July 2016

ipsogen® BCR-ABL1 Mbcr IS-MMR DX Kit Handbook

Σ 24

Version 1

IVD

Quantitative in vitro diagnostics

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

CE

REF 670823

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Contents

Intended Use

The *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit is intended for the quantification of BCR-ABL p210 b2a2 or b3a2 transcripts in bone marrow or peripheral blood samples of acute lymphoblastic leukemia (ALL) or chronic myeloid leukemia (CML) patients previously diagnosed with a BCR-ABL Mbcr fusion gene (FG) event. The test is intended to evaluate the level of molecular response; results can be used for minimal residual disease follow-up.

Summary and Explanation

Background on CML

CML belongs to the group of myeloproliferative neoplasms and is in >90% of cases characterized by the presence of the Philadelphia chromosome (Ph CHRS).

This chromosome is the product of a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22), BCR (breakpoint cluster region) being located on chromosome 22 and the c-ABL oncogene coming from chromosome 9. The corresponding fusion gene, BCR-ABL, is transcribed into an 8.5 kb mRNA, with 2 junction variants b2a2 (40% of cases) and b3a2 (55% of cases). It 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 ALL patients.

Annual incidence of CML is approximately 1–2 per 100,000, and CML accounts for 20% of adult leukemias. It is characterized clinically by an excess of myeloid cells that differentiate and function normally. CML patients will be diagnosed in 90–95% of cases in the chronic or stable phase of the disease. Historically, within an average of 4 to 6 years, patients were entering an accelerated phase leading to blastic crisis and acute leukemia, which is always fatal. The advents of imatinib and more recently, second generation tyrosine kinase inhibitors (TKI), dramatically changed the natural course of the disease: most of the patients now remain in remission and deserve long-term follow-up and disease monitoring.

Disease monitoring

To date, the 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 early relapse for each individual patient. Under TKI therapy, patients typically progress from hematologic to cytogenetic then molecular remission, corresponding to decreasing numbers of leukemic cells and BCR-ABL transcripts as detailed in the Figure 1.

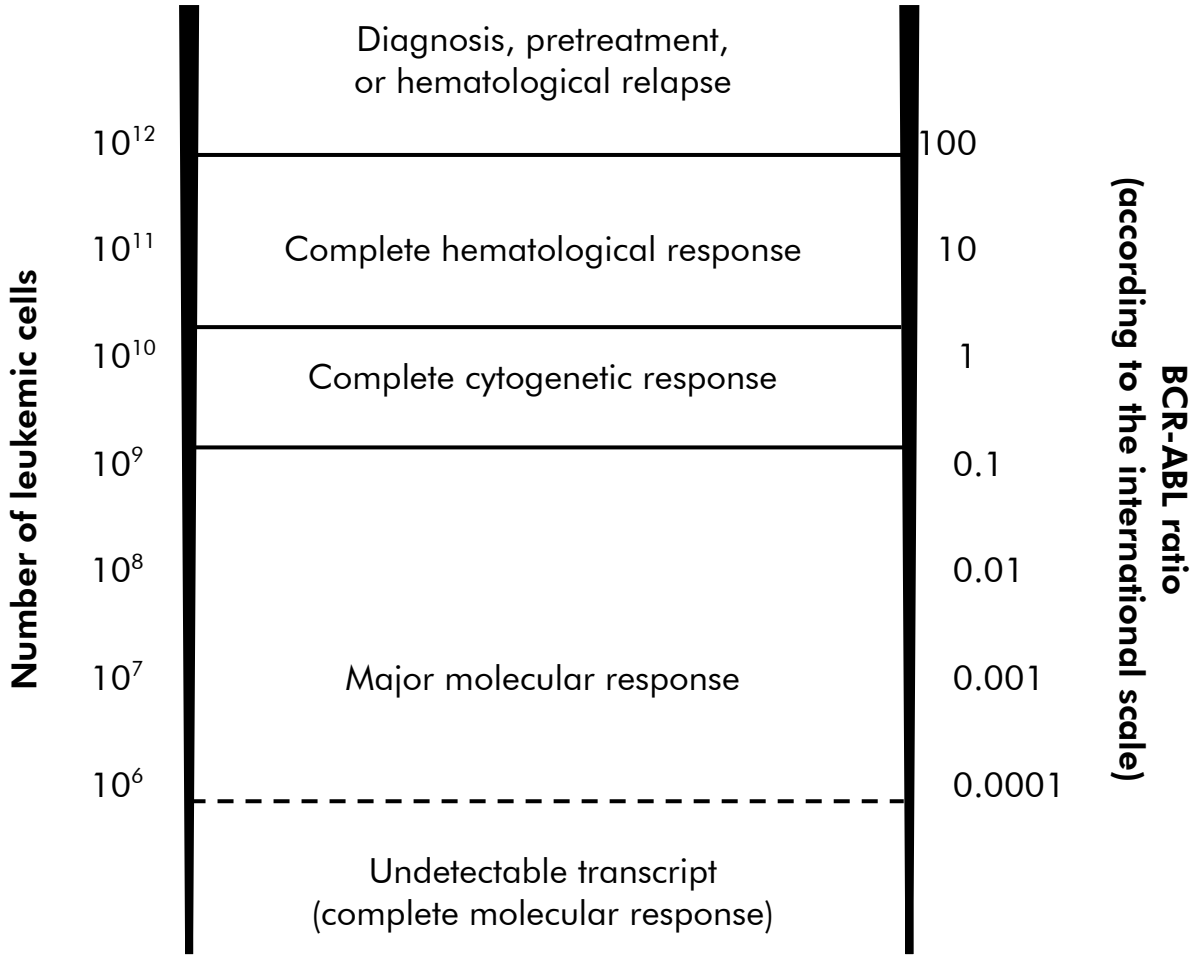

Figure 1. Adapted from reference 1.

