IVD

For In Vitro Diagnostic use

CE

BCR-ABL Mbcr Fusion*Quant*® Kit for the Real-Time Quantitative PCR Analysis of BCR-ABL Mbcr p210 Transcripts

Kit for the quantitative detection of BCR-ABL Mbcr p210 fusion gene transcripts using ABI Prism TaqMan®, LightCycler®, SmartCycler® or RotorGene™ instruments

Ref: FQPP-10-CE

Tests:	ABI* & LC480: (*7000, 7700 & 7900)	24 samples in duplicate in 3 distinct experiments
	LightCycler®*: (*1.2 and 2.0)	30 samples in duplicate in 6 distinct experiments
	SmartCycler®:	30 samples in duplicate in 6 distinct experiments
	RotorGene™:	24 samples in duplicate in 3 distinct experiments

Store at **-25°C to -15°C**

N.B. Store Primers & Probe Mixes (PPC and PPF tubes) in the dark

Instructions for use

Version 13, September 2008

IPSOGEN CANCER PROFILER

Mbcr BCR-ABL Fusion*Quant*®

Kit for the quantitative detection of BCR-ABL Mbcr fusion gene transcripts using either ABI Prism TaqMan®, LightCycler®, SmartCycler® or RotorGene™ instruments

Instructions for use

Ref: FQPP-10-CE

1. Intended Use

BCR-ABL Mbcr Fusion*Quant*® Kit is intended for the accurate quantification of BCR-ABL p210 transcripts in bone marrow or peripheral blood samples of Acute Lymphoblastic Leukaemia (ALL) or Chronic Myeloid Leukemia (CML) patients previously diagnosed with a BCR-ABL Mbcr Fusion Gene (FG) event. The results obtained can be used to monitor efficiency of treatment in patients undergoing therapy and for Minimal Residual Disease (MRD) follow-up to monitor disease relapse.

Warnings: This kit has been designed according to the "Europe Against Cancer" studies (Gabert et al., Leukemia 2003), and is compliant with the updated international recommendations (Branford et al., Leukemia 2006 ; Hugues et al., Blood 2006). It should be used following the instructions given in this manual, in combination with validated instruments and reagents. Any off label use of this product, and/or modification of the components will void IPSOGEN's liability.

2. Background

Most cases of CML are associated with the presence of t(9;22) resulting in a small derivative chromosome 22 known as the Philadelphia chromosome (Ph). Consequently, the ABL proto-oncogene on chromosome 9 is fused to the BCR gene on chromosome 22. In CML patients and approximately 35% of Ph-positive ALL adult patients, the breakpoint on chromosome 22 is located between exons 12 and 16 of the BCR gene, in the so-called major breakpoint cluster region (Mbcr). The breakpoint on chromosome 9 is located in most cases between exons 1 and 2 in the ABL gene. The transcription product of this BCR-ABL fusion gene is an 8.5-kb aberrant fusion RNA with two junction variants b2a2 and/or b3a2 that gives rise to the BCR-ABL chimeric protein (p210), a tyrosine kinase with deregulated activity (see figure 1). Rare cases with b2a3 and b3a3 BCR-ABL transcripts can be observed.

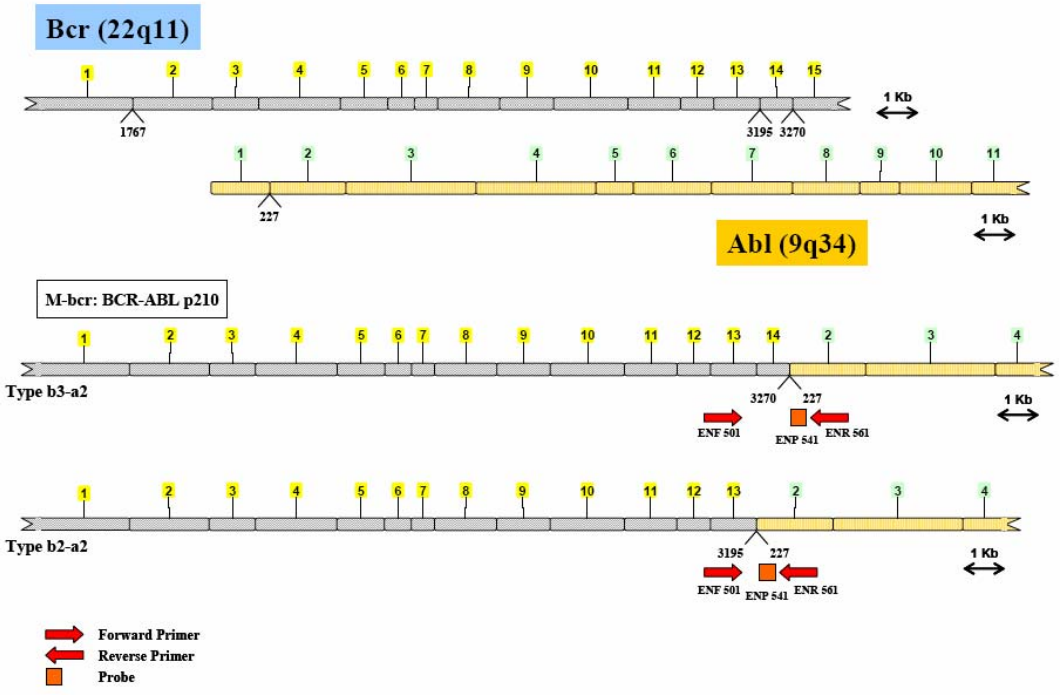


Figure 1: *Schematic diagram of the BCR-ABL FG transcript covered by the RQ-PCR primers and probe set. For Mbcr, set: ENF501–ENP541–ENR561. The number under the primers and probe refers to their nucleotide position in the normal gene transcript.*

Therapy of Ph+ CML patients has been "revolutionized" by the introduction of imatinib mesylate (IM, Glivec®, Novartis), which was used first in the clinic in 1998. IM is a tyrosine kinase inhibitor that inhibits the BCR-ABL tyrosine kinase, the constitutive abnormal tyrosine kinase created by the Philadelphia chromosome. This

treatment allows achieving 89% of overall survival after 5 years. In the IRIS trial, complete cytogenetic responses (CCyR i.e. 0% Ph+ metaphases) among patients receiving IM were 69% by 12 months and 87% by 60 months. The molecular responses (i.e. no BCR-ABL transcripts detected) at 12 and 18 months were also associated with long-term outcomes (Druker et al, NEJM 2006).

Since the frequency of CCgR is very high in IM-treated patients, it is necessary to measure the level of the BCR-ABL transcripts to determine minimal residual disease (MRD, figure 2).

