

For Life Science Research Use Only
Not Intended For Diagnostic Use

FusionQuant® Kits for Real-Time Quantitative PCR Analysis of Fusion Gene Transcripts

Catalogue Numbers:

FQPP-01	AML1-ETO
FQPP-02	CBFβ-MYH11 A
FQPP-05	PML-RARA bcr1
FQPP-06	PML-RARA bcr2
FQPP-07	PML-RARA bcr3
FQPP-09-ABL	BCR-ABL mbc / ABL
FQPP-09-GUS	BCR-ABL mbc / GUS
FQPP-09-BCR	BCR-ABL mbc / BCR
FQPP-10-ABL	BCR-ABL Mbc / ABL
FQPP-10-GUS	BCR-ABL Mbc / GUS
FQPP-10-BCR	BCR-ABL Mbc / BCR
FQPP-11	TEL-AML1
FQPP-10M-ABL	BCR-ABL Mbc Mega kit / ABL
FQPP-10M-GUS	BCR-ABL Mbc Mega kit / GUS
FQPP-10M-BCR	BCR-ABL Mbc Mega kit / BCR

Tests:

ABI* & LC 480: 24 samples in duplicate in 3 distinct experiments
*(7000, 7700 & 7900) **Mega Kits:** 64 samples in duplicate in 4 distinct experiments

LightCycler®: 30 samples in duplicate in 6 distinct experiments
(1.2; 1.5; 2.0) **Mega Kits:** 55 samples in duplicate in 11 distinct experiments

SmartCycler®: 30 samples in duplicate in 6 distinct experiments
Mega Kits: 55 samples in duplicate in 11 distinct experiments

Rotor-Gene™: 24 samples in duplicate in 3 distinct experiments
(3000 & 6000) **Mega Kits:** 52 samples in duplicate in 4 distinct experiments

Store at **-25°C to -15°C**

Note: Store Primers & Probe Mixes (PPC and PPF tubes) in the dark

Instructions for use

Version 15, January 2011



IPSOGEN
CANCER PROFILER

FusionQuant® Kits

Kits for the quantitative detection of fusion transcripts using either ABI Prism TaqMan®, LightCycler®, SmartCycler® or Rotor-Gene™ instruments.

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1. Intended Use

The FusionQuant® Kits are intended to be used in research studies for the highly sensitive quantitative detection of fusion gene transcripts in RNA preparations from cell cultures and other biological samples.

The following quantification FusionQuant® Kits are available:

AML1-ETO	REF: FQPP-01
CBFβ-MYH11 A	REF: FQPP-02
PML-RARA bcr1	REF: FQPP-05
PML-RARA bcr2	REF: FQPP-06
PML-RARA bcr3	REF: FQPP-07
BCR-ABL mbc1	REF: FQPP-09-ABL, FQPP-09-GUS, FQPP-09-BCR
BCR-ABL Mbc1	REF: FQPP-10-ABL, FQPP-10-GUS, FQPP-10-BCR, FQPP-10M-ABL, FQPP-10M-GUS, or FQPP-10M-BCR
TEL-AML1	REF: FQPP-11

Each kit determines the expression levels of the above fusion transcripts relative to the control gene ABL, **except for FQPP-09 and FQPP-10, with either ABL, GUS or BCR as control gene standards.**

2. Background

Some chromosomal translocations result in the creation of fusion gene transcripts, which can be tested with RQ-PCR. This technology combines DNA amplification with detection of the products in a single tube.

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. 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 FusionQuant® Kits, an endogenous control (ABL transcript and for FQPP-09 and FQPP-10 either GUS or BCR) is amplified from the sample as well as the fusion transcript of interest. Standard curves of known amounts of both the endogenous control and the fusion cDNA allow the calculation of the ratio of specific fusion transcript signal to endogenous control gene 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 control and fusion genes.

