

ipsogen[®] BCR-ABL1 Mbcr IS-MMR Handbook

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For quantification of BCR-ABL p210 b2a2 or b3a2 transcripts

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

For use with Rotor-Gene[®] Q, Applied Biosystems[®], ABI PRISM[®],
and LightCycler[®] instruments

REF 670713



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Intended Use

The *ipsogen* BCR-ABL1 MbcR IS-MMR Kit is intended for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Principle of the Procedure

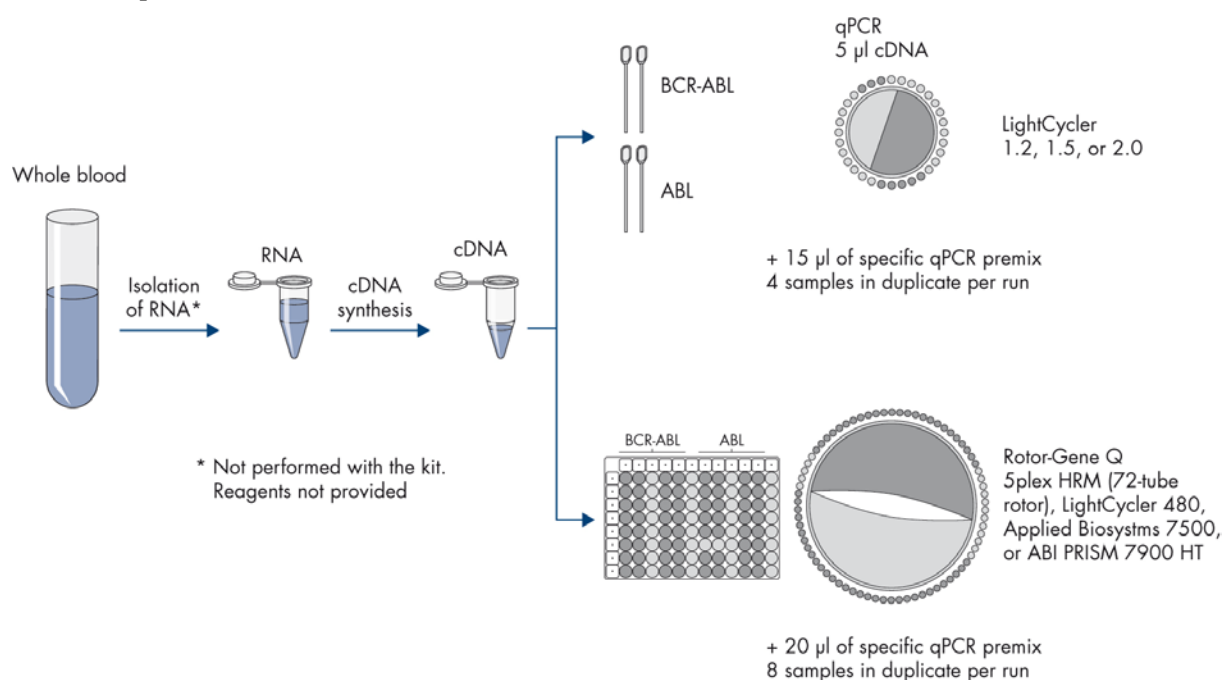


Figure 1. RNA isolation, cDNA synthesis, and qPCR.

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, 3 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.

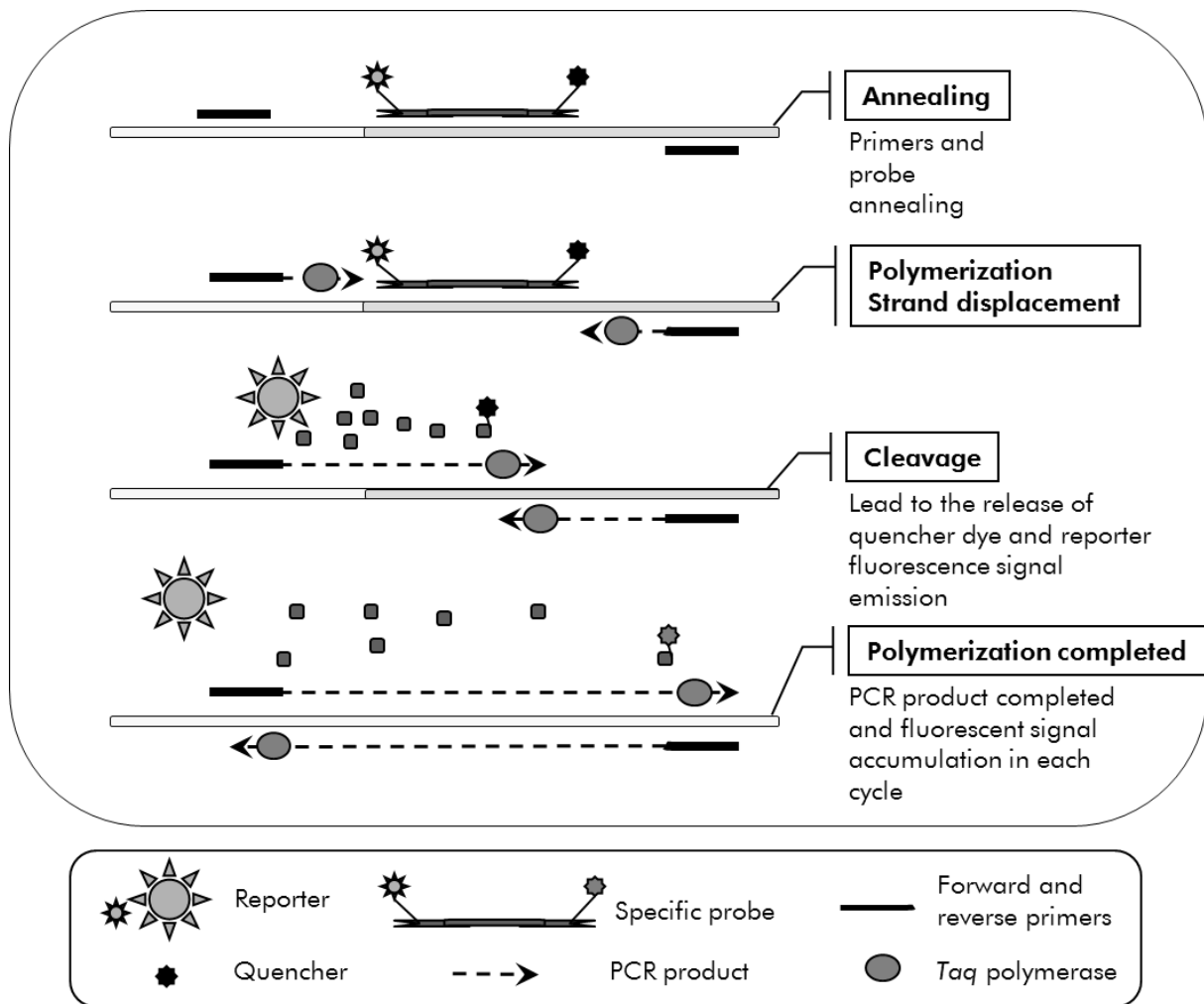


Figure 2. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–TAMRA™). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer 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 TAMRA.