The standard method to estimate the 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 on the percentage of Ph chromosome-positive metaphases (see Table 1, reference 2) However, this assessment depends on laboratory performances and has a low sensitivity, at 5% when 20 metaphases are analyzed.

Real time quantitative polymerase chain reaction (qPCR) quantifying BCR-ABL Mbcr mRNA, on peripheral blood (PB) specimens is now part of the disease monitoring techniques for CML on treatment. 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 clinical trials as well as improved disease monitoring objectives and tools. The most recent recommendations on response definition and monitoring of patients on imatinib come from the ELN experts (2).

From a technical standpoint, efforts have been made by international experts to harmonize BCR-ABL Mbcr testing and reporting (3–5). Additionally, a reference panel has been validated recently under auspices of the WHO, to allow a simple standardization of BCR-ABL quantification (6).

Principle of the Procedure

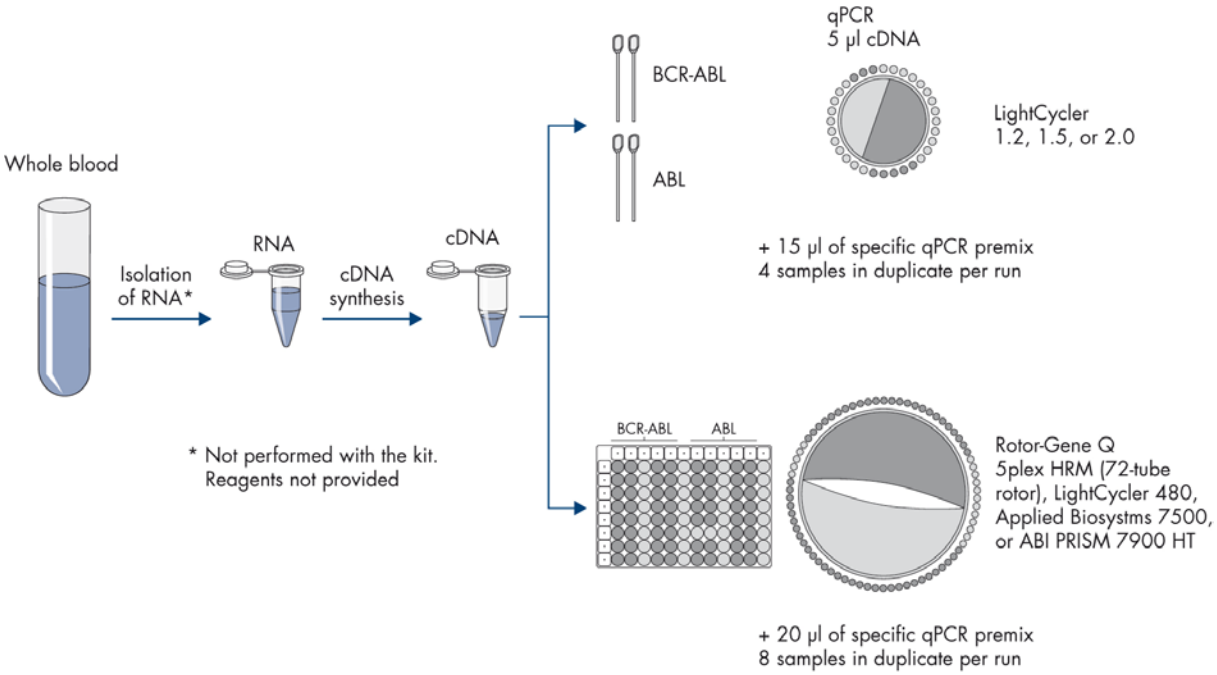

Figure 2. RNA isolation, cDNA synthesis, and qPCR.

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 3). 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.

