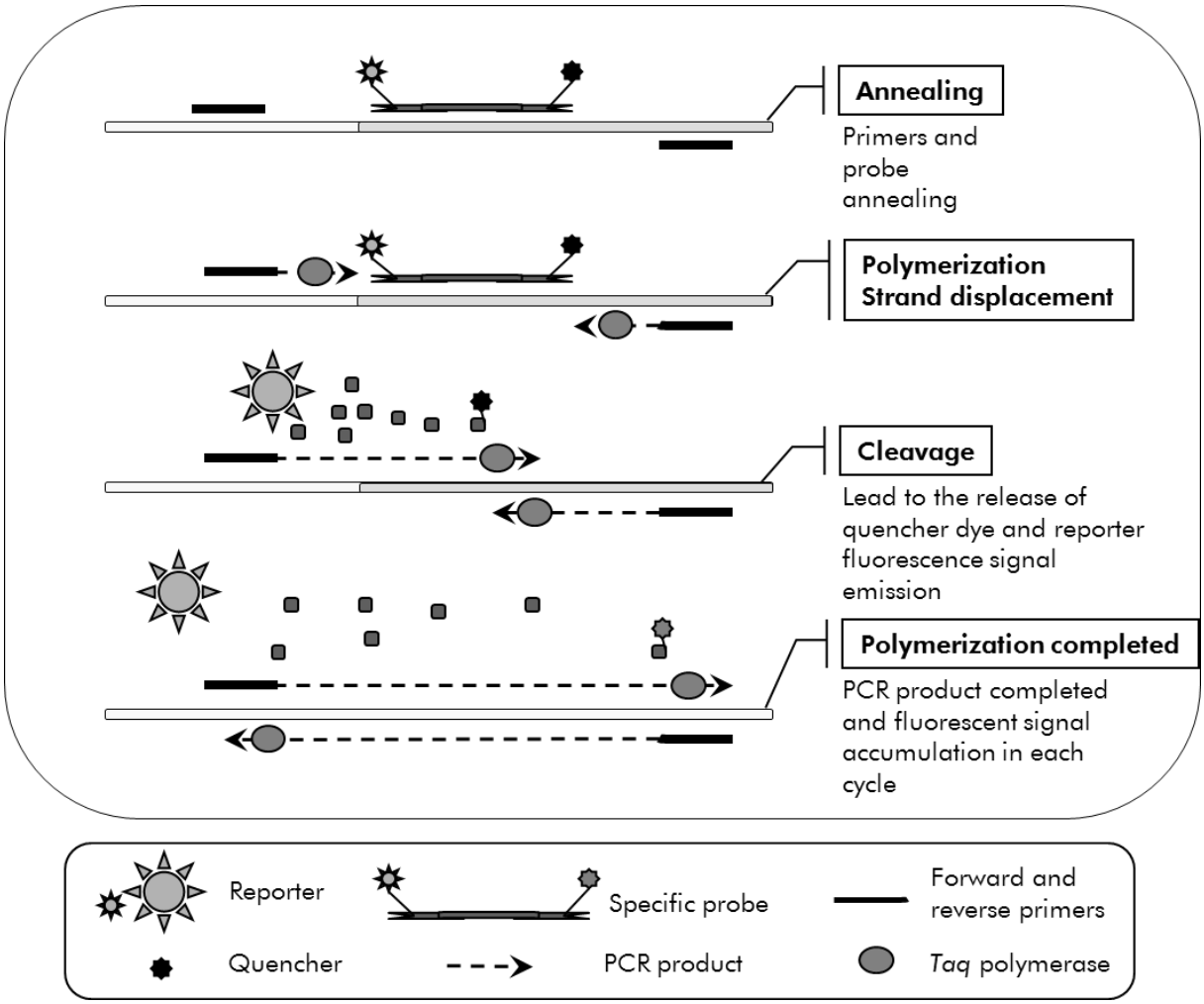

Figure 1. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–BHQ1™). The probe binds to the amplicon during each annealing step of the PCR. When the Taq extends from the primer 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 BHQ1.