Materials Provided

Kit contents

ipsogen BCR-ABL1 Mbcr IS-MMR Kit			(24)
Catalog no.			670713
Number of reactions			24
High Positive RNA Control			3 x 10 µl
IS-MMR Calibrator			3 x 10 µl
Mbcr and ABL Single Plasmid Standard Dilution (10 ¹ copies/5 µl)	SP1-BCR-ABL Mbcr & ABL		35 µl
Mbcr and ABL Single Plasmid Standard Dilution (10 ² copies/5 µl)	SP2-BCR-ABL Mbcr & ABL		35 µl
Mbcr and ABL Single Plasmid Standard Dilution (10 ³ copies/5 µl)	SP3-BCR-ABL Mbcr & ABL		70 µl
Mbcr and ABL Single Plasmid Standard Dilution (10 ⁴ copies/5 µl)	SP4-BCR-ABL Mbcr & ABL		35 µl
Mbcr and ABL Single Plasmid Standard Dilution (10 ⁵ copies/5 µl)	SP5-BCR-ABL Mbcr & ABL		70 µl
Mbcr and ABL Single Plasmid Standard Dilution (10 ⁶ copies/5 µl)	SP6-BCR-ABL Mbcr & ABL		70 µl
Primers and Probe Mix ABL*	PPC-ABL 25x		110 µl
Primers and Probe Mix BCR-ABL Mbcr Fusion Gene†	PPF-Mbcr 25x		110 µl
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* Mix of specific reverse and forward primers for the ABL control gene plus a specific FAM–TAMRA probe.

† Mix of specific reverse and forward primers for the BCR-ABL Mbcr fusion gene plus a specific FAM–TAMRA probe.

Note: Gently mix and briefly centrifuge the standards (SP1–SP6), and the primers and probe mixes before use.

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

- Reagents for RNA purification: The recommended reagents are RNeasy[®] Midi Kit (QIAGEN, cat. no. 75144) or TRIzol[®] Reagent (Thermo Fisher Scientific Inc.) cat. no. 15596-018 or 15596-026
- Nuclease-free PCR-grade water
- Buffer and Taq DNA polymerase: The recommended reagents are Premix Ex Taq[™] DNA Polymerase (Perfect Real Time) (TaKaRa, cat. no. RR039A) and Premix Ex Taq[™] DNA Polymerase (Probe qPCR) (TaKaRa, cat. no. RR390A). Both include 2x Taq DNA polymerase master mix and ROX[™] reference dyes.
- Reagents for reverse transcription: The recommended reagents are the *ipsogen* RT Kit, includes reverse transcriptase, 5x RT buffer, 100 mM DTT, RNase inhibitor, random primer, and dNTPs (QIAGEN, cat. no. 679913); or Superscript[®] III Reverse Transcriptase, includes reverse transcriptase, 5x first-strand buffer, and 100 mM DTT (Thermo Fisher Scientific Inc., cat. no. 18080-044)
- When using Superscript III, the following additional reagents are required:
 - RNase inhibitor: The recommended reagent is RNaseOUT[™] (Thermo Fisher Scientific Inc., cat. no. 10777-019)
 - Set of dNTPs, PCR grade
 - Random nonamer

Consumables

- Nuclease-free aerosol-resistant sterile PCR pipet tips with hydrophobic filters
- 0.5 ml or 0.2 ml RNase- and DNase-free PCR tubes
- Ice

Equipment

- Microtiter pipets* dedicated for PCR (1–10 μ l; 10–100 μ l; 100–1000 μ l)
- Benchtop centrifuge* with rotor for 0.2 ml/0.5 ml reaction tubes (capable of attaining 10,000 rpm)

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

- Real-time PCR instrument: * Rotor-Gene Q 5plex HRM or other Rotor-Gene instrument; LightCycler 1.2, 1.5, 2.0, or 480; Applied Biosystems 7500 Real-Time PCR System; ABI PRISM 7900HT SDS; and associated specific material
- Thermal cycler* or water bath* (reverse transcription step)

Warnings and Precautions

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.

Discard sample and assay waste according to your local safety regulations.

General precautions

qPCR tests require good laboratory practices, including equipment maintenance, that are dedicated to molecular biology and compliant with applicable regulations and relevant standards.

Reagents and instructions supplied in this kit have been tested for optimal performance. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data. PPC and PPF reagents may be altered if exposed to light. All reagents are formulated specifically for use with this test. For optimal performance of the test, no substitutions should be made.

Determining transcript levels using qPCR requires both the reverse transcription of the mRNA and the amplification of the generated cDNA by PCR. Therefore, the entire assay procedure must be performed under RNase-/DNase-free conditions.

Use extreme caution to prevent:

- RNase/DNase contamination, which might cause degradation of the template mRNA and the generated cDNA
- mRNA or PCR carryover contamination resulting in false positive signal

We therefore recommend the following.

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials) and wear gloves when performing the assay.

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

- Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Prepare pre-PCR master mix with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrixes (cDNA, DNA, plasmid) are introduced. Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).
- Handle the standards (SP1–SP6) in a separate room.

Reagent Storage and Handling

The kits are shipped on dry ice and must be stored at -15°C to -30°C upon receipt.

- Minimize exposure to light of the primers and probe mixes (PPC and PPF tubes).
- Gently mix and centrifuge the tubes before opening.
- Store all kit components in original containers.