3. Technological Principle

RQ-PCR permits accurate quantitation of PCR products during the exponential phase of the PCR amplification process, which is in full contrast to the classical PCR end point quantitation. 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: On RQ-PCR analysis using SYBR Green I Dye, or hydrolysis probes and or 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 (see Figure 1). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

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

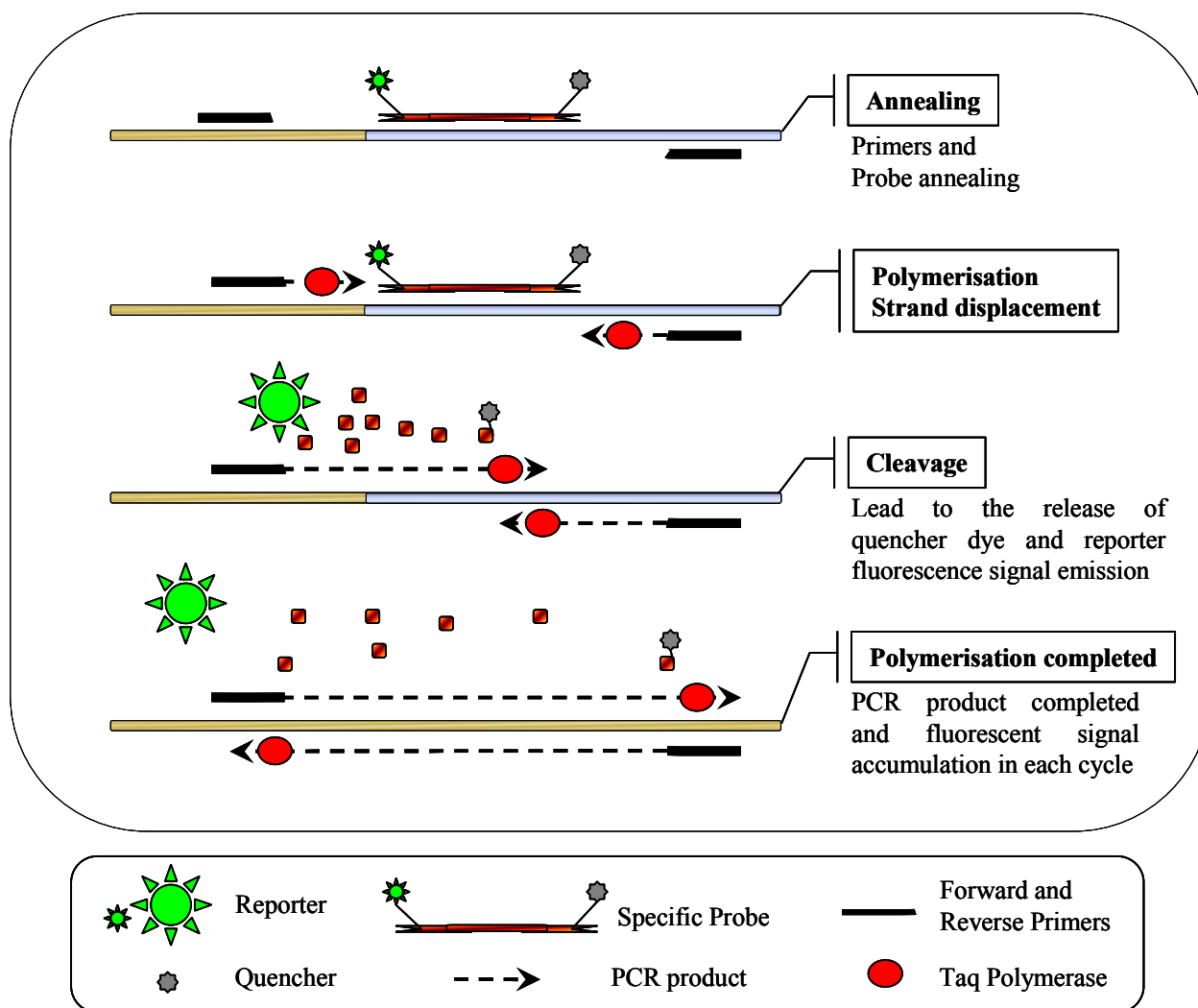


Figure 1: Total RNA is reverse transcribed and the generated cDNA amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM-TAMRA). The probe binds to the amplicon during each annealing step of the PCR. When the Taq extends from the primer bound to the amplicon it 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 FusionQuant® Kit, an endogenous control (Control Gene transcript) is amplified from the sample as well as the fusion transcript. Standard curves of known amounts of both the endogenous control gene and the fusion cDNA allow the calculation of the ratio of the fusion transcript signal to endogenous Control gene 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 control and the fusion genes.

4. Technological Specifications

The FusionQuant® Kits were developed according to the EAC network protocol:

J. Gabert et al. Standardization and quality control studies of “real-time” quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukaemia – A Europe Against Cancer Program. *Leukemia* (2003) 17, 2318 - 2357.