Materials Supplied

***ipsogen* BCR-ABL1 Mbcr RGQ RT-PCR Kit**		**(24)**
Catalog no.		**670913**
Number of reactions		**24**
Reagents for reverse transcription (RT)		
Reverse transcriptase	Purple	100 µl
RT Mix	Purple	300 µl
Reagents for calibration		
High Positive RNA Control	White	15 µl x 3
Low Positive RNA Control	White	15 µl x 3
IS-MMR Calibrator	White	15 µl x 3
SP1-BCR-ABL Mbcr and ABL: Mbcr and ABL1 Single Plasmid Standard Dilution (10^1 copies/5 µl)	Yellow	35 µl
SP2-BCR-ABL Mbcr and ABL: Mbcr and ABL1 Single Plasmid Standard Dilution (10^2 copies/5 µl)	Yellow	35 µl
SP3-BCR-ABL Mbcr and ABL: Mbcr and ABL1 Single Plasmid Standard Dilution (10^3 copies/5 µl)	Yellow	70 µl
SP4-BCR-ABL Mbcr and ABL: Mbcr and ABL1 Single Plasmid Standard Dilution (10^4 copies/5 µl)	Yellow	35 µl
SP5-BCR-ABL Mbcr and ABL: Mbcr and ABL1 Single Plasmid Standard Dilution (10^5 copies/5 µl)	Yellow	70 µl
SP6-BCR-ABL Mbcr and ABL: Mbcr and ABL1 Single Plasmid Standard Dilution (10^6 copies/5 µl)	Yellow	70 µl

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Reagents for qPCR		
Taq DNA polymerase	Mint	85 µl
qPCR Mix ABL1*	Green	720 µl x 3
qPCR Mix Mbcr†	Red	720 µl x 3

* Contains a mix of specific reverse and forward primers for the ABL1 control gene plus a specific FAM–BHQ1 probe.

† Contains a mix of specific reverse and forward primers for the BCR-ABL1 Mbcr fusion gene plus a specific FAM–BHQ1 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)

Reagents for total RNA isolation

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

* 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.

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)
- Thermomixer, heated orbital incubator, heating block, or water bath (for the reverse transcription step)†
- Rotor-Gene Q 5plex HRM† (cat. no. 9001650) and associated specific material
- Rotor-Gene Q software version 2.1.0 or higher

* 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.

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

Warnings and Precautions

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.

General precautions

- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Discard sample and assay waste according to your local safety procedures.
- Reagents for the BCR-ABL1 Mbcr RGQ RT-PCR Kit are optimally diluted. Do not further dilute reagents, as this may result in a loss of performance. Do not use reaction volumes (reaction mix plus sample) of less than 25 µl.
- All reagents supplied in the 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 BCR-ABL1 Mbcr RGQ RT-PCR Kits, as this may affect performance.
- Refer to the Rotor-Gene Q 5plex HRM instrument user manual 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.
- qPCR mixes may be altered if exposed to light.
- Use extreme caution to prevent contamination of the mixes with the Low and High Positive Control reagents.
- Use extreme caution to prevent contamination by RNase or DNase, which may cause degradation of the template RNA or cDNA.
- Use individual, dedicated pipets for setting up reaction mixes and adding templates.
- Prepare and dispense reaction mixes in an area separate from the one used for the addition of the templates.

- Do not open the Rotor-Gene Q 5plex HRM instrument until the run is finished.
- Do not open Rotor-Gene Q 5plex HRM tubes after the run is finished.
- Caution must be observed to ensure correct sample testing with respect to incorrect sample entry, loading error, and pipetting error.

Reagent Handling and Storage

The BCR-ABL1 Mbcr RGQ RT-PCR Kit is shipped on dry ice. If any component of the BCR-ABL1 Mbcr RGQ RT-PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or 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**.

The BCR-ABL1 Mbcr RGQ RT-PCR Kit should be stored immediately upon receipt at –15 to –30°C in a constant-temperature freezer and protected from light.