These storage conditions apply to both opened and unopened components. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

Expiration dates for each reagent are indicated on the individual component labels. Under correct storage conditions, the product will maintain performance until the expiration date printed on the label.

There are no obvious signs to indicate instability of this product. However, positive and negative controls should be run simultaneously with unknown specimens.

Specimen Handling and Storage

Whole blood samples should be anti-coagulated with potassium EDTA and stored at $2-8^{\circ}\text{C}$ for no more than 5 days before RNA extraction.

Procedure

Sample RNA preparation

RNA preparation from biological samples (blood or bone marrow) must have been done with a recommended procedure. The quality of the assay is largely dependent on the quality of input RNA. We therefore recommend qualifying the purified RNA by agarose* gel electrophoresis, using an Agilent® Bioanalyzer®, or spectrophotometry prior to analysis.†

Protocol: Reverse transcription using SuperScript III Reverse Transcriptase

This protocol is for reverse transcription using SuperScript III Reverse Transcriptase. When using the *ipsogen* RT Kit, follow the protocol in the *ipsogen RT Kit Handbook*.

Things to do before starting

- Prepare dNTPs, 10 mM each. Store at –20°C in aliquots.

Procedure

1. Thaw all necessary components and place them on ice.
2. Mix well (do not vortex), and centrifuge briefly (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube). Then keep on ice.
3. Adjust RNA samples to 0.1 µg/µl. Pipet 10 µl (1 µg) of each RNA sample into separate, labeled tubes. Pipet 10 µl of the high positive RNA control, 10 µl of the IS-MMR Calibrator, and 10 µl nuclease-free water (as an RT negative control) into separate, labeled tubes, and process them in parallel with the RNA samples, as described below.
4. Incubate each sample, control, and calibrator (10 µl each) for 5 min at 65°C and immediately cool on ice for 5 min.
5. Centrifuge briefly (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube). Then keep on ice.
6. Prepare the following RT mix according to the number of samples, control, and calibrator being processed (Table 1).

* 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.

Table 1. Preparation of RT mix

Component	Volume per sample (μl)	Final concentration
First-Strand Buffer, 5x (supplied with Superscript III Reverse Transcriptase)	5.0	1x
dNTPs (10 mM each, to be prepared previously and stored at –20°C in aliquots)	2.0	0.8 mM
Random nonamer (100 μM)	5.25	21 μM
RNaseOUT (40 U/μl)	0.5	0.8 U/μl
Superscript III Reverse Transcriptase (200 U/μl)	1.0	8 U/μl
DTT (supplied with Superscript III Reverse Transcriptase)	1.25	–
Heated RNA sample, control, or IS-MMR Calibrator (to be added in step 7)	10.0	40 ng/μl
Final volume	25.0	–

- 7. Pipet 15 μl of RT mix into each PCR tube. Then add 10 μl (1 μg) sample RNA, control, or calibrator (from step 4).**
- 8. Mix carefully (do not vortex) and centrifuge briefly (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**
- 9. Program the thermal cycler with the reverse transcription program as indicated in Table 2.**

Table 2. Temperature profile

Reverse transcription 1	Temperature: 25°C Time: 10 minutes
Reverse transcription 2	Temperature: 50°C Time: 60 minutes
Inactivation	Temperature: 85°C Time: 5 minutes
Cooling	Temperature: 4°C Time: 5 minutes

- 10. Place the tubes in the thermal cycler, and start the thermal cycling program, as indicated in Table 2.**
- 11. After the program is finished, centrifuge the tubes briefly (approximately 10 s, 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, according to the following protocols, according to your qPCR instrument.**

Note: For LightCycler 1.2, 1.5, and 2.0 instruments, each RT preparation provides cDNA for two qPCR runs.

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

Using this instrument, we recommend performing all measurements in duplicate, as indicated in Table 3. The kit is designed for testing of eight different cDNA samples in the same experiment three times.

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

Samples	Reactions
With the ABL primers and probe mix (PPC-ABL) (32 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA high positive control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	2 x 4 reactions (SP3, SP4, SP5, and SP6, each one tested in duplicate)
RT negative control	2 reactions
Water control	2 reactions
With the BCR-ABL MbcR primers and probe mix (PPF-MbcR) (32 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA high positive control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	2 x 5 reactions (SP1, SP2, SP3, SP5, and SP6, each one tested in duplicate)
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 and the primers and probe mixes. The rotor scheme in Figure 3 shows an example of such an experiment.