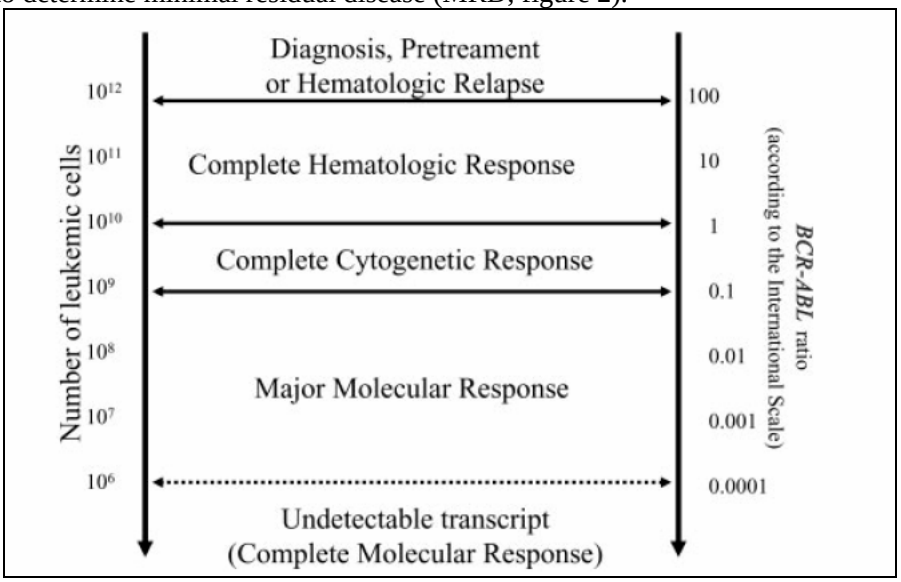


Figure 2: *From (Baccarani et al., Blood 2006). Approximate relationship between response, the putative number of leukemic cells, and the level of BCR-ABL transcripts.*

The current methodology to measure the MRD level involves using real-time quantitative polymerase chain reaction (RQ-PCR), whereby the BCR-ABL transcript numbers are related to transcript numbers of a control gene. The BCR-ABL Mbcr Fusion*Quant*® kit is based on this technique.

International recommendations for the management of CML patients are summarized in the figure bellow:

	Hematologic response	Cytogenetic response	Molecular response (BCR-ABL to control gene ratio according to the international scale)
Definitions	Complete: Platelet count $< 450 \times 10^9$/L; WBC count $< 10 \times 10^9$/L; differential without immature granulocytes and with less than 5% basophils; nonpalpable spleen	Complete: Ph^+ 0% Partial: Ph^+ 1%-35% Minor: Ph^+ 36%-65% Minimal: Ph^+ 66%-95% None: $Ph^+ > 95\%$	"Complete" indicates transcript nonquantifiable and nondetectable Major: ≤ 0.10
Monitoring	Check every 2 wk until complete response achieved and confirmed, then every 3 mo unless otherwise required	Check at least every 6 mo until complete response achieved and confirmed, hence at least every 12 mo	Check every 3 mo; mutational analysis in case of failure, suboptimal response, or transcript level increase

Complete HR, complete CgR, and major MolR should be confirmed on 2 subsequent occasions. CgR is evaluated by morphologic cytogenetics of at least 20 marrow metaphases. FISH of peripheral blood cells should be used only if marrow cells cannot be obtained. MolR is assessed on peripheral blood cells. The international scale for measuring MolR is that proposed by Hughes et al.[189]

Figure 3: *From (Baccarani et al., Blood 2006). Response definition and monitoring.*

3. Technological Principle

RQ-PCR 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 RQ-PCR techniques are available: RQ-PCR analysis using SYBR Green I Dye, RQ-PCR analysis using hydrolysis probes and RQ-PCR analysis using hybridisation probes.

This assay exploits the RQ-PCR Double Dye Oligonucleotide Hydrolysis principle. During PCR, forward and reverse primers hybridise to a specific sequence product. A Double Dye Oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labelled with a 5' reporter dye and a downstream, 3'quencher dye, hybridises to a target sequence within the PCR product. RQ-PCR analysis with hydrolysis probes exploits the 5'-3' exonuclease activity of the Thermus aquaticus (Taq) 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´ to 3´ exonuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher only if the probe hybridises 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 4). 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, non-specific amplification is not detected. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

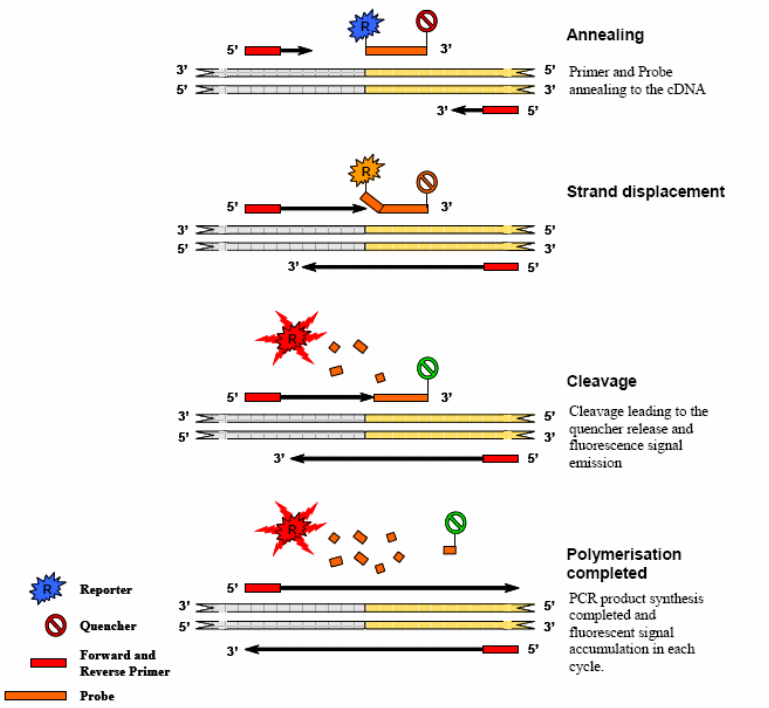


Figure 4: *Total RNA is reverse transcribed and the generated cDNA amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM-TAMRA). The probe binds to the amplicon during each annealing step of the PCR. When the Taq extends from the primer bound to the amplicon it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the Taq 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 the TAMRA.*

In our BCR-ABL Mbcr FusionQuant® Kit, an endogenous control (ABL transcript) is amplified from the sample as well as the Mbcr fusion transcript. Standard curves of known amounts of both the endogenous ABL control and the Mbcr fusion cDNA allow the calculation of the ratio of Mbcr fusion transcript signal to endogenous ABL signal in each sample. Specific primers and probe mixes and standard serial dilutions of control and fusion DNA are provided for the quantification of the ABL control and BCR-ABL Mbcr genes.

4. Technological Specifications

The BCR-ABL Mbcr Fusion*Quant*® Kit was developed according to the Europe Against Cancer (EAC) network protocol (Gabert et al., Leukemia 2003).

Performance evaluation has been performed on a 7700 ABI Prism TaqMan® instrument, in combination with reagents listed in paragraph 5.2. Equivalence studies validated its use on the following instruments: ABI 7000 and 7900, LightCycler (1.2 and 480), RotorGene and SmartCycler (Silvy et al., Leukemia 2005).