When stored under the specified storage conditions, the 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 –15 to –30°C until the stated expiration date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 3 freeze–thaw cycles.

For storage and stability 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 respective handbook.

Specimen Handling and Storage

The 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 (K_2-EDTA) and stored at 2–8°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 –15 to –30°C or lower (–65 to –90°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

RNA isolation

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

- 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* (QIAGEN, cat. no. 75144) and Buffer EL (QIAGEN, cat. no. 79217) for purification of RNA from peripheral whole blood.
- The RNA concentration in the eluate should be in the range 70–200 ng/µl.
- If the RNA concentration in the eluate is below the lower range limit, or to increase the confidence to measure 60,000 copies of ABL1, the RNA concentration in the eluate can be increased with the RNeasy MinElute Cleanup Kit (QIAGEN, cat. no. 74204).
- If RNA concentration in the eluate is above the upper range limit, it should be adjusted to 200 ng/µl with RNase-free water.
- 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‡
- Check RNA concentration after normalization

* The BCR-ABL1 Mbcr RGQ RT-PCR Kit has only been validated in combination with the RNeasy Midi Kit.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

‡ Optical density measured at 260 and 280 nm: OD of 1.0 at 260 nm is equivalent to approximately 40 µg/ml single-stranded RNA. An A_{260}/A_{280} ratio between 1.8 and 2.1 is indicative of highly purified RNA.

Reverse transcription

Things to do before starting

- Thaw all necessary components and place them on ice.
- Gently mix (do not vortex) and briefly centrifuge the reverse transcription reagents, RNA samples, controls, or IS-MMR Calibrator before use. Then, keep 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, IS-MMR Calibrator and briefly centrifuge before use. Then, 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 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 min at 65°C. Then, immediately cool on ice for 5 min.**
2. **Centrifuge briefly (approximately 10 s) at 10,000 rpm, to collect the liquid in the bottom of the tube. Then, keep on ice.**
3. **Prepare the following RT mix according to the number of samples, positive controls (high and low positive controls), water (used to generate the RT-negative control) and IS-MMR calibrator being processed (Table 1, next page).**

Table 1. Preparation of RT mix

Component	Volume per sample (µl)	Final concentration
RT Mix, 3.33x	7.5	1x
Reverse Transcriptase, 10x	2.5	1x
Final volume (to be added in step 4)	10	–

4. **Pipet 10 µl RT mix into each labeled tube containing RNA sample, control, or calibrator (from step 3).**
5. **Mix carefully (do not vortex) and centrifuge briefly (approximately 10 s) at 10,000 rpm, to collect the liquid in the bottom of the tube.**
6. **Place the tubes in the thermal cycler and program the cycler with the reverse transcription program as indicated in Table 2.**

Table 2. Temperature profile

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

7. **After the program is finished, centrifuge the tubes briefly (approximately 10 s) at 10,000 rpm to collect the liquid in the bottom of the tube. Keep the tubes on ice or at –20°C until qPCR is performed.**

qPCR on the Rotor-Gene Q 5plex HRM instrument with 72-tube rotor

We recommend performing all measurements in duplicate, as indicated in Table 3. The kit is designed to test eight different cDNA samples in the same experiment in duplicate.

Table 3. Number of reactions for Rotor-Gene Q instruments 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	2 x 4 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	2 x 5 reactions (SP1, SP2, SP3, SP5, and SP6)
Water control	2 reactions

Sample processing on Rotor-Gene Q instruments with 72-tube 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 2 (next page) shows an example experiment.