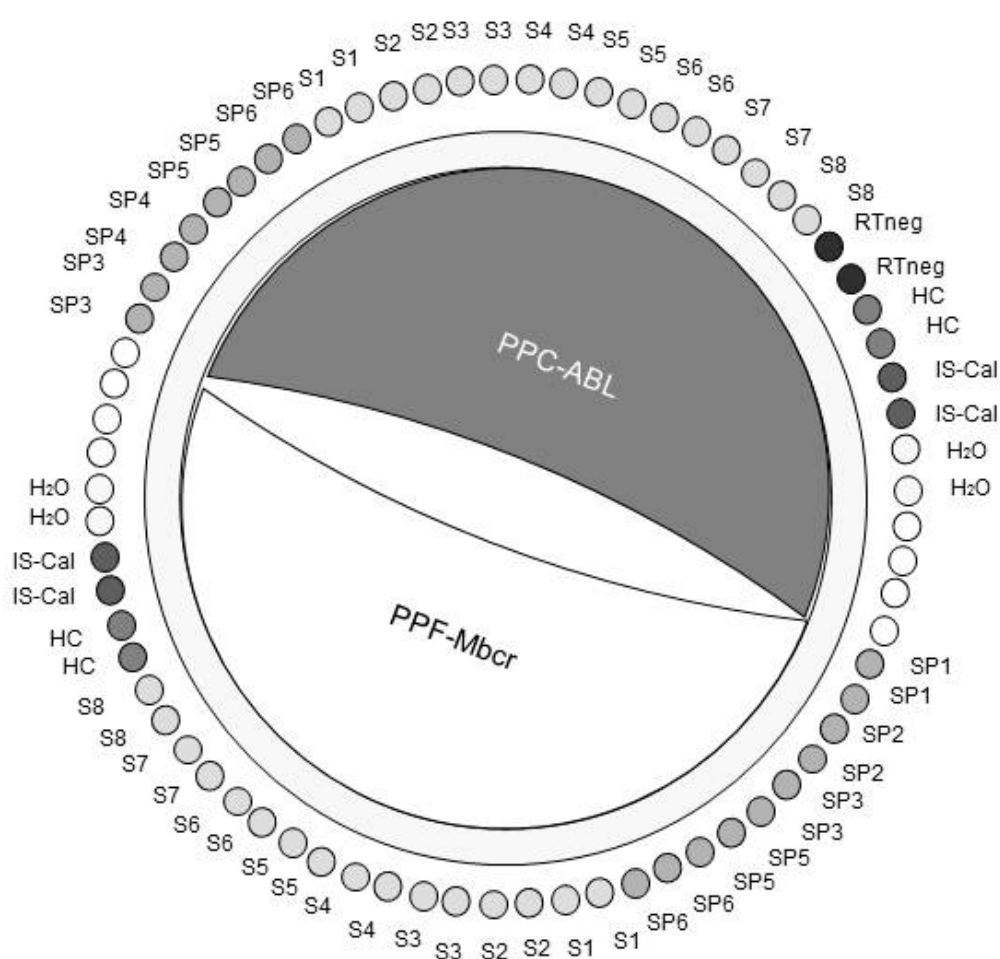


Figure 3. Suggested rotor setup for each experiment with the ipsogen BCR-ABL1 MbcR IS-MMR Kit. SP1–SP6: BCR-ABL MbcR and ABL standards; HC: High cDNA positive control; IS-Cal: IS-MMR calibrator; RTneg: RT negative control; S: cDNA sample; H₂O: water control.

Note: Take care to always place a sample to be tested in position 1 of the rotor. Otherwise, during the calibration step, the instrument will not perform calibration, and incorrect fluorescence data will be acquired.

Fill all other positions with empty tubes.

qPCR on Rotor-Gene Q instruments with 72-tube rotor

Note: Perform all steps on ice.

Procedure

1. Thaw all necessary components and place them on ice.
2. Vortex the standards, PPF-MbcR, and PPC-ABL tubes, and centrifuge briefly (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).

3. Prepare the following qPCR mix according to the number of samples being processed.

All concentrations are for the final volume of the reaction.

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

Table 4. Preparation of qPCR mix

Component	1 reaction (μl)	ABL: 32+1 reactions (μl)	BCR-ABL Mbcr: 32+1 reactions (μl)	Final concentration
Premix Ex Taq, 2x	12.5	412.5	412.5	1x
Primers and probe mix, 25x	1	33	33	1x
Nuclease-free PCR-grade water	6.5	214.5	214.5	–
Sample (to be added at step 5)	5	5 each	5 each	–
Total volume	25	25 each	25 each	–

- 4. Dispense 20 μ l of the qPCR pre-mix per tube.**
- 5. In a different lab area and with dedicated equipment, add 5 μ l of the RT product (cDNA, 200 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Reverse transcription using SuperScript III Reverse Transcriptase", page 11) in the corresponding tube (total volume 25 μ l).**
- 6. Mix gently, by pipetting up and down.**
- 7. Close all tubes, and place the tubes in the thermal cycler according to the manufacturer recommendations.**
- 8. 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 deg Time: 10 secs
Cycling	50 times 95 deg for 5 secs 60 deg for 30 secs with acquisition of FAM fluorescence in channel Green: Single
Hold 2	Temperature: 36 deg Time: 1 min

9. 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 FI"** for **"Min Reading"** to **"10 FI"** for **"Max Reading"** and the acceptable Gain range from **-10** to **10**.
10. Check the **"Perform Optimisation Before 1st Acquisition"** box, and close the **"Auto-Gain Optimisation Setup"** dialog box.
11. Start the thermal cycling program.
12. Select **"Slope Correct"** for the analysis. We recommend setting the threshold at **0.03**.

Protocol: qPCR on Applied Biosystems 7500 Real-Time PCR System, ABI PRISM 7900HT SDS, and LightCycler 480 instruments

Using 96-well-plate qPCR equipment, we recommend performing all measurements in duplicate, as indicated in Table 6. The kit is designed for testing of eight different cDNA samples in the same experiment three times.

Table 6. Number of reactions using 96-well-plate qPCR equipment

Samples	Reactions
With the ABL primers and probe mix (PPC-ABL) (32 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA high positive control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	2 x 4 reactions (SP3, SP4, SP5, and SP6, each one tested in duplicate)
RT negative control	2 reactions
Water control	2 reactions
With the BCR-ABL MbcR primers and probe mix (PPF-MbcR) (32 reactions)	
8 cDNA samples	8 x 2 reactions
1 cDNA high positive control	2 reactions
1 cDNA IS-MMR Calibrator	2 reactions
Single plasmid standards	2 x 5 reactions (SP1, SP2, SP3, SP5, and SP6, each one tested in duplicate)
Water control	2 reactions

Sample processing on Applied Biosystems, ABI PRISM, and LightCycler 480 instruments

We recommend testing at least 8 cDNA samples in the same experiment to optimize the use of the standards and the primers and probe mixes. The plate scheme in Figure 4 shows an example of such an experiment.

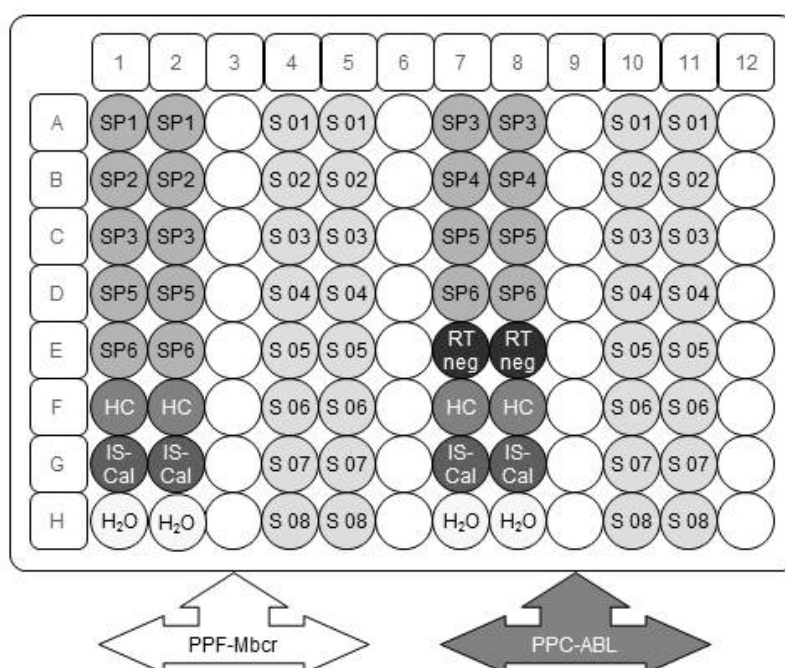


Figure 4. Suggested plate setup for one experiment with the *ipsogen* BCR-ABL1 MbcR IS-MMR Kit. SP1–SP6: BCR-ABL MbcR and ABL standards; HC: High cDNA positive control; IS-Cal: IS-MMR calibrator; RTneg: RT negative control; S: cDNA sample; H₂O: water control.

qPCR on Applied Biosystems, ABI PRISM, or LightCycler 480 instruments

Note: Perform all steps on ice.