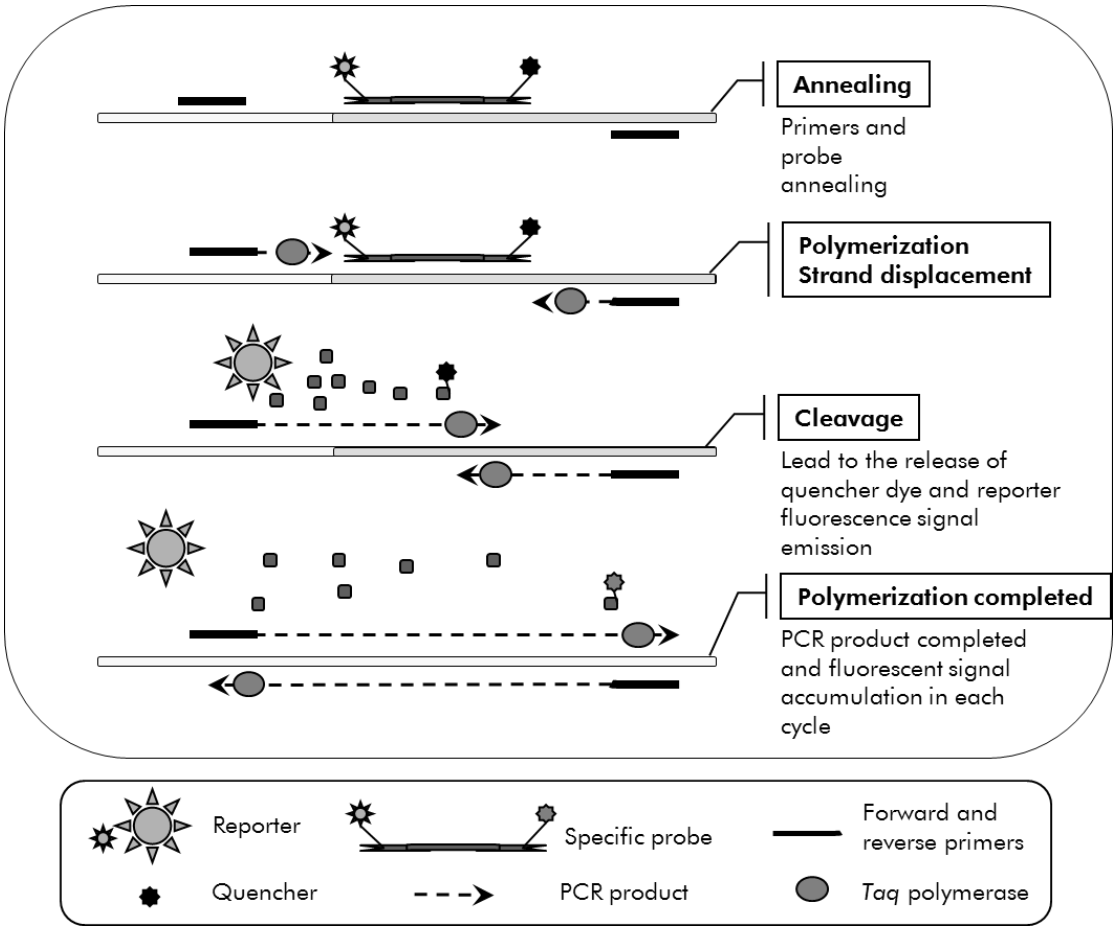

Figure 3. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–TAMRA™). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer 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 DX Kit**		**(24)**
Catalog no.		**670823**
Number of reactions		**24**
Reverse transcription		
Reverse Transcriptase		36 µl
5x RT Buffer for reverse transcription		180 µl
dNTP Mix*		72 µl
Random Primer†		190 µl
RNase Inhibitor		18 µl
DTT‡		45 µl
Calibration		
High Positive RNA Control		10 µl x 3
IS-MMR Calibrator		10 µl x 3
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

* Deoxynucleotides 10 mM each.

† Random nonamer oligonucleotide.

‡ Dithiothreitol.

Kit contents continued

Reagents for qPCR		
Master Mix for qPCR	qPCR Mix 2x	2 x 1275 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
ROX™ I fluorescent dye for ABI PRISM instruments	ROXI	102 µl
ROX II fluorescent dye for Applied Biosystems instruments	ROXII	102 µl
Nuclease-free PCR grade water		1400 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 reverse transcriptase and master mix tubes, 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 validated reagents are RNeasy Midi Kit, QIAGEN, cat. no. 75144 or TRIzol® Reagent, Thermo Fisher Scientific, cat. no. 15596-018 or 15596-026
- Nuclease-free PCR grade water

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

- Microliter pipets* dedicated for PCR (1–10 µl; 10–100 µl; 100–1000 µl)
- Benchtop centrifuge* with rotor for 0.2 ml/0.5 ml reaction tubes (capable of attaining 10,000 rpm)
- Real-time PCR instrument:* Rotor-Gene Q MDx 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)

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

Warnings and Precautions

For in vitro diagnostic use

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in 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.

The following hazard and precautionary statements apply to components of the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit:

DTT 0.1M RT

Warning! Causes mild skin irritation. If skin irritation occurs: Get medical advice/attention.

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.

This kit is intended for in vitro diagnostic use. Reagents and instructions supplied in this kit have been validated 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.
- 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°C for no more than 5 days before RNA extraction.

Procedure

Sample RNA preparation

RNA preparation from patient samples (blood or bone marrow) must have been done with a validated 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

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 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
RT Buffer, 5x (supplied with 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
RNase Inhibitor (40 U/µl)	0.5	0.8 U/µl
Reverse Transcriptase (200 U/µl)	1.0	8 U/µl
DTT (supplied with 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 min
Reverse transcription 2	Temperature: 50°C Time: 60 min
Inactivation	Temperature: 85°C Time: 5 min
Cooling	Temperature: 4°C Time: 5 min

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 Rotor Gene Q MDx 5plex HRM or Rotor-Gene Q 5plex HRM instruments 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 4 shows an example of such an experiment.