5. Reagents and Instruments

5.1. Material provided

Vial	ABL Control gene Standard Dilutions	Volume	Part Number
C1-ABL	10^3 copies/5µl	50µl	IP-PF-000050
C2-ABL	10^4 copies/5µl	50µl	IP-PF-000051
C3-ABL	10^5 copies/5µl	50µl	IP-PF-000052

Vial	BCR-ABL Mbcr Fusion gene Standard Dilutions	Volume	Part Number
F1-BCR-ABL Mbcr	10^1 copies/5µl	50µl	IP-PF-000058
F2-BCR-ABL Mbcr	10^2 copies/5µl	50µl	IP-PF-000059
F3-BCR-ABL Mbcr	10^3 copies/5µl	50µl	IP-PF-000060
F4-BCR-ABL Mbcr	10^5 copies/5µl	50µl	IP-PF-000061
F5-BCR-ABL Mbcr	10^6 copies/5µl	50µl	IP-PF-000062

Primers and Probe Mixes	Content	Volume	Part Number
PPC-ABL 25X Amber tube *Supplied ready-to-use*	Mix of specific reverse and forward primers for the ABL control gene plus a specific FAM-TAMRA probe	90µl	IP-PF-000068
PPF-Mbcr 25X Amber tube *Supplied ready-to-use*	Mix of specific reverse and forward primers for the Mbcr fusion gene plus a specific FAM-TAMRA probe	110µl	IP-PF-000070

N.B. Spin the tubes before opening

Using in the ABI instrument, the kit is sufficient to quantify 24 samples in duplicate, with a final reaction volume of 25µl each. The kit is optimised for 3 runs of 52 reactions each (8 samples plus associated controls in duplicate, see experiment design, corresponding to 156 reactions maximum). See sections 6.3 to 6.5 for other instrument protocols.

N.B. Additional runs may result in depletion of some reagents. Taking in account pipetting errors, volumes are provided in excess.

5.1.1. Handling and Storage

Store at -15°C to -25°C in a constant-temperature freezer (N.B. kits are shipped at room temperature but should be stored at -15°C to -25°C immediately on receipt). Keep the Primers & Probe Mixes (PPC and PPF tubes) away from light as this product is photosensitive
Vortex and centrifuge the tubes before opening.
Expiration dates for each reagent are indicated on the individual component labels. These storage conditions apply to both opened and un-opened components. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.
Store all kit components in original containers.

5.1.2. Kit stability

The kit will remain stable until the expiration date printed on the label under correct storage conditions. The product will maintain performance through the expiry date printed on the label.

5.1.3. Quality Control

This kit is manufactured according to ISO 13485:2003 standard, which requires stringency in validation and documentation of manufacturing procedures.
Each batch is tested on a Light Cycler® 480 (Roche). Certificates of Analyses are available upon request at: support@ipsogen.com.

5.1.4. Warnings

The users must have been trained and be familiar with this technology prior to the use of this device. Perform the test according to the “Good Laboratory Practice” (GLP) guidelines for PCR-based diagnostic applications.

5.2. Reagents and material required but not provided

5.2.1. RNA preparation

RNA preparation from patient samples 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 or by using Agilent Bioanalyzer® prior to downstream analysis.

5.2.2. Reagents

Warning: this test does not allow the synthesis of cDNA from purified RNA.
Complementary DNA is obtained from:

- Control cell lines (ref. PCCL-01, 03 and 05 corresponding to High, Low and Negative controls for BCR-ABL Mbcr p210 detection).
- Peripheral blood or bone marrow.

Additional reagents to be provided by the user are:

- Buffer and Taq Polymerase (we recommend the use of the TaqMan® Universal PCR Master Mix, Applied Biosystems® or Light Cycler TaqMan Master mix)
- General Laboratory Equipment
- Specific material and reagents for LightCycler® use
- Nuclease-free PCR grade H_2O

Validated Reagents:

Reagent	**Validated reagent (Brand name)**	**Validated reagent (Provider, reference in Europe)**
Reverse transcriptase (200U/µl)	Superscript™ or Superscript II™	
5X First-Strand Buffer	5X Buffer (supplied with Invitrogen® RT)	
DTT 100 mM	DTT (supplied with Invitrogen® RT)	
RNase Inhibitor (40U/µl)	RnaseOut™.	Invitrogen® # 10777-019
100mM dNTP	Set of dNTP, PCR Grade.	Any
PCR grade H_2O , RNase, DNase free	Any	Any
PCR Master Mix 2X	TaqMan® Universal PCR Master Mix	Applied Biosystems® # 4304437
LightCycler TaqMan Master kit	Freshly prepared 5X Master Mix	Roche # 4535286001

5.2.3. Equipment

To perform the assay, you will need the following equipment:

- Real-time PCR instrumentation (TaqMan® ABI or any equivalent equipment)
- 0.5ml or 0.2ml RNase- and DNase free PCR tubes
- Nuclease free aerosol-resistant sterile PCR pipette tips with hydrophobic filters
- Sterile reaction cups (Eppendorf) to prepare dilutions
- Microcentrifuge equipped for 0.2 ml/0.5ml tubes. Max speed: 13 000 / 14 000 rpm
- Microliter pipettor dedicated for PCR (1-10µl; 10-100µl; 100-1000µl)
- Ice

5.3. Warnings and precautions

N.B. Reagents and instructions supplied in this kit have been validated for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may result in erroneous or discordant data. Differences in sample processing and technical procedures in the user's laboratory may invalidate the assay results. Determining transcript levels using RQ-PCR 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 free conditions.

Use extreme caution to prevent:

- RNAse/DNAse contaminations, that might cause degradation of the template mRNA and the generated cDNA
- mRNA or PCR carry-over contamination resulting in false positive signal

We therefore recommend the following:

- Prepare appropriate aliquots of the kit solutions or additional reagents and keep them separate from other reagents in the laboratory.
- Use nuclease-free labware (e.g. pipettes, pipettes tips, reactions vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipette tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- To avoid carry-over contamination, transfer the required solutions for one experiment into a fresh tube.
- Manipulate the standard dilutions (C1-3 and F1-5) in a separate room.
- Minimise microbial contamination of reagents to avoid non-specific reactions.
- Incubation times, temperatures, or methods other then those specified may give erroneous results.
- Reagents have been optimally diluted. Further dilutions may result in loss of performances or erroneous results.
- PPC and PPF reagents may be altered if exposed to light. Do not store components or perform experiment in strong light, such as direct sunlight.
- Wear appropriate personal protective equipment to avoid contact with eyes and skin. Refer to the Materials Safety Data Sheet (MSDS) for additional information.

- Human tissues must be handled as if capable of transmitting infections and disposed of with proper precautions, and in compliance with OSHA and/or CAP (or EU equivalent) guidelines.
- Never pipette kit reagents by mouth and avoid contact with skin and mucous membranes. If reagents are exposed to sensitive areas, wash thoroughly with copious amount of water and contact a physician.
- All reagents are formulated specifically for use with this test. No substitutions should be made for optimal performance of this test.

6. Instructions for Use

Before starting: the user should read these instructions carefully and become familiar with all components prior to use.