Procedure

1. Thaw all necessary components and place them on ice.
2. Vortex the standards, ROX, PPF-MbcR, and PPC-ABL tubes, and centrifuge briefly (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).

3. Prepare the following qPCR mix according to the number of samples being processed. If using 96-well-plate qPCR equipment, we recommend performing all measurements in duplicate.

All concentrations are for the final volume of the reaction.

Table 7 describes the pipetting scheme for the preparation of one reagent mix for Applied Biosystems and ABI PRISM instruments, calculated to achieve a final reaction volume of 25 μ l. Table 8 describes the pipetting scheme for the preparation of one reagent mix for the LightCycler 480 instrument, calculated to achieve a final reaction volume of 25 μ l. A pre-mix can be prepared, according to the number of reactions, using the same primers and probe mix (either PPC-ABL or PPF-Mbcr). Extra volumes are included to compensate for pipetting error.

Table 7. Preparation of qPCR mix for Applied Biosystems and ABI PRISM instruments

Component	1 reaction (μl)	ABL: 32+1 reactions (μl)	BCR-ABL Mbcr: 32+1 reactions (μl)	Final concentration
Premix Ex Taq, 2x	12.5	412.5	412.5	1x
Primers and probe mix, 25x	1	33	33	1x
ROX I dye, 50x (ABI PRISM 7900HT) or ROX II dye, 50x (Applied Biosystems 7500)	0.5	16.5	16.5	1x
Nuclease-free PCR-grade water	6	198	198	–
Sample (to be added at step 5)	5	5 each	5 each	–
Total volume	25	25 each	25 each	–

Table 8. Preparation of qPCR mix for LightCycler 480

Component	1 reaction (μl)	ABL: 32+1 reactions (μl)	BCR-ABL Mbc: 32+1 reactions (μl)	Final concentration
Premix Ex Taq, 2x	12.5	412.5	412.5	1x
Primers and probe mix, 25x	1	33	33	1x
Nuclease-free PCR-grade water	6.5	214.5	214.5	–
Sample (to be added at step 5)	5	5 each	5 each	–
Total volume	25	25 each	25 each	–

4. Dispense 20 μ l of the qPCR pre-mix per well.
5. In a different lab area and with dedicated equipment, add 5 μ l of the RT product (cDNA, 200 ng RNA equivalent) obtained in the reverse transcription (see “Protocol: Reverse transcription using SuperScript III Reverse Transcriptase”, page 11) in the corresponding well (total volume 25 μ l).
6. Mix gently, by pipetting up and down.
7. Close the plate and briefly centrifuge (300 x g, approximately 10 s).
8. Place the plate in the thermal cycler according to the manufacturer’s recommendations. Program the thermal cycler with the thermal cycling program as indicated in Table 9 for Applied Biosystems and ABI PRISM instruments, or Table 10 for the LightCycler 480 Instrument.

Table 9. Temperature profile for Applied Biosystems and ABI PRISM instruments

Mode of analysis	Standard Curve — Absolute Quantitation
Hold 1	Temperature: 95°C Time: 10 seconds
Cycling	50 times 95°C for 5 seconds 60°C for 30 seconds with acquisition of FAM fluorescence: Single; quencher: TAMRA
Hold 2	Temperature: 36°C Time: 1 minute

Table 10. Temperature profile for LightCycler 480 Instrument

Mode of analysis	Absolute Quantification ("Abs Quant")
Detection formats	Select "Simple Probe" in the Detection formats window
Hold 1	Temperature: 95°C Time: 10 seconds
Cycling	50 times 95°C for 5 seconds 60°C for 30 seconds with acquisition of FAM fluorescence corresponding to (483–533 nm) for LC version 01 and (465–510 nm) for LC version 02
Hold 2	Temperature: 36°C Time: 1 minute

- 9. For the Applied Biosystems 7500 and ABI PRISM 7900HT SDS, follow step 9a. For the LightCycler 480 Instrument, follow step 9b.**
- 9a. Applied Biosystems and ABI PRISM: We recommend a threshold set at 0.1 in the analysis step on the instrument. Start the cycling program, as indicated in Table 9.**
- 9b. LightCycler 480 Instrument: We recommend a Fit point analysis mode with background at 2.0 and threshold at 2.0. Start the thermal cycling program, as indicated in Table 10.**

Protocol: qPCR on LightCycler 1.2, 1.5, and 2.0 Instruments

Using capillary instruments, we recommend measuring samples in duplicate and controls in only once, as indicated in Table 11. The kit is designed for testing of four different cDNA samples in the same experiment six times.

Table 11. Number of reactions for LightCycler 1.2, 1.5, and 2.0 instruments

Samples	Reactions
With the ABL primers and probe mix (PPC-ABL) (16 reactions)	
4 cDNA samples	4 x 2 reactions
1 cDNA high positive control	1 reaction
1 cDNA IS-MMR Calibrator	1 reaction
Single plasmid standards	1 x 4 reactions (SP3, SP4, SP5, and SP6)
RT negative control	1 reaction
Water control	1 reaction
With the BCR-ABL Mbcr primers and probe mix (PPF-Mbcr) (16 reactions)	
4 cDNA samples	4 x 2 reactions
1 cDNA high positive control	1 reaction
1 cDNA IS-MMR Calibrator	1 reaction
Single plasmid standards	1 x 5 reactions (SP1, SP2, SP3, SP5, and SP6)
Water control	1 reaction

Sample processing on LightCycler 1.2, 1.5, and 2.0 Instruments

We recommend testing at least four cDNA samples in the same experiment to optimize the use of the standards and primers and probe mixes. The capillary scheme in Figure 5 shows an example of an experiment.