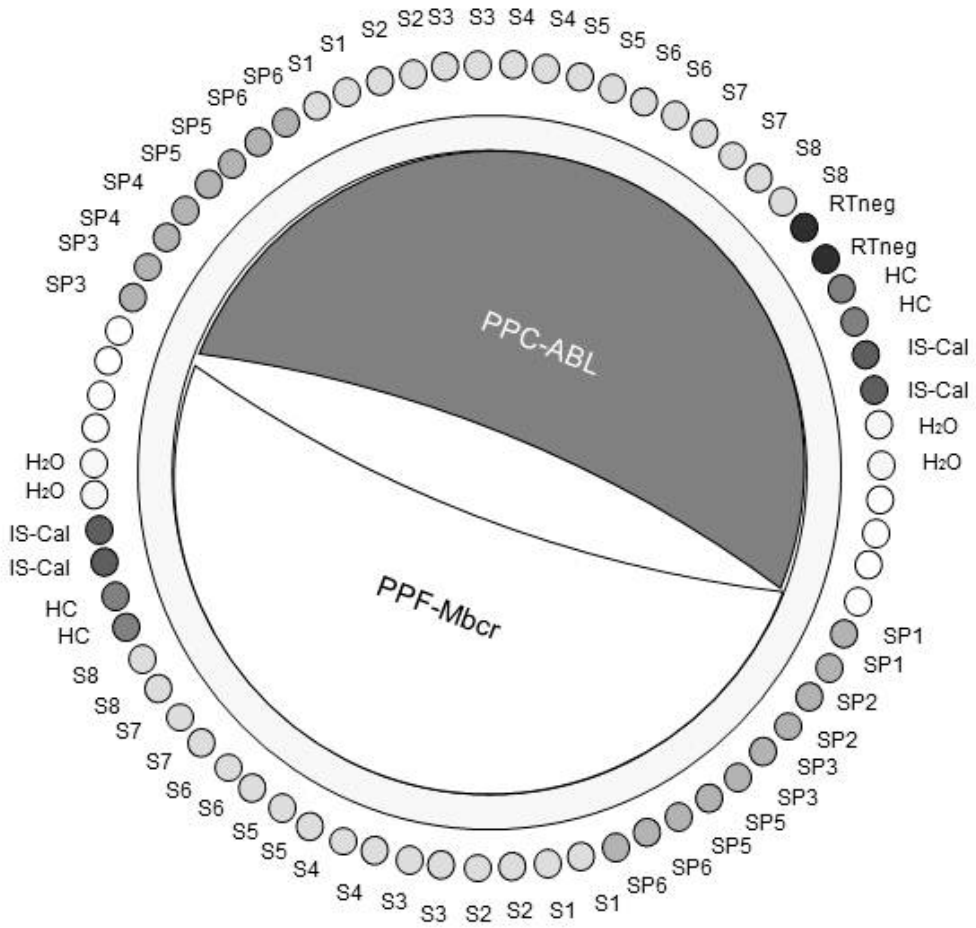


Figure 4. Suggested rotor setup for each experiment with the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit. SP1–SP6: BCR-ABL Mbcr and ABL standards; **HC**: High cDNA positive control; **IS-Cal**: IS-MMR calibrator; **RTneg**: RT negative control; **S**: cDNA sample; **H_2O**: water control.

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 µl. 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)	BCR-ABL ABL: 32+1 reactions (µl)	BCR-ABL Mbcr: 32+1 reactions (µl)	Final concentration
qPCR Mix, 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 µl of the qPCR pre-mix per tube.**
5. **Add 5 µl of the RT product (cDNA, 200 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Reverse transcription", page 14) in the corresponding tube (total volume 25 µl).**
6. **Mix gently, by pipetting up and down.**
7. **Place the tubes in the thermal cycler according to the manufacturer's 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°C Time: 10 s
Cycling	50 times 95°C for 5 s 60°C for 30 s with acquisition of FAM fluorescence in channel Green: Single
Hold 2	Temperature: 36°C 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 Fl" for "Min Reading" to "10 Fl" 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 eight cDNA samples in the same experiment to optimize the use of the standards and the primers and probe mixes. The plate scheme in Figure 5 shows an example of such an experiment.

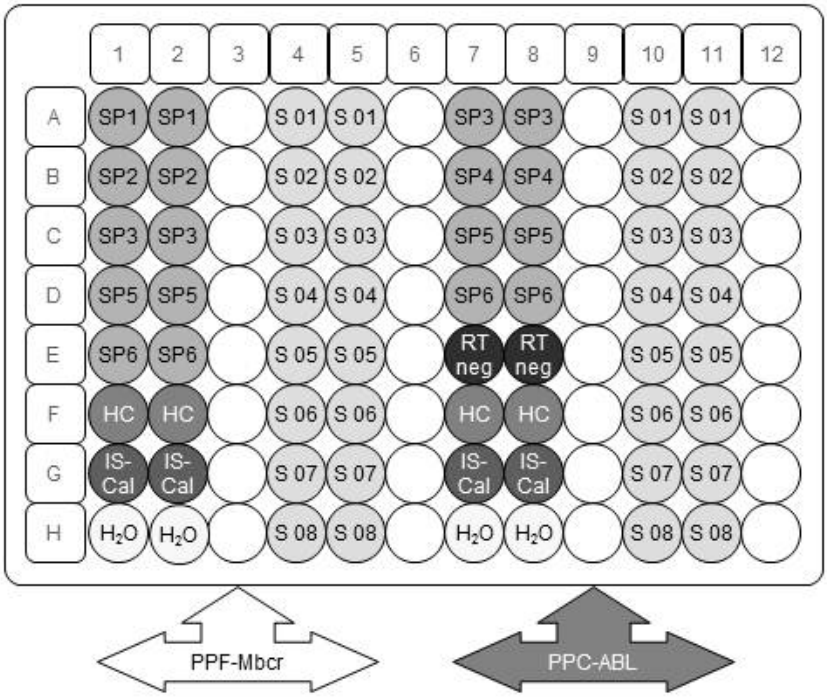


Figure 5. Suggested plate setup for one experiment with the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit. SP1–SP6: BCR-ABL Mbcr and ABL standards; **HC**: High cDNA positive control; **IS-Cal**: IS-MMR calibrator; **RTneg**: RT negative control; **S**: cDNA sample; **H_2O**: water control.

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, 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 µl. 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 µl. 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
qPCR Mix, 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 Mbcr: 32+1 reactions (µl)	Final concentration
qPCR Mix, 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 µl of the qPCR pre-mix per well.**
5. **Add 5 µl of the RT product (cDNA, 200 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Reverse transcription", page 14) in the corresponding well (total volume 25 µl).**
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 s
Cycling	50 times 95°C for 5 s 60°C for 30 s with acquisition of FAM fluorescence: Single; quencher: TAMRA
Hold 2	Temperature: 36°C Time: 1 min

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 s
Cycling	50 times 95°C for 5 s 60°C for 30 s 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 min

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 6 shows an example of an experiment.