6.1. Recommended Standardised EAC Reverse Transcription protocol

- Thaw all necessary components and place them on ice.
- Incubate 1µg of RNA (1 to 4µl) for 10 min at 70°C and immediately cool on ice for 5 min.
- Spin briefly (~10sec, 10,000 rpm, to collect the liquid in the bottom of the tube) and keep on ice.
- Prepare the following RT pre-mix according to the number of samples being processed:

RT Premix	Vol. for 1 sample	Final Conc.
5X Expand™ reverse transcriptase buffer (first-strand)	4.0 µl	1X
MgCl2 (50 mM)	2.0 µl	5 mM
dNTP (10 mM each, to be prepared previously and stored at -20°C in aliquots)	2.0 µl	1 mM
DTT (100 mM)	2.0 µl	10 mM
RNAse Inhibitor (40 U/µl)	0.5 µl	20 U
Random hexamer	5.0 µl	25 µM
MMLV or Superscript II (200 U)	0.5µl	100 U
RT Premix Volume	16 µl	
Heated Sample RNA to be tested (1 µg)	1 to 4 µl	50 ng/µl
PCR grade nuclease free water	Adjust vol. to 20 µl	

- Mix well and spin briefly (~10sec, 10,000 rpm, to collect the liquid in the bottom of the tube).
- Incubate at 20°C for 10 min.
- Incubate at 42°C on a thermal cycler for 45 min, then immediately at 99°C for 3 min.
- Cool on ice (to stop the reaction) for 5 min.
- Briefly spin (~10sec, 10,000 rpm, to collect the liquid in the bottom of the tube) the obtained cDNA (keep on ice).
- Dilute the final cDNA with 30 µl of H2O. Total volume = 50µl
- Process the following steps according to your RQ-PCR instrument.

6.2. ABI Prism TaqMan® instrument (7000, 7700 and 7900) and LC 480

To test n cDNA samples we recommend measuring the following points in duplicate:
With the ABL Primers & Probe Mix
n cDNA samples n x 2 reactions
ABL standard 6 reactions (3 dilutions, each one tested in duplicate)
Water control 2 reactions
With the BCR-ABL Mbcr Primers & Probe Mix
n cDNA samples n x 2 reactions
Fusion Gene standard 10 reactions (5 dilutions, each one tested in duplicate)
Water control 2 reactions

6.2.1. Sample processing

We recommend testing at least 8 cDNA samples in the same experiment, to optimise the use of the Standards and Primers & Probe mixes. The plate scheme below shows an example of such an experiment:

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C1**	**C1**		**cDNA 1**	**cDNA 1**			***F1***	***F1***		***cDNA 1***	***cDNA 1***
B	**C2**	**C2**		**cDNA 2**	**cDNA 2**			***F2***	***F2***		***cDNA 2***	***cDNA 2***
C	**C3**	**C3**		**cDNA 3**	**cDNA 3**			***F3***	***F3***		***cDNA 3***	***cDNA 3***
D	**H2O**	**H2O**		**cDNA 4**	**cDNA 4**			***F4***	***F4***		***cDNA 4***	***cDNA 4***
E				**cDNA 5**	**cDNA 5**			***F5***	***F5***		***cDNA 5***	***cDNA 5***
F				**cDNA 6**	**cDNA 6**			***H2O***	***H2O***		***cDNA 6***	***cDNA 6***
G				**cDNA 7**	**cDNA 7**						***cDNA 7***	***cDNA 7***
H				**cDNA 8**	**cDNA 8**						***cDNA 8***	***cDNA 8***

with PPC-ABL ***with PPF-Mbcr***

N.B. Each BCR-ABL Mbcr Fusion*Quant*® Kit provides enough reagents to perform this 8 cDNA samples experiment 3 times.

6.2.2. RQ-PCR for ABI and LC480 instruments

- Thaw all necessary components and place them on ice.
- Prepare the following RQ-PCR premix according to the number of samples being processed:

RQ-PCR premix Reagents	Final Conc.	1 Reaction	26 Reactions	30 Reactions
TaqMan Universal PCR Master Mix 2X Applied Biosystems (Not Provided)	1X	12.5 µl	325 µl	375 µl
IPSOGEN Primers & Probe mix 25X	1X	1 µl	26 µl	30 µl
Adjust vol. to 20 µl with nuclease-free H_2O		6.5 µl	169µl	195 µl
	Total volume	= 20 µl	520 µl	600 µl
Material to be quantified (Sample cDNA, Standard or Water control)		5 µl		

All mentioned concentrations are for the final volume of the reaction.

The above table describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final PCR reaction volume of 25µl. A pre-mix can be prepared, according to the number of reactions using the same Primer & Probe mix (either PPC-ABL or PPF-Mbcr).

To be performed on ice:

- Dispense 20 µl of the RQ-PCR pre-mix well.
- Add 5 µl of the RT product (cDNA, 100ng RNA equivalent) obtained in step 6.1 above in the corresponding well (total volume 25µl).
- Mix gently, by pipetting up and down.
- Place the plate in the thermal cycler.
- Run the following program:

RQ-PCR program		
Temperature	**Time**	**Cycles**
50°C	2 min	**X 1**
95°C	10 min	**X 1**
95°C	15 sec	**X 50**
60°C	1 min	

We recommend a threshold set at 0.1 as describe in the EAC protocol in the analysis step on the ABI Prism® 7700 and 7900 instruments.

6.3. LightCycler® instruments (1.2 & 2.0)

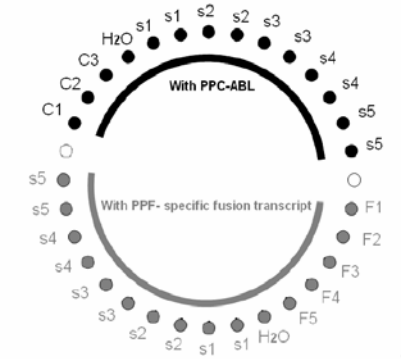
To test n cDNA samples we recommend measuring cDNA samples in duplicate:

With the ABL Primers & Probe Mix
n cDNA samples n x 2 reactions
ABL standard 3 reactions (3 standard dilutions)
Water control 1 reaction

With the Fusion transcript Primers & Probe Mix
n cDNA samples n x 2 reactions
Fusion Gene standard 5 reactions (5 standard dilutions)
Water control 1 reaction

6.3.1. Sample processing.

We recommend testing at least 5 cDNA samples in the same experiment, to optimise the use of the standards and Primers & Probe mixes. The capillaries scheme below shows an example of experiment:



S = cDNA sample

N.B. Each BCR-ABL Mbcr Fusion*Quant*® Kit provides enough reagents to perform this 5 cDNA sample experiment 6 times.

6.3.2. RQ-PCR For LightCycler® instruments

N.B. Because of particular technological requirements, LightCycler experiments must be performed using specific reagents. We recommend to use the LightCycler® TaqMan Master Kit and to follow the manufacturer's instructions to prepare the Master Mix 5X.

- Thaw all necessary components and place them on ice.
- Prepare the following RQ-PCR premix according to the number of samples being processed:

RQ-PCR premix Reagents	Final Conc.	1 Reaction	16 Reactions	18 Reactions
Freshly prepared Master Mix 5X LightCycler TaqMan Master (Not Provided)	1X	4 µl	64 µl	72 µl
IPSOGEN Primers & Probe mix 25X	1X	0.8 µl	12.8 µl	14.4 µl
Adjust vol. to 15 µl with nuclease-free H_2O		10.2 µl	163.2 µl	183.6 µl
	Total volume	= 15 µl	240 µl	270µl
Material to be quantified (Sample cDNA, Standard or Water control)		5 µl		

Ready-to-use dilution of the IPSOGEN standard.
All mentioned concentrations are for the final volume of the reaction.
The above table describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final PCR reaction volume of 20µl. A pre-mix can be prepared, according to the number of reactions using the same Primers & Probe mix (either PPC-ABL or PPF-Mbcr).