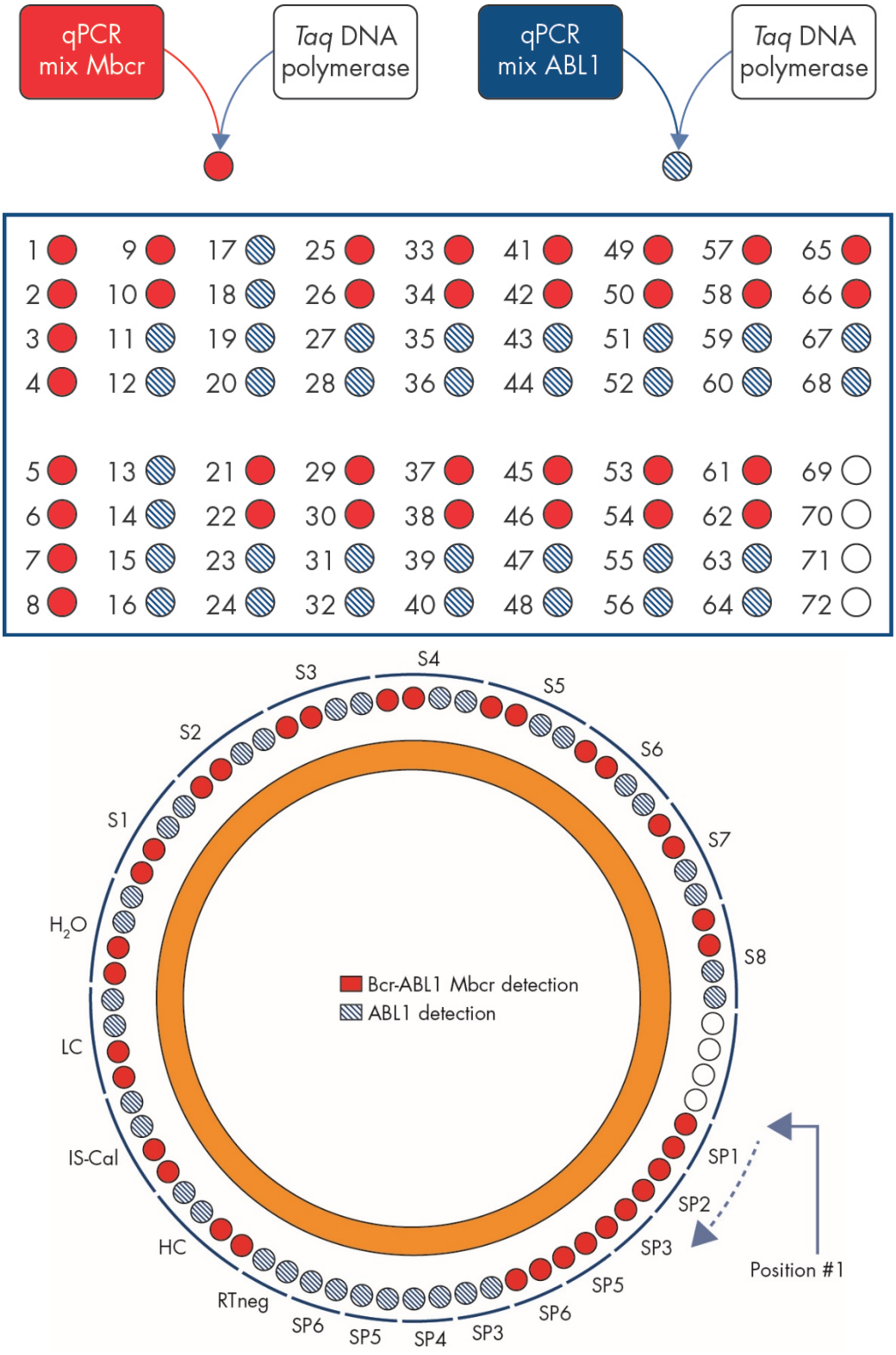

Figure 2. Rotor setup for each experiment. SP1–SP6: BCR-ABL1 Mbcr and ABL1 standards; **HC**: High Positive Control; **LC**: Low Positive Control; **IS-Cal**: IS-MMR calibrator; **RTneg**: RT negative control; **S1-S8**: cDNA samples; **H_2O**: water control.

Note: Fill all empty positions with empty tubes.

Things to do before starting

- Thaw all necessary components and place them on ice.
- Gently mix (do not vortex) and briefly centrifuge the qPCR Mix ABL1 and qPCR Mix Mbcr before use. Then keep on ice.