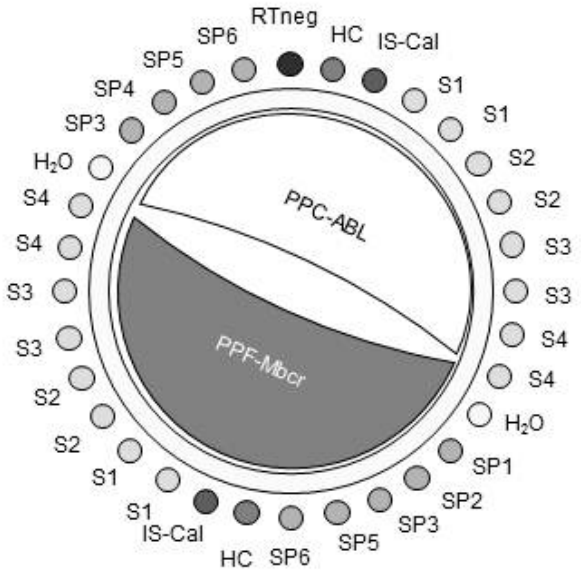


Figure 6. Suggested rotor setup for each experiment with the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit. SP1–SP6: BCR-ABL Mbcr and ABL standards; **HC**: High cDNA positive control; **IS-Cal**: IS-MMR calibrator; **RTneg**: RT negative control; **S**: cDNA sample; **H_2O**: water control.

qPCR on LightCycler 1.2, 1.5, and 2.0 Instruments

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 12 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 20 µl. 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 12. Preparation of qPCR mix for LightCycler 1.2, 1.5, and 2.0 instruments

Component	1 reaction (µl)	BCR-ABL ABL: 16+1 reactions (µl)	BCR-ABL Mbcr: 16+1 reactions (µl)	Final concentration
qPCR Mix, 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. **Add 5 µl of the RT product (cDNA, 200 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Reverse transcription", page 14) 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 s Ramp: 20
Cycling	50 times 95°C for 5 s; ramp: 20 60°C for 30 s; ramp: 20; with acquisition of FAM fluorescence: Single
Hold 2	Temperature: 36°C Time: 1 min 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.

Interpretation of 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 7 and 8 show examples of TaqMan amplification curves similar to those obtained for standards, the IS-MMR Calibrator, and the high positive RNA control with the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit.

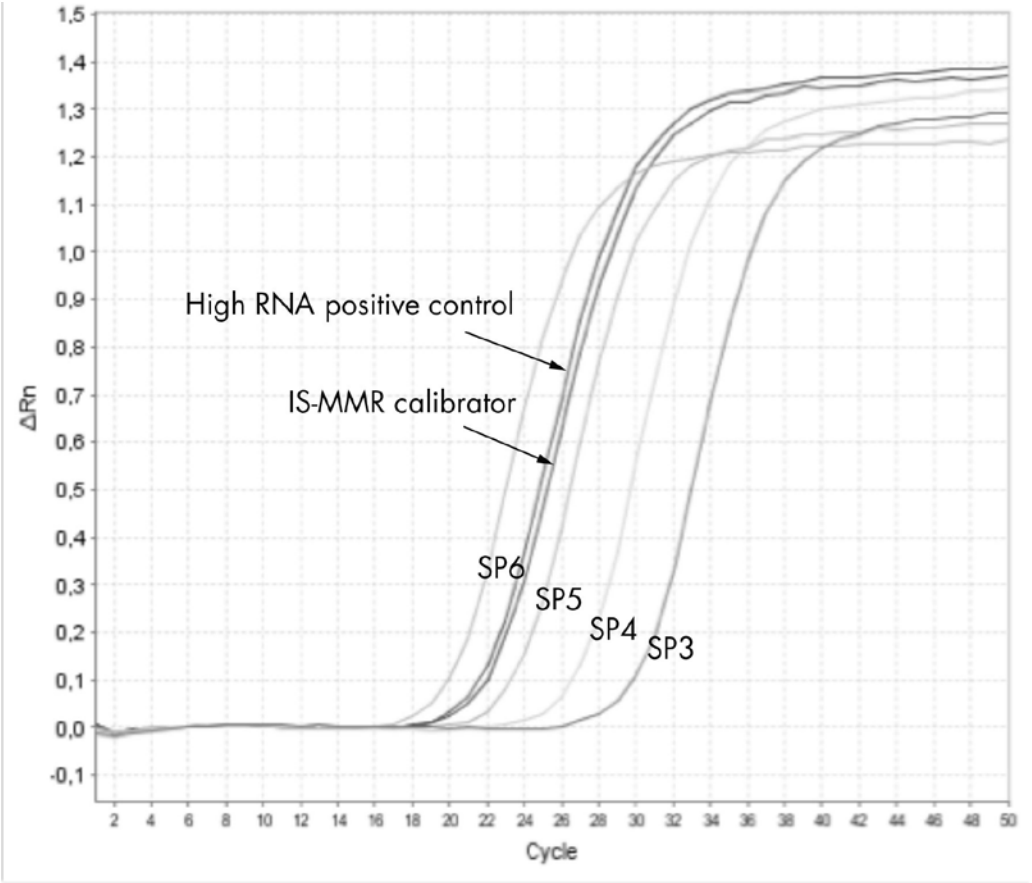


Figure 7. Detection of ABL with standards SP3, SP4, SP5, and SP6.. 10^3, 10^4, 10^5, and 10^6 copies/5 µl.