To be performed on ice:

- Dispense 15 µl of the RQ-PCR pre-mix per capillary.
- Add 5 µl of the RT product (cDNA, 100ng RNA equivalent) obtained in step 6.1 above in the corresponding capillary (total volume 20µl).
- Mix gently, by pipetting up and down.
- Load the samples in the apparatus according to the manufacturer recommendations.
- Run the following PCR program: (recommended on a LightCycler® Instrument):

LightCycler RQ-PCR program for TaqMan Probe				
Temperature	**Time**	**Cycles**	**Ramp**	**Acquisition**
95°C	10 min	**X 1**	**20**	**None**
95°C	10 sec	**X 50**	**20**	**None**
60°C	1 min		**20**	**Single**
45°C	1 min	**X 1**	**20**	**None**

We recommend using Automated (F''max) analysis on LightCycler II software version 4.0 to obtain reproducible results.

6.4. SmartCycler® instrument

To test n cDNA samples we recommend measuring the following points in duplicate:

With the ABL Primers & Probe Mix
n cDNA samples n x 2 reactions
ABL standard 3 reactions (3 different dilutions)
Water control 1 reaction

With the Fusion transcript Primers & Probe Mix
n cDNA samples n x 2 reactions
Fusion Gene standard 5 reactions (5 different dilutions)
Water control 1 reaction

6.4.1. Sample processing

We recommend testing at least 5 cDNA samples in the same experiment, to optimise the use of the standards and Primers & Probe mixes. The two block scheme below shows an example:

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**All the assays on this first block are performed with PPC-ABL**
C1	**C2**	**C3**	**H20**	**cDNA 1**	**cDNA 1**	**cDNA 2**	**cDNA 2**	
9	**10**	**11**	**12**	**13**	**14**	**15**	**16**	
cDNA 3	**cDNA 3**	**cDNA 4**	**cDNA 4**	**cDNA 5**	**cDNA 5**			

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**All the assays on this second block are performed with PPF - " Mbcr Fusion Gene"**
F1	**F2**	**F3**	**F4**	**F5**	**H20**	**cDNA 1**	**cDNA 1**	
9	**10**	**11**	**12**	**13**	**14**	**15**	**16**	
cDNA 2	**cDNA 2**	**cDNA 3**	**cDNA 3**	**cDNA 4**	**cDNA 4**	**cDNA 5**	**cDNA 5**	

N.B. Each BCR-ABL Mbcr Fusion*Quant*® Kit provides enough reagents to perform this 5 cDNA samples experiment 6 times.

6.4.2. RQ-PCR For SmartCycler® instrument

- Thaw all necessary components and place them on ice.
- Prepare the following RQ-PCR premix according to the number of samples being processed:

RQ-PCR premix Reagents	Final Conc.	1 Reaction	16 Reactions	18 Reactions
PCR Master Mix 2X (Not Provided)	1X	12.5 µl	200 µl	225 µl
IPSOGEN Primers & Probe mix 25X	1X	1 µl	16 µl	18 µl
Adjust vol. to 20 µl with nuclease-free H_2O		6.5 µl	104 µl	117 µl
	Total volume	= 20 µl	320 µl	360 µl
Material to be quantified (Sample cDNA, Standard or Water control)		5 µl		

All mentioned concentrations are for the final volume of the reaction.
The above table describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final PCR reaction volume of 25µl. A pre-mix can be prepared, according to the number of reactions using the same Primers & Probe mix (either PPC-ABL or PPF-Mbcr).
To be performed on ice:

- Dispense 20 µl of the RQ-PCR pre-mix per well.
- Add 5 µl of the RT product (cDNA, 100ng RNA equivalent) obtained in step 6.1 above in the corresponding well (total volume 25µl).
- Mix gently, by pipetting up and down.
- Load the samples in the apparatus according to the manufacturer recommendations.
- Run the following program:

RQ-PCR program		
Temperature	**Time**	**Cycles**
50°C	2 min	**X 1**
95°C	10 min	**X 1**
95°C	15 sec	**X 50**
60°C	1 min	

We recommend a threshold set at 30 on a SmartCycler® instrument.

6.5. Corbett RotorGene™ 3000 instrument

To test n cDNA samples we recommend measuring the following points in duplicate:

With the ABL Primers & Probe Mix
n cDNA samples n x 2 reactions
ABL standard 6 reactions (3 dilutions, each one tested in duplicate)
Water control 2 reactions

With the 'Fusion Gene' Primers & Probe Mix
n cDNA samples n x 2 reactions
Fusion Gene standard 10 reactions (5 dilutions, each one tested in duplicate)
Water control 2 reactions

6.5.1. Sample processing.

We recommend testing at least 8 cDNA samples in the same experiment, to optimise the use of the Standards and Primers & Probe mixes.
N.B. Each BCR-ABL Mbcr Fusion*Quant*® Kit provides enough reagents to perform an 8 cDNA samples experiment 3 times using the 72 Tubes Rotor.

6.5.2. RQ-PCR for RotorGene™ 3000 instrument

- Thaw all necessary components and place them on ice.
- Prepare the following RQ-PCR premix according to the number of samples being processed:

RQ-PCR premix Reagents	Final Conc.	1 Reaction	26 Reactions	30 Reactions
TaqMan Universal PCR Master Mix 2X (Not Provided) Applied Biosystems	1X	12.5 µl	325 µl	375 µl
IPSOGEN Primers & Probe mix 25X	1X	1 µl	26 µl	30 µl
Adjust vol. to 20 µl with nuclease-free H_2O		6.5 µl	169µl	195 µl
	Total volume	= 20 µl	520 µl	600 µl
Material to be quantified (Sample cDNA, Standard or Water control)		5 µl		

All mentioned concentrations are for the final volume of the reaction.
The above table describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final PCR reaction volume of 25µl. A pre-mix can be prepared, according to the number of reactions using the same Primer & Probe mix (either PPC-ABL or PPF-Fusion Gene).
To be performed on ice:

- Dispense 20 µl of the RQ-PCR pre-mix per tube
- Add 5 µl of the RT product (cDNA, 100ng RNA equivalent) obtained in step 6.1 above in the corresponding tube (total volume 25µl).
- Mix gently, by pipetting up and down.
- Place the tubes in the thermal cycler.
- Run the following program:

RQ-PCR program		
Temperature	**Time**	**Cycles**
50°C	2 min	**X 1**
95°C	10 min	**X 1**
95°C	15 sec	**X 50**
60°C	1 min	

We recommend using Automatic threshold analysis on RotorGene™ 3000 instrument to obtain reproducible results.

7. Test Interpretation

7.1. Data analysis principle

In the TaqMan® technology, the number of PCR cycles necessary to detect a signal above the threshold is called the Cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the reaction. We recommend a threshold set at 0.1 on a TaqMan® machine, and a baseline set between cycles 3 and 15.

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. IPSOGEN's standard curves are plasmid-based; we use three plasmid standard dilutions for the CG, and five standard dilutions for the FG, in order to ensure accurate standard curves.