5. Reagents and Instruments

5.1. Material provided

a. For the following References: FQPP-01, FQPP-02, FQPP-05, FQPP-06, FQPP-07, FQPP-09-ABL, FQPP-10-ABL, FQPP-10M-ABL and FQPP-11

ABL Standard Dilutions	Dilution	Volume
C1-ABL	10 ³ copies/5µl	50µl
C2-ABL	10 ⁴ copies/5µl	50µl
C3-ABL	10 ⁵ copies/5µl	50µl

b. For the following References: FQPP-09-GUS, FQPP-10-GUS, FQPP-10M-GUS

GUS Standard Dilutions	Dilution	Volume
C1-GUS	10 ³ copies/5µl	50µl
C2-GUS	10 ⁴ copies/5µl	50µl
C3-GUS	10 ⁵ copies/5µl	50µl

c. For the following References: FQPP-09-BCR, FQPP-10-BCR, FQPP-10M-BCR

BCR Standard Dilutions	Dilution	Volume
C1-BCR	10 ³ copies/5µl	50µl
C2-BCR	10 ⁴ copies/5µl	50µl
C3-BCR	10 ⁵ copies/5µl	50µl

d. For all References

Fusion Transcript Standard Dilutions	Dilution	Volume
F1-« <i>specific fusion transcript</i> »	10 ¹ copies/5µl	50µl
F2-« <i>specific fusion transcript</i> »	10 ² copies/5µl	50µl
F3-« <i>specific fusion transcript</i> »	10 ³ copies/5µl	50µl
F4-« <i>specific fusion transcript</i> »	10 ⁵ copies/5µl	50µl
F5-« <i>specific fusion transcript</i> »	10 ⁶ copies/5µl	50µl

Primers & Probe Mixes	Content	Volume
PPC-ABL or PPC-GUS or PPC-BCR Amber tube <i>Supplied ready-to-use</i>	Mix of specific reverse and forward primers for the ABL control gene Plus a specific FAM-TAMRA probe.	90µl 2 x 90µl for Mega kits
PPF-« <i>specific fusion transcript</i> » Amber tube <i>Supplied ready-to-use</i>	Mix of specific reverse and forward primers for the fusion gene Plus a specific FAM-TAMRA probe.	110µl 2 x 110µl for Mega kits

Note: Spin the “Standard dilutions” and the “Primers and Probe” tubes before use.

5.1.1. Handling and Storage

Note: kits can be shipped at room temperature but must be stored at -25°C to -15°C in a constant-temperature freezer immediately upon receipt.

Keep the Primers & Probe Mixes (PPC and PPF tubes) away from light as this product is photosensitive. These storage conditions apply to both opened and un-opened components. 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. Vortex and centrifuge the tubes before opening.

5.1.2. Kit stability

Expiration dates for each reagent are indicated on the individual component labels.

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

These reagents are manufactured according to ISO 13485:2003 standard, which requires stringency in validation and documentation of manufacturing procedures.

Quality control of the complete kits have been performed on a LightCycler® 480 apparatus.

Certificates of Analyses are available upon request at support@ipsogen.com

5.1.4. Warnings

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

5.2. Reagents and material required but not provided.

5.2.1. Sample RNA preparation

RNA preparation from human samples (peripheral blood or bone marrow cells) 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.

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 the specific LightCycler® TaqMan® Probe Master Mix)
- Specific reagent for LightCycler® use
- Nuclease-free PCR grade H₂O

Recommended Validated Reagents

	Reagent	Validated reagent (Brand name)	Validated reagent (Provider, reference in Europe)
For the Reverse Transcription step	Ready to use set containing all reagents for Reverse Transcription step	RT Kit	IPSOGEN / RT-01
	Reverse transcriptase (200U/μl)	Superscript® or Superscript® II	Invitrogen® # 18064-022
	5X Buffer	5X First-Strand Buffer (supplied with Invitrogen® RT)	
	DTT 100 mM	DTT (supplied with Invitrogen® RT)	
	MgCl ₂ 50mM	MgCl ₂ (supplied by Invitrogen®)	Invitrogen® # 10342-020
	random hexamer / nonamer	Any	Any
	RNase Inhibitor (40U/μl)	RNaseOut™.	Invitrogen® # 10777-019