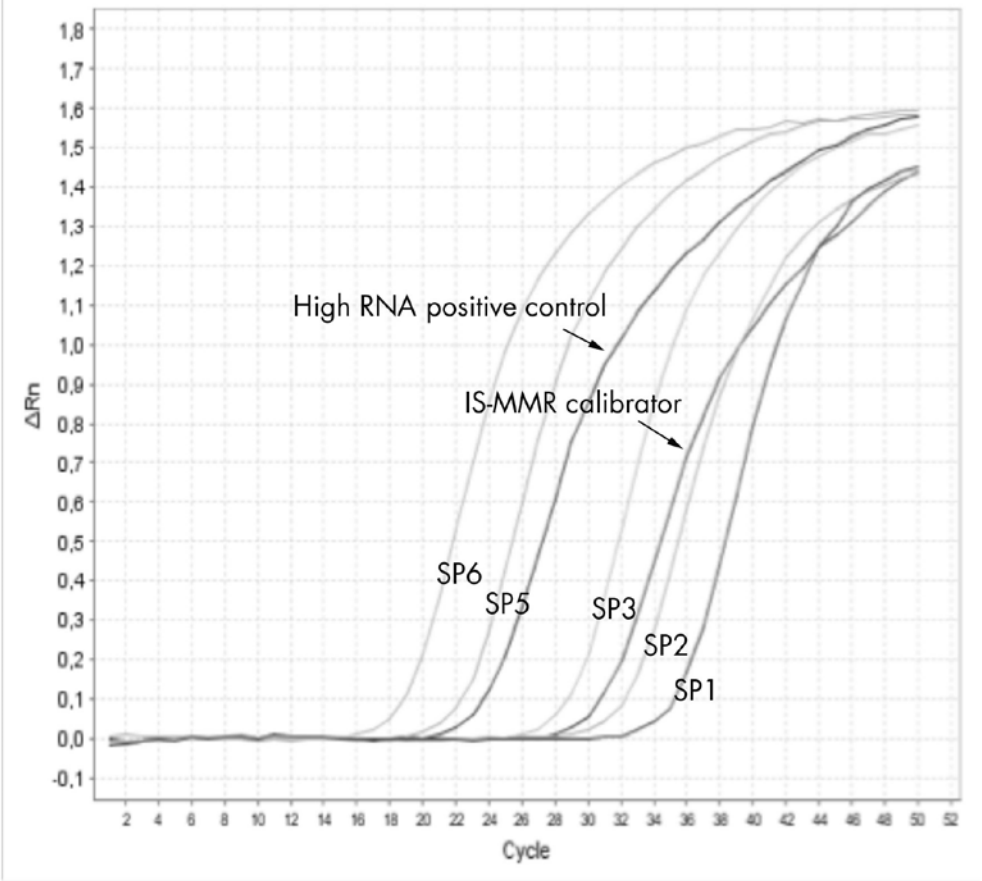


Figure 8. Detection of BCR-ABL Mbcr with standards SP1, SP2, SP3, SP5, and SP6. 10^1, 10^2, 10^3, 10^5, 10^6 copies/5 µl.

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 (7).

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 9 shows an example of a theoretical ABL curve calculated on 4 standard dilutions. Figure 10 shows an example of a theoretical BCR-ABL Mbcr curve calculated on five standard dilutions.

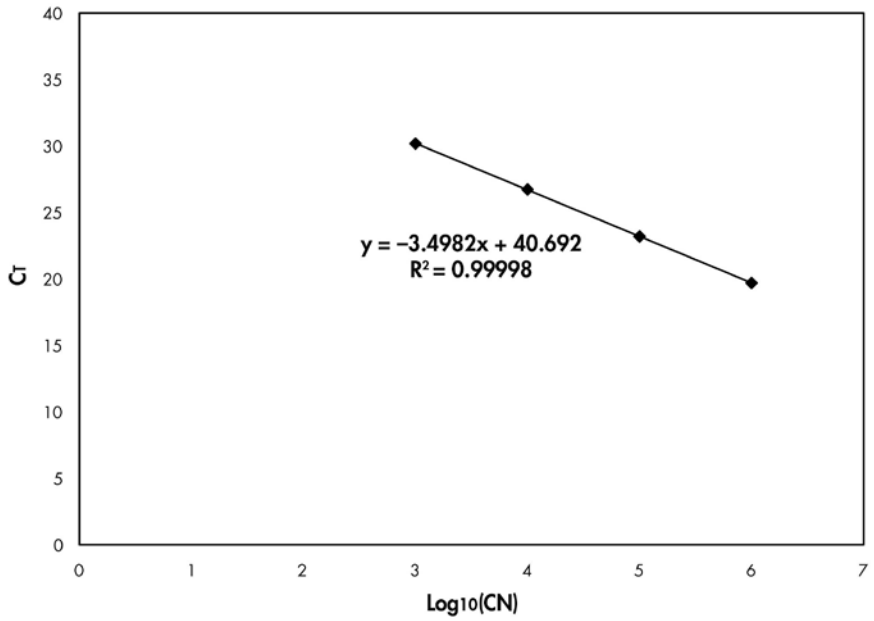


Figure 9. Theoretical curve for ABL calculated from 4 standard dilutions. A linear regression curve (y = ax + b) is calculated, where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (R^2) are printed on the graph.