Table 12. Preparation of qPCR mix for LightCycler 1.2, 1.5, and 2.0 instruments

Component	1 reaction (μl)	ABL: 16+1 reactions (μl)	BCR-ABL Mbc: 16+1 reactions (μl)	Final concentration
Premix Ex Taq, 2x	10	170	170	1x
Primers and probe mix, 25x	0.8	13.6	13.6	1x
Nuclease-free PCR-grade water	4.2	71.4	71.4	–
Sample (to be added at step 5)	5	5 each	5 each	–
Total volume	20	20 each	20 each	–

4. Dispense 15 μl of the qPCR pre-mix per capillary.
5. In a different lab area and with dedicated equipment, add 5 μl of the RT product (cDNA, 200 ng RNA equivalent) obtained in the reverse transcription (see “Protocol: Reverse transcription using SuperScript III Reverse Transcriptase”, page 11) in the corresponding capillary (total volume 20 μl).
6. Mix gently, by pipetting up and down.
7. Place the capillaries in the adapters provided with the apparatus, and briefly centrifuge (700 x g, approximately 10 s).
8. Load the capillaries into the thermal cycler according to the manufacturer recommendations.
9. Program the LightCycler 1.2, 1.5, or 2.0 Instrument with the thermal cycling program as indicated in Table 13.

Table 13. Temperature profile

Mode of analysis	Quantification
Hold 1	Temperature: 95°C Time: 10 seconds Ramp: 20
Cycling	50 times 95°C for 5 seconds; ramp: 20 60°C for 30 seconds; ramp: 20; with acquisition of FAM fluorescence: Single
Hold 2	Temperature: 36°C Time: 1 minute Ramp: 20

10. For the LightCycler 1.2 and 1.5, follow step 10a. For the LightCycler 2.0, follow step 10b.

10a. LightCycler 1.2 and 1.5: The F1/F2 and “2nd derivative analysis” mode is recommended. Start the thermal cycling program, as indicated in Table 13.

10b. LightCycler 2.0: We recommend using Automated (F''max) analysis on LightCycler 2.0 Software version 4.0 to obtain reproducible results. Start the thermal cycling program, as indicated in Table 13.

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 *ipsogen* standard curves are plasmid-based. In order to ensure accurate standard curves, we use four standard dilutions for ABL, and five standard dilutions for Mbcr. The kit also includes an IS-MMR calibrator allowing conversion of results to the international scale. Figures 6 and 7 show examples of TaqMan amplification curves obtained for standards, the IS-MMR Calibrator, and the high positive RNA control with the *ipsogen* BCR-ABL1 Mbcr IS-MMR Kit.

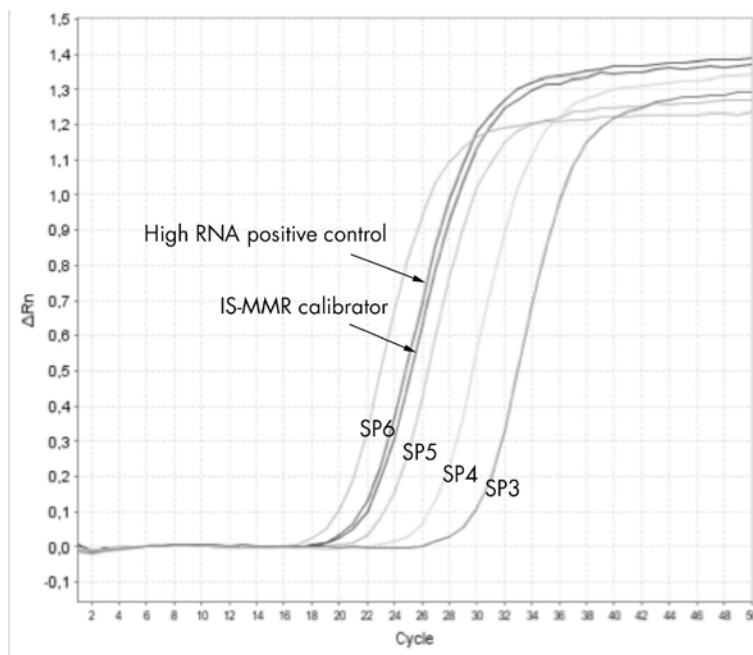


Figure 6. Detection of ABL with standards SP3, SP4, SP5, and SP6. 10^3 , 10^4 , 10^5 , and 10^6 copies/ $5\ \mu\text{l}$.

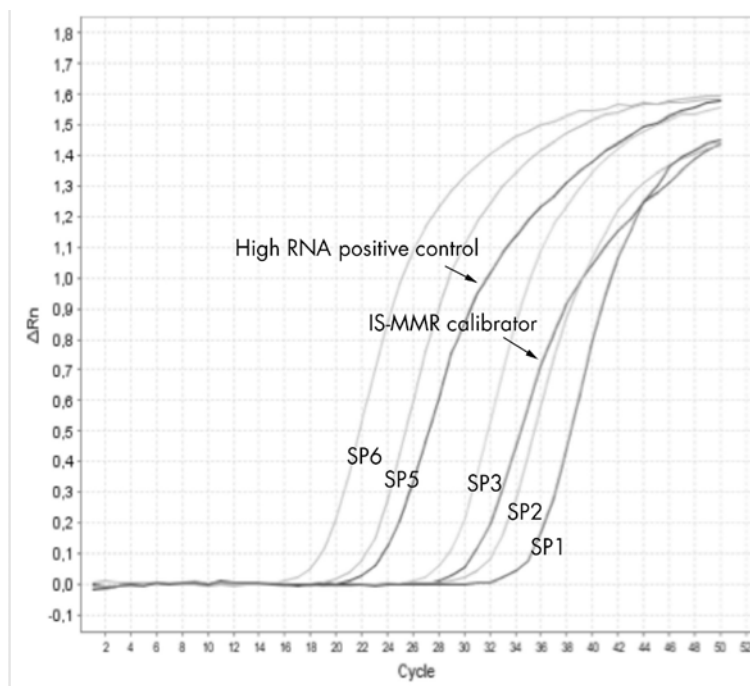


Figure 7. Detection of BCR-ABL Mbc with standards SP1, SP2, SP3, SP5, and SP6. 10^1 , 10^2 , 10^3 , 10^5 , 10^6 copies/5 μ l.