The figures below show an example of TaqMan® results obtained with IPSOGEN BCR-ABL Mbcr Fusion*Quant*® Kit:

Figure 3 :

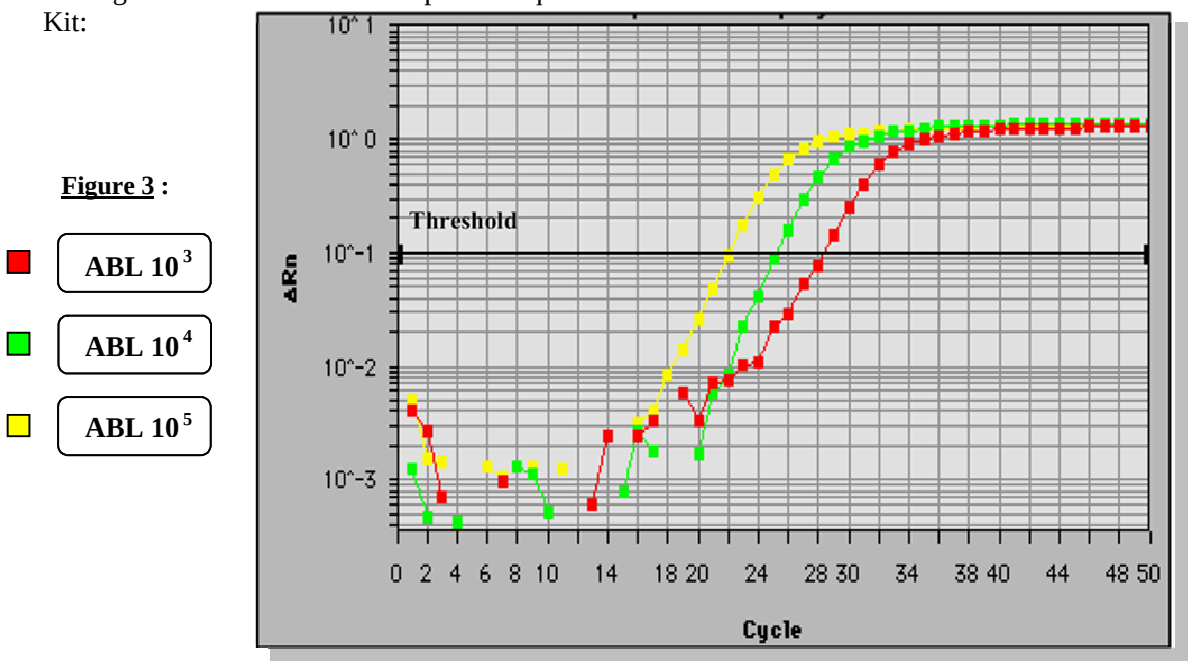
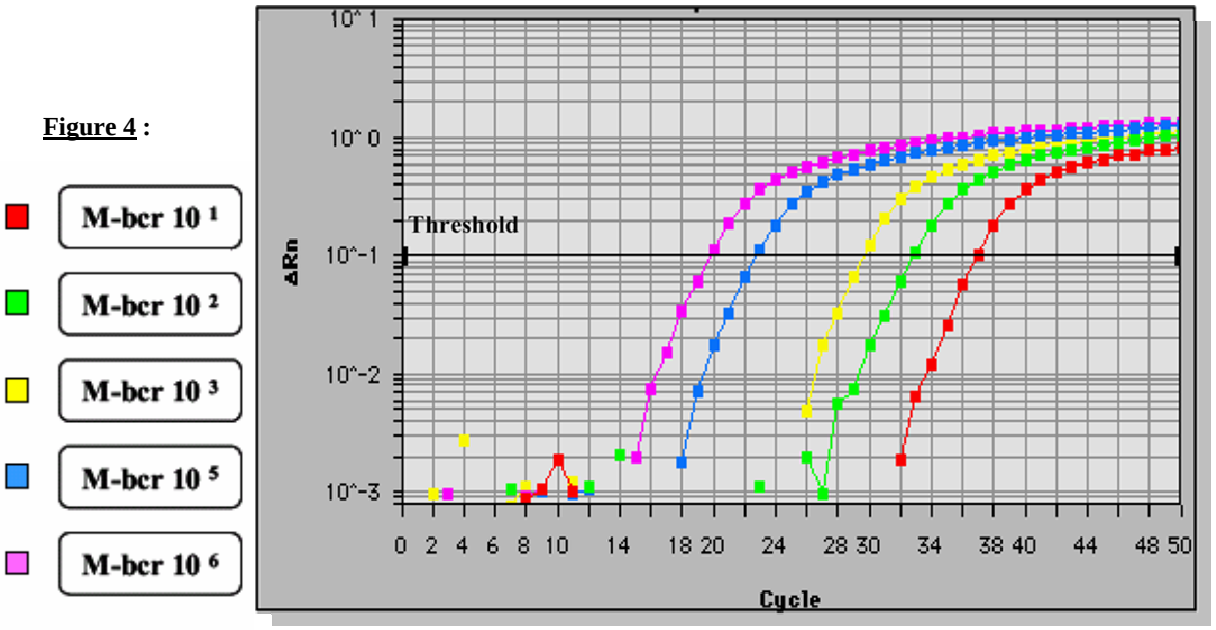


Figure 4 :

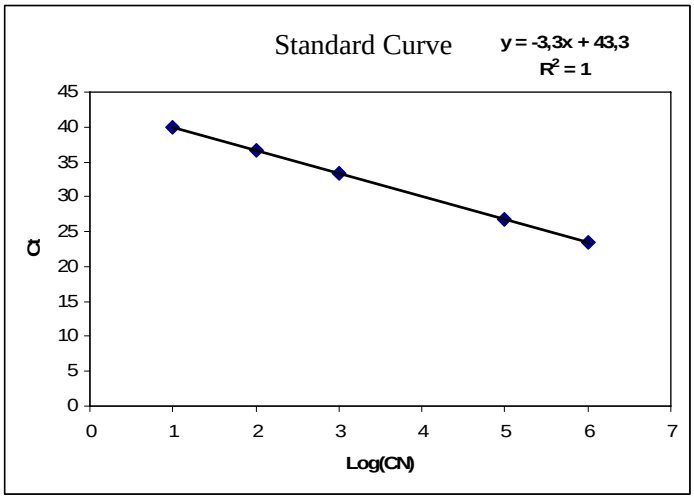


7.2. Expression of the results

Results are relative to approximately 100 ng of total RNA, corresponding to 5 µl ($1/10^{th}$) of the RT reaction.

7.2.1. Standard curve and quality criteria

Raw data can be pasted in an Excel file to be analysed.
For each gene (ABL and BCR-ABL), raw Ct values obtained on plasmid standard dilutions are plotted according to the Log Copy Number (3, 4 and 5 for C1, C2 and C3; 1, 2, 3, 5 and 6 for F1, F2, F3, F4 and F5). The figure below shows an example of the theoretical curve calculated on 5 standard dilutions. A linear regression curve ($y = ax + b$) is calculated for each gene (ABL and BCR-ABL), 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. According to Van der Velden et al., Leukemia 2003, a slope between -3.0 and -3.9 is acceptable as long as the r^2 is > 0.95. However, a value for r^2 of greater than 0.98 is desirable for precise results (Branford et al., Leukemia 2006).
- The F1 standard should be detected and taken into account in the curve calculation.