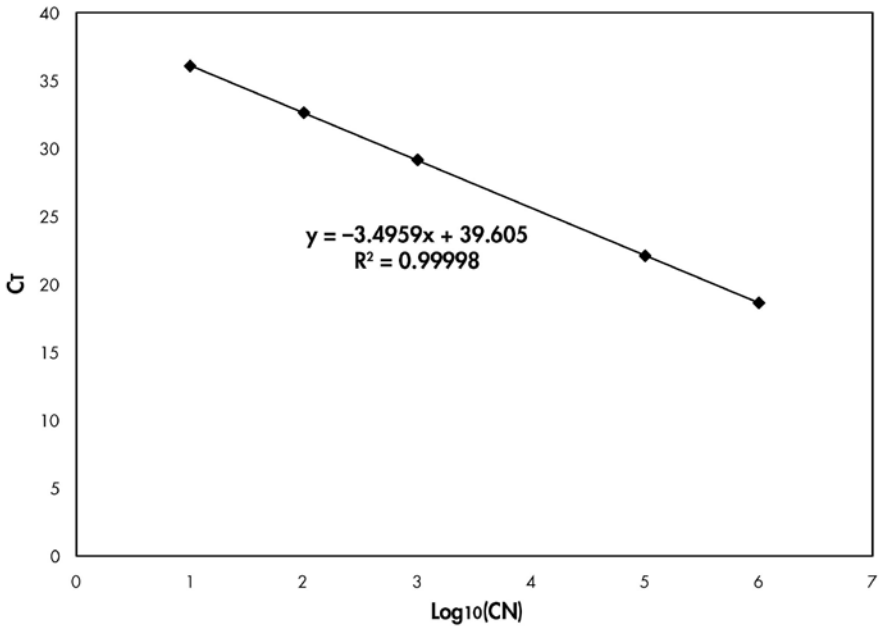


Figure 10. Theoretical curve for BCR-ABL calculated from 5 standard dilutions. A linear regression curve (y = ax + b) is calculated, where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (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 (7). However, a value for R^2 >0.98 is desirable for precise results (3).

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} ≥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 Mbcr 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):

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

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 DX Kit in combination with validated reagents and instruments (see "Materials Provided", page 9, and "Materials Required but Not Provided", page 11), 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 ($IS\text{-}NCN_{sample}$).

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

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

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

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

Figure 11 shows an example of patient monitoring using NCN and IS-NCN results.

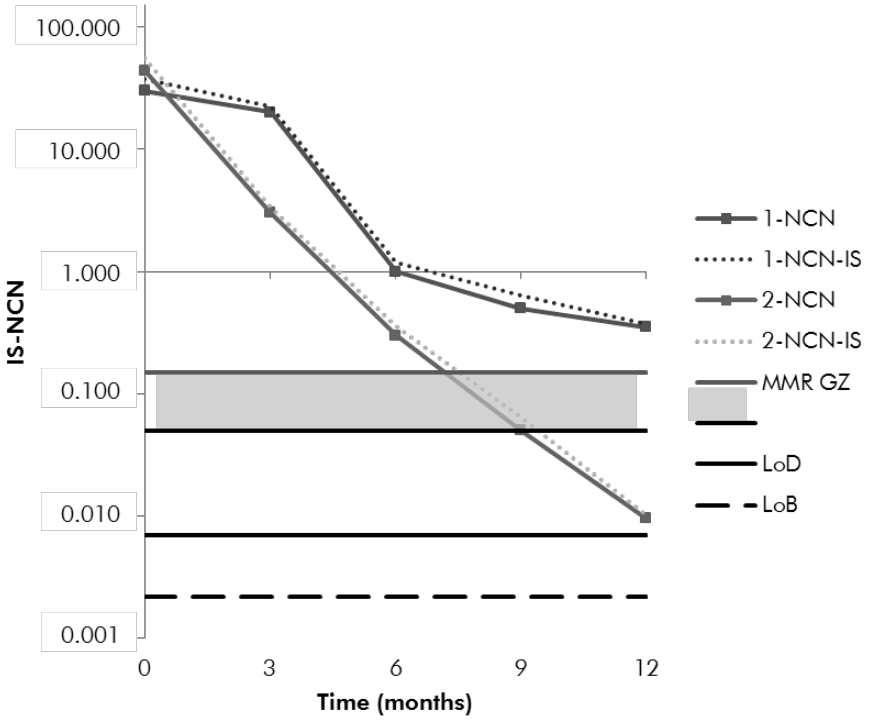


Figure 11. Monitoring curves for patient MMR status with the *ipsogen* BCR-ABL1 Mbcr IS-MMR DX Kit. NCN: normalized copy number; **NCN-IS**: normalized copy number international scale; **MMR GZ**: MMR gray zone (GZ) inconclusive result; **LoD**: limit of detection; **LoB**: background level.

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	≤2 C_T if mean C_T value >36 ≤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 patient 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 42).

Quality Control

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

Limitations

The users must be trained and familiar with this technology prior to the use of this device.

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 which are not covered by the QIAGEN performance studies.

Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

Note: The kit has been designed according to the "Europe Against Cancer" (EAC) studies (8, 9) and is compliant with the updated international recommendations. The kit contains an IS-MMR Calibrator, standardized to the international scale, allowing to convert NCN results to the international scale and report MMR (major molecular response) status.

Each lot of the IS-MMR Calibrator has an assigned value derived directly from a calibration against the NIBSC WHO certified primary reference material (International Genetic Reference Panel for the quantitation of BCR-ABL translocation by RQ-PCR (1st I.S.), ref. 09/138).

A certificate of analysis indicating the assigned value of the IS-MMR Calibrator is provided with each kit.

The kit should be used following the instructions given in this manual, in combination with validated reagents and instruments (see "Materials Required but Not Provided", page 11). Any off-label use of this product and/or modification of the components will void QIAGEN's liability.

Performance Characteristics

Note: The performance characteristics were established using the Applied Biosystems 7500 Real-Time PCR System in combination with the *ipsogen* BCR-ABL Mbcr IS-MMR Kit and validated additional reagents (see "Materials Required but Not Provided", page 11).

Limit of blank and limit of detection

Limit of blank (LoB) and limit of detection (LoD) were determined following CLSI/NCCLS EP17-A guideline.