	100mM dNTP	Set of dNTP, PCR Grade.	Any
For the RQ-PCR step	PCR grade H ₂ O, RNase, DNase free	Any	Any
	PCR Master Mix	TaqMan® Universal PCR Master Mix	Applied Biosystems® # 4304437
	Light Cycler TaqMan® Master	Freshly prepared 5X Master Mix	Roche # 4535286001

Complementary Ipsogen reagents

- Control kits consisting in cell lines with negative, high and low positive expression of the transcript of interest for the qualitative validation of the RNA extraction and the reverse transcription (see Ipsogen references CLCK-01, CLCK-02 and CLCK-03 which provide negative, high and low positive controls available respectively for BCR-ABL Mbc, BCR-ABL mbc and PML-Rara fusion genes)

5.2.3. Equipment

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

- ✓ Real-time PCR instrumentation (TaqMan® ABI or any equivalent equipment)
- ✓ General Laboratory Equipment
- ✓ Specific material for LightCycler® use
- ✓ 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 (ependorf) for preparing 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)
- ✓ Thermal Cycler with heated lid
- ✓ 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 than 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

The user should read these instructions carefully and become familiar with all components prior to use.

The quality of the assay is largely dependent on the quality of input RNA. We therefore recommend analysing the purified RNA by agarose gel electrophoresis or Agilent Bioanalyser® prior to analysis.

Complementary DNA can be obtained thanks to Ipsogen's RT Kit for Reverse Transcription (RT-01). When using this kit, please refer to the specific protocol provided and go directly to paragraph 6.2, 6.3, 6.4 or 6.5, depending on the instrument used, for the RQ-PCR step. Otherwise see paragraph 6.1 below.

Note: Controls can be used to perform qualitative validation of RNA extraction and Reverse Transcription (see paragraph 5.2.2 Recommended validated reagents / Complementary Ipsogen reagents).

6.1. Standardised EAC Reverse Transcription protocol

- ❑ Thaw all necessary components and place them on ice.
- ❑ Incubate 1 µg of RNA (1 to 4 µl) **for 10 min at 70°C** and immediately cool on ice for 5 min.
- ❑ Spin briefly (~10sec, 10,000rpm, 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
MgCl ₂ (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)	0.5 µl	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, 10,000rpm, 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, 10,000rpm, 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 = 50 µl**
- ❑ Process the following steps according to your RQ-PCR instrument.

6.2. ABI Prism TaqMan® (7000, 7700 and 7900) & LightCycler® 480 instruments

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

With the Control Gene Primers & Probe Mix

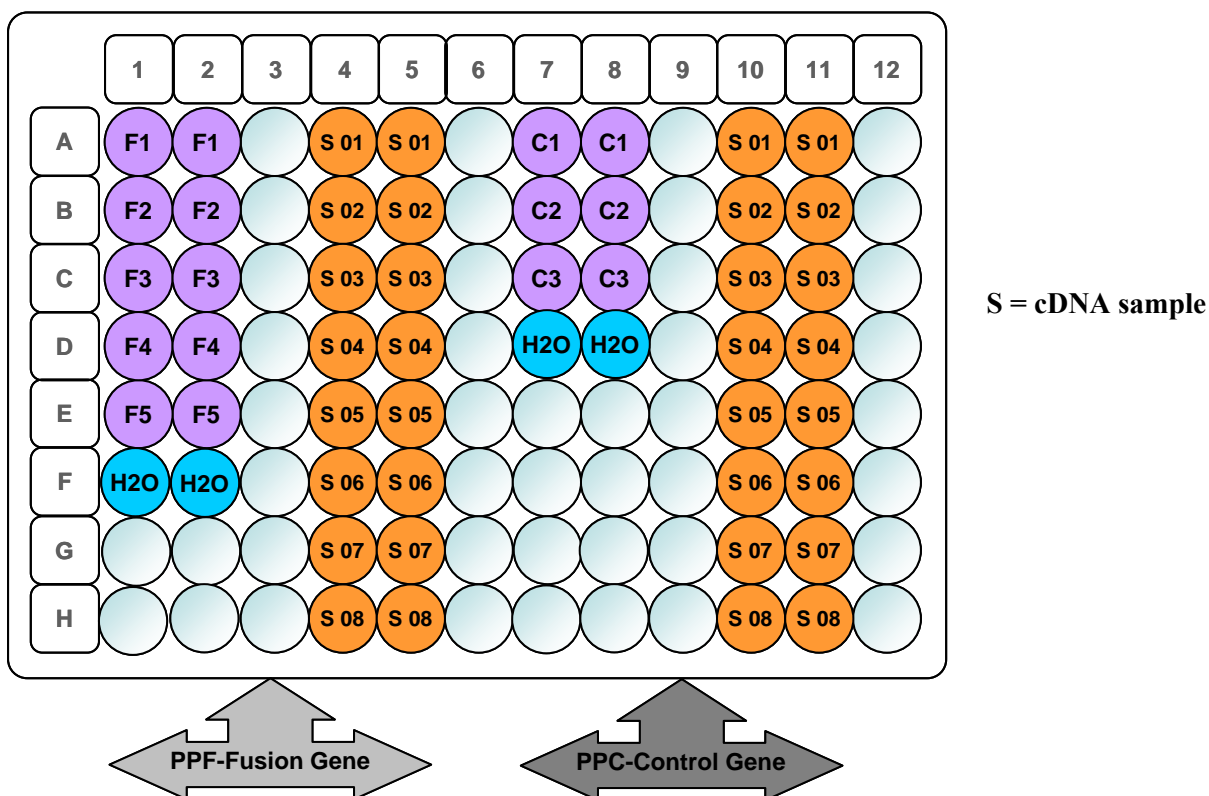
n cDNA samples n x 2 reactions
 Control Gene standard 6 reactions (3 dilutions, each one tested in duplicate)
 Water control 2 reactions