Standard curves and quality criteria applicable to raw data

Reproducibility between replicates

The variation in C_T values between replicates should be <2 , corresponding to a fourfold change in copy number values.

Variation in C_T values between replicates is generally <1.5 if the mean C_T value of the replicates is <36 (1).

Note: Each user should measure their own reproducibility in their laboratory.

Standard curves

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

For each gene (ABL and BCR-ABL), raw 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 8 shows an example of a theoretical ABL standard curve calculated on four standard dilutions. Figure 9 shows an example of a theoretical BCR-ABL standard curve calculated on five standard dilutions.

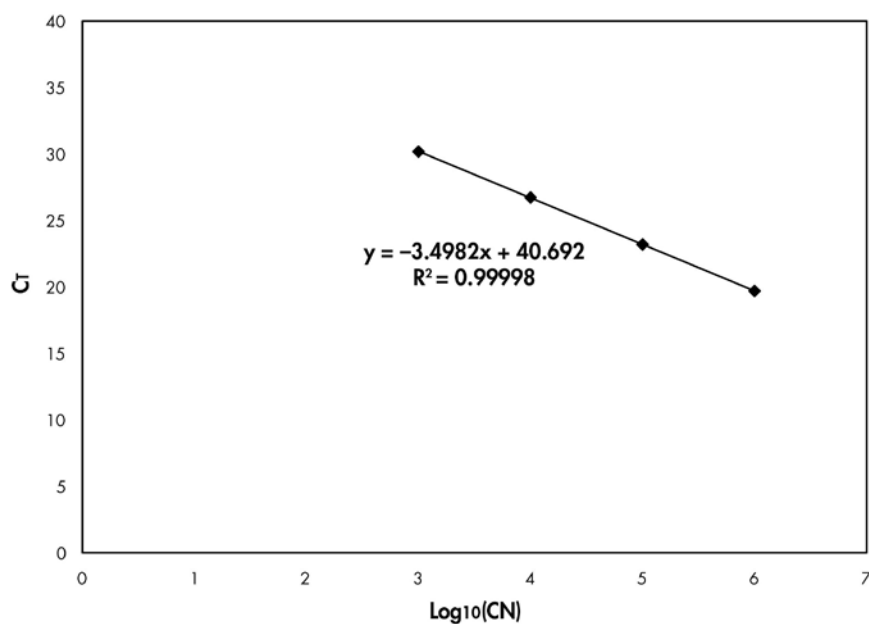


Figure 8. Theoretical standard curve for ABL calculated from 4 standard dilutions. A linear regression curve ($y = ax + b$) is calculated, where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (R^2) are printed on the graph.

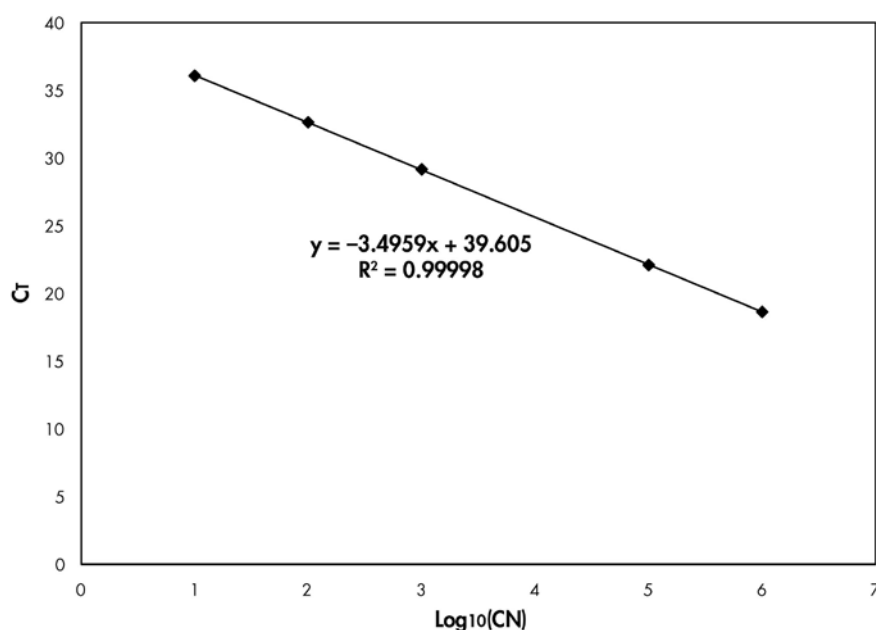


Figure 9. Theoretical standard curve for BCR-ABL calculated from 5 standard dilutions. A linear regression curve ($y = ax + b$) is calculated, where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (R^2) are printed 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 (1). However, a value for $R^2 >0.98$ is desirable for precise results (2).

Note: The SP1 standard dilution (BCR-ABL plasmid, 10 copies) must be detected and included in the BCR-ABL standard curve.

Quality control on all ABL values

Poor quality of the RNA or problems during the qPCR steps result in low ABL copy numbers (ABL_{CN}). Optimal sensitivity is achieved with samples giving $ABL_{CN} \geq 10,000$ copies. This criterion on ABL_{CN} also applies to the high 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 ABL and BCR-ABL Mbcr. A positive result for these NTCs indicates cross-contamination during reverse transcription and/or qPCR.

Normalized copy number (NCN)

The ABL standard curve equation should be used to transform raw C_T values (obtained with PPC-ABL) for the unknown samples into ABL copy numbers (ABL_{CN}).

The BCR-ABL standard curve equation should be used to transform raw C_T values (obtained with PPF-Mbcr) for the unknown samples, into BCR-ABL copy numbers ($BCR-ABL\ Mbcr_{CN}$).

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

$$NCN = \frac{BCR-ABL\ Mbcr_{CN}}{ABL_{CN}} \times 100$$

Calculate the NCN result for the high positive RNA control (NCN_{HC}), the IS-MMR calibrator (NCN_{cal}) and each sample (NCN_{sample}).

High positive RNA control and IS-MMR Calibrator

These controls allow the monitoring of the reverse transcription and amplification steps of ABL and BCR-ABL Mbcr during transcript quantification.