The ABL standard curve equation should be used to transform raw Ct values (obtained with ABL-PPC) for the unknown samples, into ABL copy numbers (ABL_{CN}).
The BCR-ABL standard curve equation should be used to transform raw Ct values (obtained with BCR-ABL-PPF) for the unknown samples, into BCR-ABL copy numbers ($BCR\text{-}ABL_{CN}$).
The ratio of these CN values gives the normalised copy number (NCN):

$$NCN = BCR\text{-}ABL_{CN} \ / \ ABL_{CN}$$

The minimal residual disease (MRD) value is the ratio between the CG normalized expression of the FG in follow-up $(FG_{CN}/CG_{CN})_{FUP}$ and diagnostic samples $(FG_{CN}/CG_{CN})_{DX}$.

$$MRD\ value\ (MRDv) = (FG_{CN} / CG_{CN})_{FUP} / (FG_{CN}/CG_{CN})_{DX}$$

SENSv is calculated according to the relative expression of the FG at diagnosis $(FG_{CN}/CG_{CN})_{DX}$ and CG expression ($CG_{CN,FUP}$) in the follow-up sample.

$$Sensitivity\ (SENSv) = CG_{CN,DX} / (CG_{CN,FUP} \times FG_{CN,DX})$$

7.2.2. Quality control on ABL values

Poor quality of the RNA or troubles during the RQ-PCR steps result in low ABL_{CN}. We recommend discarding results from samples giving $ABL_{CN} < 4246.2$ (lower value of the 95% CI from CML patient samples in the EAC study, Gabert et al., Leukemia 2003).

7.2.3. Reproducibility between replicates

The variation in Ct values between replicates should be less than 2, corresponding to a 4-fold change in copy number values.
According to Van der Velden et al., Leukemia 2003, the variation in Ct values between replicates is generally less than 1.5 if the mean Ct value of the replicates is below 36.
N.B. Each user should measure their own reproducibility in their laboratory.

7.2.4. H_2O controls

Negative controls should give zero CN.
A positive H2O control results from a cross-contamination. See « Troubleshooting Guide » at the end of the document to find a solution.

7.2.5. Positive (high and low) and negative controls (optional)

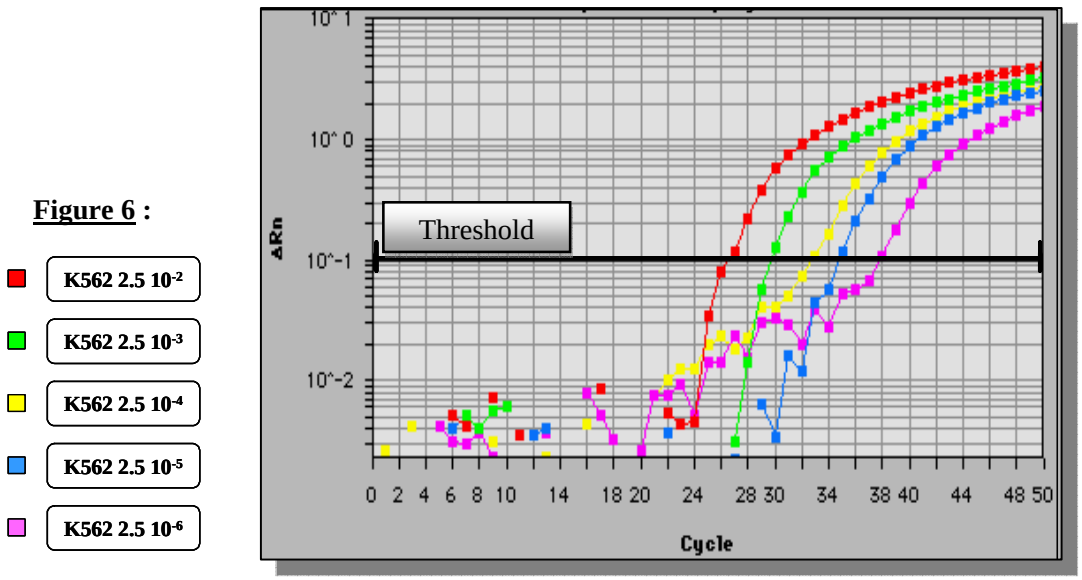
We recommend the use of PCCL-01, 03 and 05 controls which allow monitoring RNA extraction and reverse transcription steps for BCR-ABL Mbcr transcripts detection.

8. Performance Characteristics: Non-clinical studies

8.1. Material and method

Non-clinical studies were conducted to establish the analytical performance of the BCR-ABL Mbcr Fusion*Quant*® Kit. These non-clinical laboratory studies were performed on total RNA from K562 cell line diluted in constant final amount of MV4-11 cell line total RNA.
To determine the repeatability of the assay five different concentrations of K562 total RNA (5ng, 500pg, 50pg, 5pg and 0.5pg) diluted in MV4-11 total RNA, in a constant final total amount of 200ng, were analysed in 5 replicates per run and in 4 different runs.

Figure 6 :



Amplification plots of $2.5x10^{-2}$ (5ng), $2.5x10^{-3}$ (0.5ng), $2.5x10^{-4}$ (0.05ng), $2.5x10^{-5}$ (0.005ng) and, $2.5x10^{-6}$ (0.0005ng) dilution of K562 total RNA in MV4-11 negative total RNA.

8.2. Analytical data

		Inter-Assay analysis			
Cell lines	**BCR-ABL Mbcr**	**Mean Ct**	**SD**	**n**	**CV(%)**
	2.5 10^{-2} (5ng/200ng)	26,18	0,40	20,00	1,54
	2.5 10^{-3} (0.5ng/200ng)	29,32	0,53	19,00	1.82
	2.5 10^{-4} (0.05ng/200ng)	32,62	0,62	20,00	1.91
	ABL	23,59	0,20	95,00	0,83

Plasmids	**BCR-ABL Mbcr**	**Mean Ct**	**SD**	**n**	**CV(%)**
	F1- 10^{1} copies	34,47	1,25	8,00	3,64
	F2- 10^{2} copies	31,48	0,54	8,00	1,71
	F3- 10^{3} copies	28,17	1,11	7,00	3,95
	F4- 10^{5} copies	21,20	0,65	8,00	3,06
	F5- 10^{6} copies	18,22	0,09	6,00	0,49
	ABL	**Mean Ct**	**SD**	**n**	**CV(%)**
	C1- 10^{3} copies	28,47	0,34	8,00	1,18
	C2- 10^{4} copies	25,25	0,31	8,00	1,22
	C3- 10^{5} copies	21,92	0,70	8,00	3,19

		Inter-Assay analysis			
Cell lines	**BCR-ABL Mbcr**	**Mean CN**	**SD**	**n**	**CV(%)**
	2.5 10^{-2} (5ng/200ng)	4134,27	2512,40	20	60,77
	2.5 10^{-3} (0.5ng/200ng)	512,8	479,51	19	93,51
	2.5 10^{-4} (0.05ng/200ng)	42,94	22,05	20	51,36
	ABL	**33831,51**	**13637,7**	**94**	**40,31**

		Inter-Assay analysis			
Cell lines	**BCR-ABL Mbcr**	**Mean NCN***	**SD**	**n**	**CV(%)**
	2.5 10^{-2} (5ng/200ng)	1263,38	532.79	20	42,17
	2.5 10^{-3} (0.5ng/200ng)	116,05	94,69	19	81,61
	2.5 10^{-4} (0.05ng/200ng)	17,82	10,73	20	60,23

* NCN = (Mbcr CN / (ABL CN /10000))

9. Performance Characteristics: Clinical Studies

A group of 26 laboratories, in 10 European countries, organised in a Europe Against Cancer (EAC) concerted action used plasmids provided by IPSOGEN to establish a standardised protocol for RQ-PCR analysis of the major leukemia-associated fusion genes in the clinical setting. The BCR-ABL p210 transcript was one of the Fusion Genes included in the study. We present here a summary of this validation study; full results have been published (Beillard et al., Gabert et al., Leukemia 2003).