Procedure

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

   Table 4 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 25 µl. A pre-mix can be 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.

Table 4. Preparation of PCR master mix

Component	1 reaction	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
Taq DNA Polymerase	0.25	9	1x
Sample, Standard, Control or IS-MMR Calibrator (to be added at step 5)	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 the RT product (cDNA), standards and water in the corresponding well according to the sample layout (total volume 25 µl).**
4. **Mix gently, by pipetting up and down.**
5. **Place the tubes in the adapter provided with the instrument.**
6. **Unused positions must be filled with empty tubes.**

7. **Place the locking ring above tubes and press to lock.**
8. **Load the full adapter into the Rotor-Gene Q instrument.**
9. **Program the Rotor-Gene Q instrument with the thermal cycling program as indicated in Table 5.**

Table 5. Temperature profile

Mode of analysis	Quantitation
Hold 1	Temperature: 95°C Time: 15 min
Cycling	50 cycles 94°C; 15 s 60°C; 60 s with acquisition of FAM fluorescence in the Green channel. Acquisition mode: "Single"

10. **Click "Gain Optimisation" in the "New Run Wizard" dialog box to open the "Auto-Gain Optimisation Setup" dialog. Set the range for the Green channel from "5 Fl" for "Min Reading" to "10 Fl" for "Max Reading" and the acceptable Gain range from –10 to 10.**
11. **Check the "Perform Optimisation Before 1st Acquisition" box, and close the "Auto-Gain Optimisation Setup" dialog box.**
12. **Start the thermal cycling program.**
13. **Create both ABL1 and Mbcr subsets, filling "Edit samples" window.**
14. **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".**
15. **For both ABL1 and Mbcr, analyze 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 the raw data is recorded and proceed with analysis of results.

Results

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. In order 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. Figures 3 and 4 show examples of TaqMan amplification curves similar to those obtained for standards, the IS Calibrator, the High Positive RNA Control and the Low Positive RNA Control with the BCR-ABL1 Mbcr RGQ RT-PCR Kit.

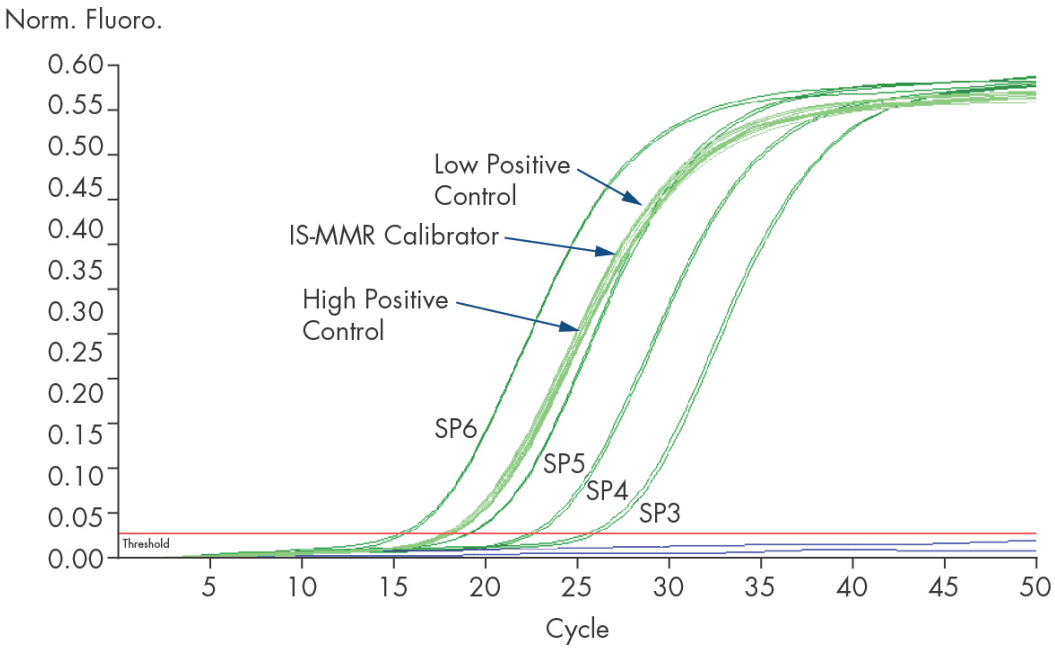

Figure 3. Detection of ABL1 with controls and standards SP3, SP4, SP5, and SP6. 10^3, 10^4, 10^5, and 10^6 copies/reaction.