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

6.2.1. Sample processing:

The plate scheme and the mix preparation below show an example of an experiment using FQPP-XX references (“normal kits”). Adapt the scheme and the volumes when using Mega kits FQPP-10M-ABL (or GUS or BCR).



N.B: Each FusionQuant® Kit provides enough reagents to perform this 8 cDNA samples experiment 3 times.

N.B: Each BCR-ABL Mbc FusionQuant® Mega Kit provides enough reagents to perform 16 cDNA samples experiment 4 times.

6.2.2. RQ-PCR for ABI PRISM® and LightCycler® 480 instruments

- Thaw all necessary components and place them on ice.
- Spin briefly all the tubes (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube)
- Prepare the following **RQ-PCR premix** according to the number of samples being processed.

RQ-PCR premix Reagents	Final Conc.	1 Reaction	24 Reactions	28 Reactions
TaqMan® Universal PCR Master Mix 2X Applied Biosystems (Not Provided)	1X	12.5 µl	312.5µl	362.5µl
IPSOGEN Primers & Probe mix 25X	1X	1 µl	25µl	29µl
Adjust vol. to 20 µl with nuclease-free H ₂ O		6.5 µl	162.5µl	188.5µl
	Total volume	= 20 µl	500µl	580µl
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-Control Gene or PPF-Fusion Gene). Extra volumes are included to compensate pipetting error.

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) in the corresponding well (total volume 25µl).**
- Mix gently, by pipetting up and down.
- Close the plate and briefly centrifuge (300g, ~10 sec)
- Place the plate in the thermal cycler.

- ☐ Run the following program:

RQ-PCR program			
Temperature	Time	Cycles	Acquisition
50°C	2 min	X 1	None
95°C	10 min	X 1	None
95°C	15 sec	X 50	None
60°C	1 min		Single

- ☐ We recommend a threshold set at 0.1 as describe in the EAC protocol in the analysis step on the ABI Prism® 7000, 7700 and 7900 instruments.