9.1. Inter laboratory reproducibility for CG and FG plasmids standards

Eleven laboratories performed an inter-laboratory reproducibility experiment to assess variability in the measurement of CG and FG plasmid standard dilutions. Dilutions were performed in duplicate at each facility. The following table reports the mean, standard deviation, and CV (%) for each dilution.

ABL Control Gene			
	Ct		
	Mean	**SD**	**CV(%)**
C1	29.59	1.34	4.54
C2	26.33	1.02	3.90
C3	22.75	1.59	6.97

BCR-ABL p210 Fusion Gene			
	Ct		
	Mean	**SD**	**CV(%)**
F1	41.11	2.26	5.50
F2	37.43	1.51	4.04
F3	33.76	1.28	3.81
F4	26.50	1.03	3.90
F5	22.98	0.97	4.21

Ct: Cycle threshold; SD: standard deviation; CV: Coefficient of variation

9.2. Expression values of the BCR-ABL Mbcr FG transcript in cell line and CML patients at diagnosis

Expression values of the BCR-ABL Mbcr FG transcript and ABL CG, for K562 cell line, CML and ALL patients at diagnosis and normal patients:

	Ct values	
	BCR-ABL Mbcr	**ABL**
K562 cell line	20.5	20.7
CML Patient samples		
BM (n=15)	25.1 [21.5 – 27.0]	25.2 [20.7 – 26.8]
PB (n=14)	23.1 [21.9 – 25.8]	23.7 [22.6 – 26.7]
ALL Patient samples		
BM and PB (n=17)	24.1 [21.5 – 29.9]	24 [21.6 – 26.4]
Negative patient samples		
BM (n=26)		25.35 [24.68 – 26.02]
PB (n=74)		25.15 [24.83 – 25.48]

ABL Ct values did not differ significantly between normal and leukemia samples, nor between samples types (PB or BM) or leukemia samples (ALL, AML, CML).

	CN values [95% Range]		**NCN values [95% Range]**
	Mbcr	**ABL**	**CN Mbcr/ CN ABL**
CML Patient samples			
BM (n=15)	8710 [2089-112202]	10115.8 [4786.3-37153.52]	0.86 [0.44– 3.02]
PB (n=14)	17783 [2042-112202]	15237 [4246.2-25568.3]	1.17 [0.48 – 4.41]
Negative patient samples			
BM (n=26)		19201 [12922–25480]	
PB (n=74)		21136 [17834–24437]	

9.3. False positive and false negative rates

False negative and false positive rates were computed using the following method:
Positive controls: K562 cells, a cell line well known for its positivity for BCR-ABL p210 fusion gene; patients samples already assessed for p210 positivity.
Negative controls: negative RNA samples, no amplification controls (NAC) made of E. Coli RNA instead of human RNA to check for PCR contamination, and no template controls (NTC) which contained water instead of human RNA.
Amplification on RNA samples of the FG was done in triplicate and in duplicate for the CG.
A false-negative sample was defined as a positive RNA sample with less than 50% of positive wells (0/2, 0/3 or 1/3).
A false-positive sample was defined as a negative sample with at least 50% of positive wells (1/2, 2/3 or 3/3).

False negativity		False positivity	
10^{-3}	10^{-4}	FG neg control	NAC/NTC
0% (0/33)	6.1% (2/33)	10.9% (6/55)	4.1% (14/340)

10. Troubleshooting guide

Problem	Probable cause(s)	Suggested Corrective Action(s)
Negative result for the control gene (ABL) and for BCR-ABL Mbcr in all the samples – Standard OK	• Poor RNA sample quality • RT step failure	• Always check the RNA quality and concentration before starting. • Run a cell line RNA positive control (PCCL-01) in parallel
Negative result for the control gene (ABL) in the samples – Standard OK	• Poor RNA sample quality • RT step failure	• Always check the RNA quality and concentration before starting • Run a cell line RNA positive control (PCCL-01) in parallel
Standard signal negative	• Pipetting • Inappropriate storage of kit components	• Check pipetting sheme and the set-up of the reaction • Repeat the PCR run • Aliquots reagents • Store the BCR-ABL Mbcr Fusion*Quant*® Kit at -15 to -25°C and keep Primers & Probes mixes (PPC and PPF) protected from light • Avoid repeated freezing and thawing
Negative (H2O) control is positive	• Cross contamination	• Replace all critical reagents • Repeat the experiment with new aliquots of all reagents. • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination
No signal, even in standard controls	• LightCycler®: Incorrect detection channel has been chosen	• Set Channel Setting to F1/F2 or 530nm/640nm
	• Pipetting errors or omitted reagents	• Check pipetting sheme and the set-up of the reaction • Repeat the PCR run
	• LightCycler®: No data acquisition programmed	• Check the cycle programs • Select acquisition mode "single" at the end of each annealing segment of the PCR program.
	• Inhibitory effects of the sample material, caused by insufficient purification	• Repeat RNA preparation
Absent or low signal in samples, but standard controls OK	• RNA quality or concentration • RT reaction failure	• Always check the RNA quality and concentration before starting • Run a cell line RNA positive control (PCCL-01, High BCR-ABL Control) in parallel.
Fluorescence intensity too low	• Inappropriate storage of kit components	• Aliquot reagents • Store the BCR-ABL Mbcr Fusion*Quant*® Kit at -15 to -25°C and keep Primers & Probes mixes (PPC and PPF) protected from light. • Avoid repeated freezing and thawing
	• Very low initial amount of target RNA	• Increase the amout of sample RNA. Note: Depending of the chosen method of RNA preparation, inhibitory effects may occur

Negative control samples are positive	• Carry-over contamination	• Replace all critical reagents • Repeat the experiment with new aliquots of all reagents • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination
Fluorescence intensity varies	• Pipetting	• LightCycler®: Variability, caused by so-called "pipetting error" can be reduced by analysing data in th F1/F2 or 530nm/640nm mode
	LightCycler®: no, or insufficient centrifugation of the capillaries • Prepared PCR mix is still in the upper vessel of the capillary • Air bubble is trapped in the capillary tip	• Always centrifuge capillaries loaded with the reaction mix as described in the specific operating manual of the apparatus
	Outer surface of the capillary tip dirty	• Always wear gloves when handling the capillaries
LightCycler®: Error of standard curve	• Pipetting	• LightCycler®: variability, caused by so-called "pipetting error" can be reduced by analysing data in the F1/F2 or 530nm/640nm mode

N.B. If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please contact IPSOGEN Technical Service at support@ipsogen.com for further assistance.

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