Quality control on NCN_{cal} result

Note: The NCN result obtained for the IS-MMR-Calibrator, tested with the *ipsogen* BCR-ABL Mbcr IS-MMR Kit in combination with recommended reagents and instruments (see “Materials Provided”, page 7, and “Materials Required but Not Provided”, page 8), must be within the interval 0.05–0.3. Otherwise, NCN values cannot be converted to the International Scale. Furthermore, the whole experiment must be rejected if the high positive RNA control is not detected.

IS conversion and MMR reporting

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 ($IS-NCN_{sample}$).

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

Determine the MMR status of each sample according to the following criteria.

- **$IS-NCN_{sample} \leq 0.05$:** Major molecular response
- **$0.05 < IS-NCN_{sample} < 0.15$:** Gray zone around the MMR cutoff, inconclusive result
- **$IS-NCN_{sample} \geq 0.15$:** No major molecular response

The $IS-NCN_{HC}$ result (NCN on the international scale for the high positive RNA control) should give no major molecular response.

Figure 10 shows an example using NCN and IS-NCN results.

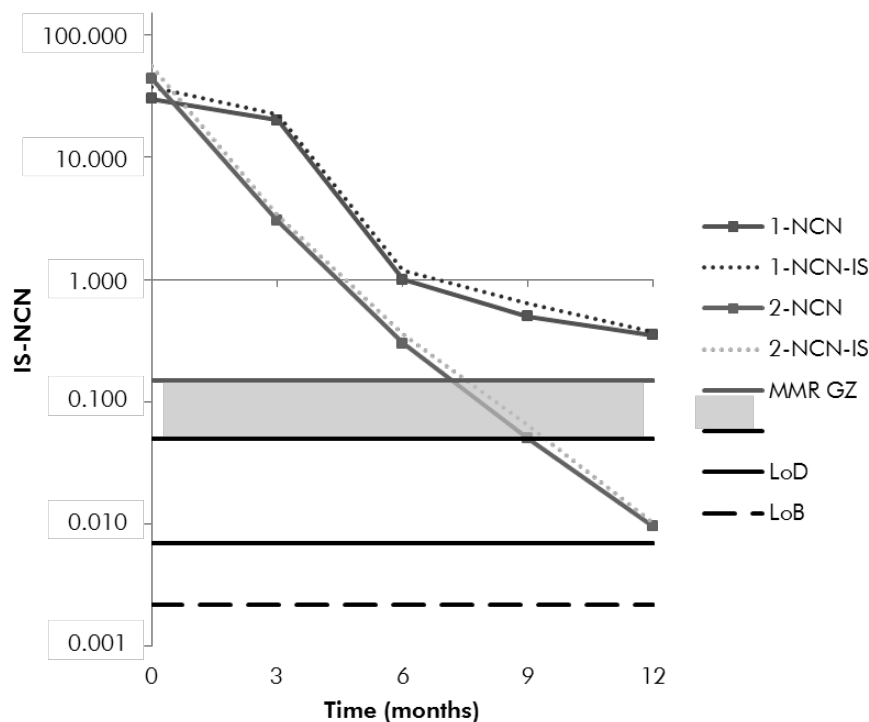


Figure 10. Monitoring curves for MMR status with the ipsogen BCR-ABL1 MbcR IS-MMR Kit. NCN: normalized copy number; NCN-IS: normalized copy number international scale; MMR GZ: MMR gray zone (GZ) inconclusive result; LoD: limit of detection; LoB: background level.

Summary of quality criteria

Table 14 summarizes the various quality criteria and associated values or results.

Table 14. Quality criteria summary

Criteria	Acceptable values/results
Variations in C_T values between replicates	$\leq 2 C_T$ if mean C_T value > 36 $\leq 1.5 C_T$ if mean C_T value ≤ 36
Slope for standard curves	Between -3.0 and -3.9
R^2 for standard curves	At least > 0.95 better if > 0.98
SP1 standard dilution (BCR-ABL 10 copies plasmid)	Must be detected and included in the standard curve
Quality control on ABL_{CN} value for biological samples, high positive RNA control, and the IS-MMR-Calibrator	$ABL_{CN} > 10,000$ copies of ABL to reach the optimal sensitivity
PCR (water) and reverse transcription (RT negative) controls	For each $ABL_{CN} = 0$ and $Mbcr_{CN} = 0$
NCN obtained for IS-MMR Calibrator (NCN_{cal})	Must be within the interval $0.05-0.3$
High positive RNA control	Must be detected
NCN obtained for the high positive RNA control converted to the international scale ($IS-NCN_{HC}$)	Status: No major molecular response

Troubleshooting

For more information, see 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 protocol in this handbook or sample and assay technologies (for contact information, see "Contact Information", page 35).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *ipsogen* BCR-ABL1 Mbcr IS-MMR Kit is tested against predetermined specifications to ensure consistent product quality. Certificates of analysis are available on request at www.qiagen.com/support/.

References

1. van der Velden, V.H., Hochhaus, A., Cazzaniga, G., Szczepanski, T., Gabert, J., and van Dongen, J.J. (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* **17**, 1013.
2. Branford, S. et al. (2006) Rationale for the recommendations for harmonizing current methodology for detecting BCR-ABL transcripts in patients with chronic myeloid leukaemia. *Leukemia* **20**, 1925.

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

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Ordering Information

Product	Contents	Cat. no.
<i>ipsogen</i> BCR-ABL1 Mbcr IS-MMR Kit (24)	For 24 reactions: Mbcr and ABL Single Plasmid Standards, High RNA Positive Control, IS-MMR Calibrator, Primers and Probe Mix ABL, Primers and Probe Mix BCR-ABL Mbcr Fusion Gene	670713
Rotor-Gene Q – for outstanding performance in real-time PCR		
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, 1-year warranty on parts and labor, installation and training not included	9001580
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, 1-year warranty on parts and labor, installation and training	9001650
<i>ipsogen</i> RT Kit – for reverse transcription		
<i>ipsogen</i> RT Kit	Reverse Transcriptase, Random Primer, DTT, dNTP, RNase Inhibitor, RT Buffer	679913
RNeasy Kits – for purification of total RNA		
RNeasy Midi Kit (50)	For 50 RNA preps: 50 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75144

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Notes

Notes

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HB-1414-003

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