6.3. LightCycler® instruments (1.2, 1.5 and 2.0)

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

• With the Control Gene Primers & Probe Mix

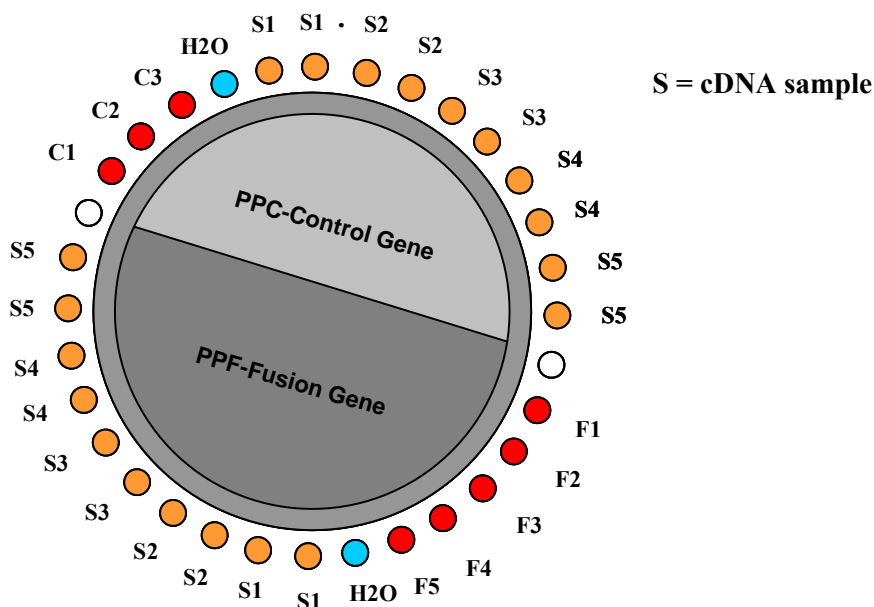
n cDNA samples n x 2 reactions
 Conto Gene standard 3 reactions (3 standard dilutions)
 Water control 1 reaction

• 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:

The capillaries scheme and the mix preparation just after show an example of an experiment using FQPP-XX references (“normal kits”). Adapt the scheme and the volumes when using Mega kits FQPP-10M-ABL (or GUS or BCR)



N.B. Each FusionQuant® Kit provides enough reagents to perform this 5 cDNA samples experiment 6 times.

N.B. Each BCR-ABL Mbc FusionQuant® Mega Kit provides enough reagents to perform this 5 cDNA sample experiment 11 times.

6.3.2. RQ-PCR For LightCycler® instruments

NB: Because of particular technological requirements, LightCycler experiments must be performed using specific reagents. We recommend to use the Light Cycler TaqMan® Probe Master kit and to follow the manufacturer's instructions to prepare the Master Mix 5X.

- ☐ Thaw all necessary components and place them on ice.

- Spin briefly all the tubes (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube)
- Prepare the following **RQ-PCR premix** according to the number of samples being processed.

RQ-PCR premix Reagents	Final Conc.	1 Reaction	14 Reactions	16 Reactions
Freshly prepared Master Mix 5X LightCycler TaqMan® Master (Not Provided)	1X	4 µl	60µl	68µl
IPSOGEN Primers & Probe mix 25X	1X	0.8 µl	12µl	13.6µl
Adjust vol. to 15 µl with nuclease-free H ₂ O		10.2 µl	153µl	173.4µl
	Total volume	= 15 µl	225µl	255µl
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-Control Gene or PPF-Fusion Gene). Extra volumes are included to compensate pipetting error.

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) in the corresponding capillary (total volume 20µl).**
- Mix gently, by pipetting up and down.
- Place the capillaries in the adapter provided with the instrument and centrifuge briefly (700g, ~10sec.)
- 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 Control Gene Primers & Probe Mix

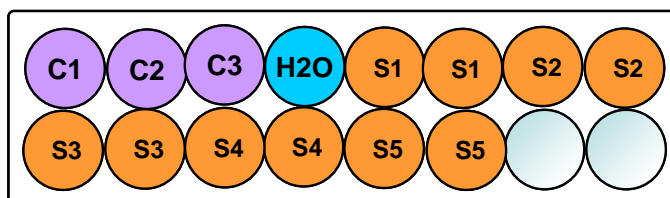
n cDNA samples n x 2 reactions
 Control standard 3 reactions (3 different dilutions)
 Water control 1 reaction

• With the Fusion transcript Primers & Probe Mix

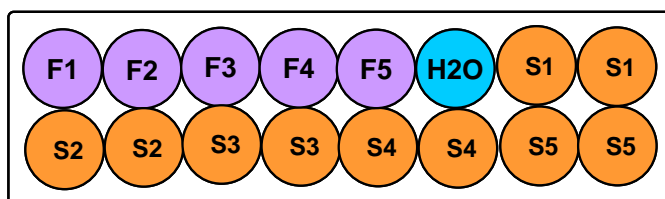
n cDNA samples n x 2 reactions
 Fusion Gene standard 5 reactions (5 different dilutions)
 Water control 1 reaction

6.4.1. Sample processing

The two-block scheme and the mix preparation below show an example of experiment using FQPP-XX references (normal kits). Adapt the scheme and the volumes when using Mega kits FQPP-10M-ABL (or GUS or BCR).