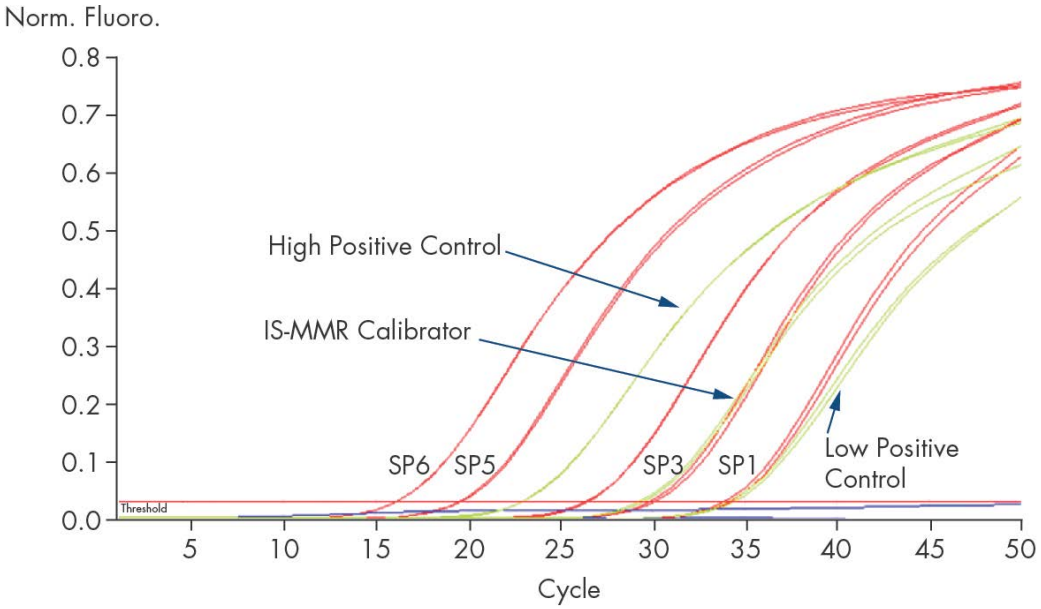

Figure 4. Detection of BCR-ABL1 Mbcr with controls and standards SP1, SP2, SP3, SP5, and SP6. 10^1, 10^2, 10^3, 10^5, and 10^6 copies/reaction.

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 ≥36 or if C_{Ta} ≥36 and C_{Tb} is "not detected" then ΔC_T criteria do not apply; the duplicate conforms.

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 Mbcr), 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 5 shows an example of an ABL1 curve calculated on four standard dilutions. Figure 6 shows an example of a BCR-ABL1 Mbcr curve calculated on five standard dilutions.