The background level (LoB) was determined on negative samples from healthy donors (11 samples, 69 measurements), and was found to be equal to 0.0022 BCR-ABL Mbcr NCN.

The limit of detection (LoD or analytical sensitivity) was determined on known low positive samples (n = 8, 74 measurements), and was found to be equal to 0.0069 BCR-ABL Mbcr NCN.

- **NCN ≤LoB**: BCR-ABL Mbcr not detected
- **LoB <NCN <LoD**: BCR-ABL Mbcr detected but not quantified
- **NCN ≥LoD**: BCR-ABL Mbcr quantified

Linearity

Linearity was determined following CLSI/NCCLS EP6-A guideline.

The study was performed on mixtures of positive and negative RNA extracted from cell lines. Eleven different levels were tested in triplicates. The results obtained on these samples show that the *ipsogen* BCR-ABL Mbcr IS-MMR assay is linear in a range from 0.003 to 65 BCR-ABL Mbcr NCN.

Inputs

Five different RNAs with various NCN BCR-ABL Mbcr levels were selected for the study. Different RNA and cDNA amounts were tested to evaluate the input impact on NCN results. Results showed that the RNA input variation had a limited impact on NCN results, while cDNA input was a more sensitive factor if more or less material is used. As a consequence, an input of 1 µg of RNA and 5 µl of cDNA are recommended to run the test.

Precision

Precision was determined following CLSI/NCCLS EP5-A2 guideline.

The precision study was performed on 13 different samples tested 42 times in duplicates (n = 84). These samples were representative of the different level of BCR-ABL Mbcr expression in patients' samples around and above the MMR value. The global coefficient of variation around the MMR value was found to be equal to 25%.

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

The most recent working definitions of BCR-ABL1 Mbcr molecular response in CML are given by ELN/EUTOS Molecular Monitoring Steering Group, recommending the use of the ERM-AD623 BCR-ABL1 plasmid (IRMM, Belgium): Cross, N.C., et al. Laboratory recommendations for scoring deep molecular responses following treatment for chronic myeloid leukemia (2015) Leukemia. **29**, 999.

To comply with this recommendation, QIAGEN conducted a concordance study to compare the *ipsogen* multitarget single plasmid, used in *ipsogen* BCR-ABL1 Mbcr IS-MMR Kit (24) CE (cat. no. 670723), to the ERM-AD623 BCR-ABL1 plasmid (IRMM).

The comparison was based on BCR-ABL1 Mbcr/ABL1 normalized copy number ratio (NCN), assessed using either of both standards dilutions (*ipsogen* or ERM-AD623 BCR-ABL1), on control samples included in *ipsogen* kits and on certified reference material from the NIBSC; White, H.E., et al. (2010) Establishment of the first World Health Organization International Genetic Reference Panel for quantitation of BCR-ABL mRNA. Blood **116**, e111.

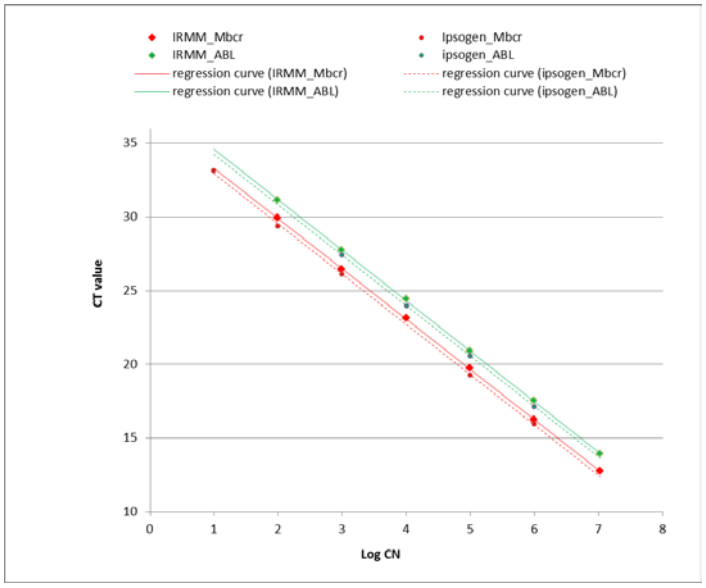
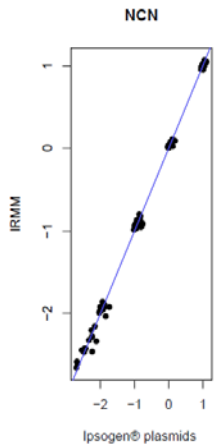


Figure 12. *ipsogen* and ERM-AD623 BCR-ABL1 plasmid standard curves are aligned.



ipsogen BCR-ABL1 Mbcr IS-MMR Kit.

Figure 13. ERM-AD623 BCR-ABL1 versus *ipsogen* NCN values.

The QIAGEN study concludes there is no statistical 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:

	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	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.
ipsogen BCR-ABL1 Mbcr IS-MMR DX Kit (24)	For 24 reactions: Reverse transcriptase, 5x RT buffer, dNTP mix, Random primer, RNase Inhibitor, DTT, qPCR Master mix, Mbcr and ABL Single Plasmid Standards, High RNA Positive Control, IS-MMR Calibrator, ROX I fluorescent dye, ROX II fluorescent dye, Primers and Probe Mix ABL, Primers and Probe Mix BCR-ABL Mbcr Fusion Gene	670823
Rotor-Gene Q MDx – for IVD-validated real-time PCR analysis in clinical applications		
Rotor-Gene Q MDx 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	9002032
Rotor-Gene Q MDx 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	9002033
RNeasy Kits – for purification of total RNA		
RNeasy Mini Kit (50)	For 50 RNA preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
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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