All the assays on this first block are performed with PPC-Control Gene
S = cDNA sample



All the assays
on this second block are performed
with PPF-“Fusion Gene”

S = cDNA sample

N.B: Each FusionQuant® Kit provides enough reagents to perform this 5 cDNA samples experiment 6 times.
N.B: Each BCR-ABL Mber FusionQuant® Mega Kit provides enough reagents to perform this 5 cDNA samples experiment 11 times.

6.4.2. RQ-PCR For SmartCycler® instrument

- Thaw all necessary components and place them on ice.
- Spin briefly all the tubes (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube)
- Prepare the following **RQ-PCR premix** according to the number of samples being processed.

RQ-PCR premix Reagents	Final Conc.	1 Reaction	14 Reactions	16 Reactions
PCR Master Mix 2X (Not Provided)	1X	12.5 µl	187.5µl	212.5µl
IPSOGEN Primers & Probe mix 25X	1X	1 µl	15µl	17µl
Adjust vol. to 20 µl with nuclease-free H ₂ O		6.5 µl	97.5µl	110.5µl
	Total volume	= 20 µl	300µl	340µl
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-Control Gene or PPF-Fusion Gene). Extra volumes are included to compensate pipetting error.

To be performed on ice:

- Dispense **20 µl of the RQ-PCR pre-mix**.
- Add **5 µl of the RT product (cDNA, 100ng RNA equivalent) in the corresponding well (total volume 25µl)**.
- Mix gently, by pipetting up and down.
- Close the plate(s) and briefly centrifuge (300g, ~10 sec)
- Load the samples in the apparatus according to the manufacturer recommendations.
- Run the following program:

RQ-PCR program			
Temperature	Time	Cycles	Acquisition
50°C	2 min	X 1	None
95°C	10 min	X 1	None
95°C	15 sec	X 50	None
60°C	1 min		Single

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

6.5. Rotor-Gene™ 3000 / 6000 instrument

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

With the Control Gene Primers & Probe Mix

n cDNA samples n x 2 reactions
Control gene 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

N.B: Each FusionQuant® Kit provides enough reagents to perform this 8 cDNA samples experiment 3 times using the 72 tubes rotor.

N.B: Each FusionQuant® Mega Kit provides enough reagents to perform 13 cDNA samples experiment 4 times using the 72 Tubes Rotor.

6.5.2. RQ-PCR for Rotor-Gene™ 3000 / 6000 instruments (72 tubes Rotor)

- Thaw all necessary components and place them on ice.
- Spin briefly all the tubes (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube)
- Prepare the following **RQ-PCR premix** according to the number of samples being processed.

RQ-PCR premix Reagents	Final Conc.	1 Reaction	24 Reactions	28 Reactions
TaqMan® Universal PCR Master Mix 2X Applied Biosystems (Not Provided)	1X	12.5 µl	312.5µl	362.5µl
IPSOGEN Primers & Probe mix 25X	1X	1 µl	25µl	29µl
Adjust vol. to 20 µl with nuclease-free H ₂ O		6.5 µl	162.5µl	188.5µl
	Total volume	= 20 µl	500µl	580µl
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). Extra volumes are included to compensate pipetting error.

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) in the corresponding tube (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	Acquisition
50°C	2 min	X 1	None
95°C	10 min	X 1	None
95°C	15 sec	X 50	None
60°C	1 min		Single

- We recommend using Automatic threshold analysis on Rotor-Gene™ 3000 and 6000 instruments to obtain reproducible results.

7. Test Interpretation

7.1. The NCN method based on Fusion Gene (FG) and Control Gene (CG) standard curves

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. This methodology has the advantage that degradation of probes can be compensated, and data generated on different types of RQ-PCR instrument can be compared.

The Control Gene standard curve equation should be used to transform raw Ct values (obtained with CG-PPC) for the unknown samples, into CG copy numbers (CG_{CN}).

The Fusion Gene standard curve equation should be used to transform raw Ct values (obtained with FG-PPF) for the unknown samples, into Fusion Gene copy numbers (FG_{CN}).