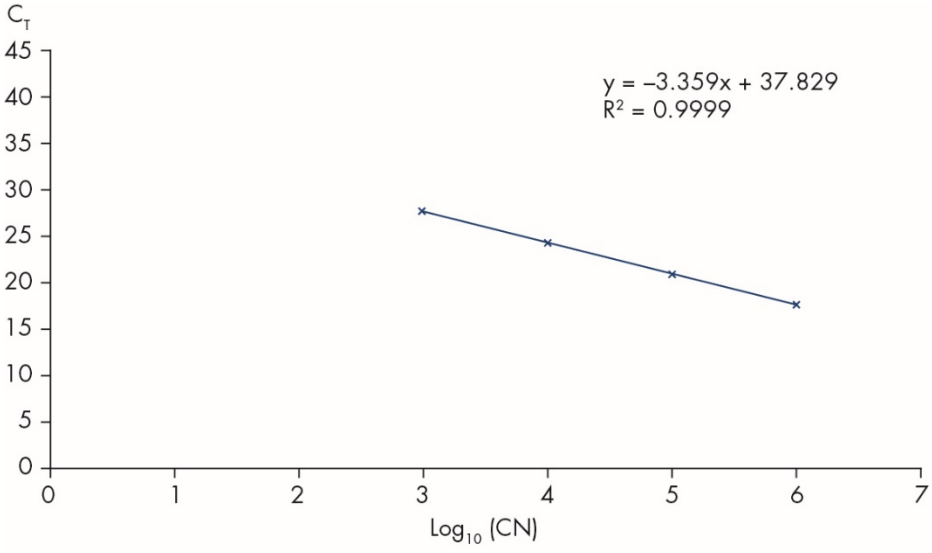

Figure 5. Standard curve for ABL1 calculated from 4 standard dilutions. A linear regression curve (y = ax + b) is calculated, where "a" is the slope of the line and "b" is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. The equation and coefficient of determination (R^2) are shown on the graph.

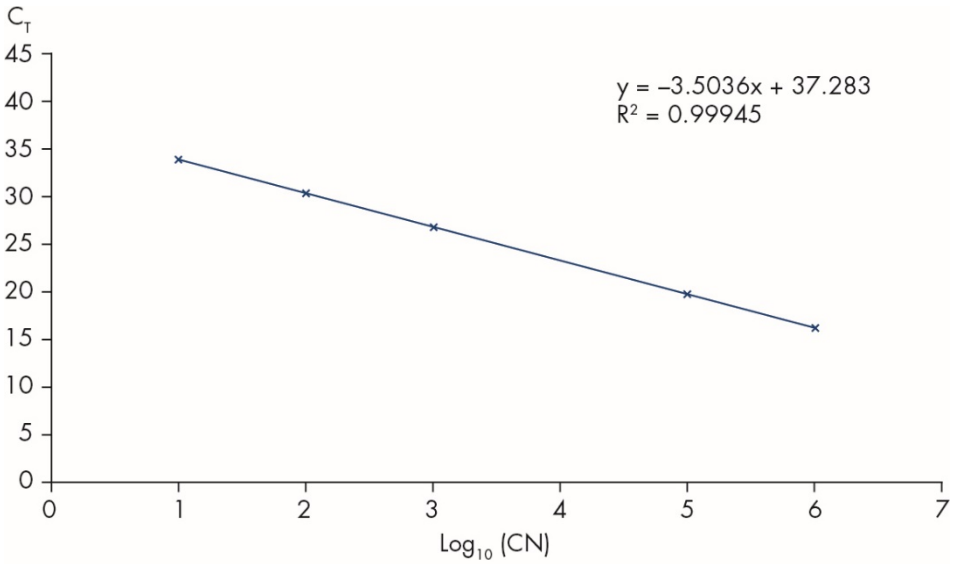

Figure 6. Standard curve for BCR-ABL1 Mbcr calculated from 5 standard dilutions. A linear regression curve (y = ax + b) is calculated, where "a" is the slope of the line and "b" is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. The equation and coefficient 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 –3.0 and –3.9 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 and included in the BCR-ABL1 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 $Mbcr_{CN}$).

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

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

Quality control on all $ABL1_{CN}$ values

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

The sensitivity of the test at low BCR-ABL1 Mbcr copy number greatly depends on ABL_{CN}. For optimal test sensitivity, $ABL1_{CN}$ should be >100,000 for the High Positive RNA Control, Low Positive RNA Control and IS-MMR Calibrator.

RT negative and water controls

No template controls (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 Mbcr. 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 ABL or BCR-ABL Mbcr standard curve equation should be used to transform raw C_T values (obtained with PPC-ABL or PPF-Mbcr) for the unknown samples into ABL or BCR-ABL copy numbers (ABL_{CN} or BCR-ABL $Mbcr_{CN}$).