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

$$\text{NCN} = \text{FG}_{\text{CN}} / \text{CG}_{\text{CN}}$$

8. Troubleshooting guide

Problem	Probable cause(s)	Suggested Corrective Action(s)
Negative result for the control gene (Control Gene) and Fusion gene samples – Standard OK	<ul style="list-style-type: none"> Poor RNA sample quality RT step failure 	<ul style="list-style-type: none"> Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel
Negative result for the control gene (Control Gene) in the samples – Standard OK	<ul style="list-style-type: none"> Poor RNA sample quality RT step failure 	<ul style="list-style-type: none"> Always check the RNA quality and concentration before starting Run a cell line RNA positive control in parallel
Standard signal negative	<ul style="list-style-type: none"> Pipetting Inappropriate storage of kit components 	<ul style="list-style-type: none"> Check pipetting scheme and the set-up of the reaction Repeat the PCR run Aliquots reagents Store the FusionQuant® Kit at -25 to -15°C and keep Primer & Probes mixes (PPC and PPF) protected from light Avoid repeated freezing and thawing
Negative (H2O) control is positive.	<ul style="list-style-type: none"> Cross contamination 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> LightCycler®: Incorrect detection channel has been chosen 	<ul style="list-style-type: none"> Set Channel Setting to F1/F2 or 530nm/640nm
	<ul style="list-style-type: none"> Pipetting errors or omitted reagents 	<ul style="list-style-type: none"> Check pipetting scheme and the set-up of the reaction Repeat the PCR run
	<ul style="list-style-type: none"> LightCycler®: No data acquisition programmed 	<ul style="list-style-type: none"> Check the cycle programs Select acquisition mode "single" at the end of each annealing segment of the PCR program.
	<ul style="list-style-type: none"> Inhibitory effects of the sample material, caused by insufficient purification 	<ul style="list-style-type: none"> Repeat RNA preparation
Absent or low signal in samples, but standard controls OK	<ul style="list-style-type: none"> RNA quality or concentration RT reaction failure 	<ul style="list-style-type: none"> Always check the RNA quality and concentration before starting Run a cell line RNA positive control in parallel.
Fluorescence intensity too low	<ul style="list-style-type: none"> Inappropriate storage of kit components 	<ul style="list-style-type: none"> Aliquot reagents Store the FusionQuant® Kit at -25 to -15°C and keep Primer & Probes mixes (PPC and PPF) protected from light. Avoid repeated freezing and thawing
	<ul style="list-style-type: none"> Very low initial amount of target RNA 	<ul style="list-style-type: none"> Increase the amount of sample RNA. Note: Depending of the chosen method of RNA preparation, inhibitory effects may occur
Negative control samples are positive.	<ul style="list-style-type: none"> Carry-over contamination 	<ul style="list-style-type: none"> 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.	<ul style="list-style-type: none"> Pipetting 	<ul style="list-style-type: none"> LightCycler®: Variability, caused by so-called "pipetting error" can be reduced by analysing data in the F1/F2 or 530nm/640nm mode
	<ul style="list-style-type: none"> 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 	<ul style="list-style-type: none"> Always centrifuge capillaries loaded with the reaction mix as described in the specific operating manual of the apparatus
	<ul style="list-style-type: none"> Outer surface of the capillary tip dirty 	<ul style="list-style-type: none"> Always wear gloves when handling the capillaries

LightCycler®: Error of standard curve	<ul style="list-style-type: none">• Pipetting	<ul style="list-style-type: none">• LightCycler®: Variability, caused by so-called "pipetting error" can be reduced by analysing data in the F1/F2 or 530nm/640nm mode
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NOTE: 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.

9. References

- 1- VHJ van der Velden et al. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* (2003) **17**, 1013–1034
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- 3- J. Gabert et al. Standardization and quality control studies of “real-time” quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukaemia – A Europe against Cancer Program. *Leukemia* (2003) **17**, 2318 – 2357

10. Contact information

Produced by: **IPSOGEN**

IPSOGEN SA – Manufacturing site

Luminy Biotech Entreprises
Case 923, 163 Avenue de Luminy
13288 Marseille Cedex 9
France
Tel: +33 (0)4 91 29 30 90
Fax: +33 (0)4 91 29 30 99
Email: support@ipsogen.com
infos@ipsogen.com
Web: www.ipsogen.com

IPSOGEN Inc.

700 Canal Street
Fifth Floor
Stamford, CT 06902
USA
Tel: +1 203-504-8585
Fax: +1 203-504-8590
Email: support-us@ipsogen.com
infos-us@ipsogen.com
Web: www.ipsogen.com

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FusionQuant® Kits

Instructions for use

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