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

$$\text{NCN} = \frac{\text{BCR-ABL Mbcr}_{CN}}{\text{ABL}_{CN}} \times 100$$

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 Mbcr during transcript quantification.

- The NCN result obtained for the IS-MMR-Calibrator, tested with the BCR-ABL1 Mbcr RGQ RT-PCR kit, must be within the interval 0.05–0.3. Otherwise, NCN values cannot be converted to the International Scale.
- The whole experiment must be rejected if the High Positive RNA Control is not detected.
- The sensitivity of the experiment can be assessed only if the Low Positive RNA Control is detected.

IS 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.

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 ($IS\text{-}NCN_{sample}$).

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

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 6.

Summary of quality criteria

Table 7 summarizes the various quality criteria and associated values or results.

Table 1. Molecular response reporting

Case	ABL CN	BCR-ABL1 Mbcr 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
		≤LOB	–	Not detected/MR4.5
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.

Table 7. Quality criteria summary

Criteria	Acceptable values/results
Variations in C_T values between replicates	≤2 C_T Except if mean C_T ≥36 or if C_{Ta} ≥36 and C_{Tb} is "not detected": the duplicate conforms. CN calculated for C_{Ta} should be divided by 2.
Slope for standard curves	Between –3.0 and –3.9
R^2 for standard curves	At least >0.95 (better >0.98)
SP1 standard dilution (BCR-ABL1 10 copies plasmid)	Must be detected and included in the standard curve
Quality control on ABL_{CN} value for biological samples	$ABL1_{CN}$ >10,000 to establish MMR status with confidence $ABL1_{CN}$ >33,000 to establish MR4 status with confidence $ABL1_{CN}$ >100,000 to establish MR4.5 status with confidence
High Positive RNA Control, Low Positive RNA Control, and the IS-MMR-Calibrator	$ABL1_{CN}$ >100,000
PCR (water) and reverse transcription (RT negative) controls	For each ABL_{CN} = 0 and $Mbcr_{CN}$ = 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} ≤0.01 (MR4) Must be detected to interpret MR4 and below

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.

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 sample. Consider pooling both eluates and concentrating RNA using the RNeasy MinElute Cleanup Kit (cat. no. 74204).
Insufficient RNA concentration in the eluate	
RNA concentration must be 70–200 ng/µl for optimal sensitivity	Use the RNeasy MinElute Cleanup Kit, (cat. no. 74204) to concentrate the sample, then adjust concentration to 200 ng/ml.
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 BCR-ABL1 Mbcr RGQ RT-PCR Kit at –15°C to –30°C and keep qPCR Mix ABL1 and qPCR Mix Mbcr protected from light. Do not exceed a maximum of 3 freeze–thaw cycles.
c) The 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 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 BCR-ABL1 Mbcr RGQ RT-PCR Kit at –15°C to –30°C and keep primers and probe mixes protected from light. Do not exceed a maximum of 3 freeze–thaw cycles.
d) The 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 16. 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 BCR-ABL1 Mbcr RGQ RT-PCR Kit at –15°C to –30°C and keep qPCR Mix ABL1 and qPCR Mix Mbcr protected from light. Do not exceed a maximum of 3 freeze–thaw cycles.

	Comments and suggestions
b) The 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.
Negative control (H_2O) 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.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of BCR-ABL1 Mbcr RGQ RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Symbols

The following symbols may appear on the packaging and labeling:

 <N>	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Consult instructions for use

Contact Information

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

Ordering Information

Product	Contents	Cat. no.
BCR-ABL1 Mbcr RGQ RT-PCR Kit (24)	For 24 reactions: ABL1 and BCR-ABL1 Mbcr quantitative Standards, Low and High Positive Control, Is-Cal, qPCR mix ABL1, QPCR mix Mbcr, Reverse Transcription and qPCR reagents	670913
Rotor-Gene Q and accessories		
Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9001650
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9001580
Loading Block 72 x 0.1ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106

Product	Contents	Cat. no.
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

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

Notes

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