March 2015

ipsogen® WT1 Profile*Quant*® (ELN*) Handbook

Σ 24

For quantification of Wilm's tumor (WT) gene transcripts in total RNA

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

For use with Rotor-Gene® Q, ABI PRISM® 7900HT SDS, Applied Biosystems® 7500 Real-Time PCR System, and LightCycler® instruments

REF 676913

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

ELN European LeukemiaNet

R2

QIAGEN®

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.

Contents

Intended Use

The *ipsogen* WT1 Profile*Quant* Kit is intended for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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

Principle of the Procedure

The qPCR technique permits the accurate quantification of PCR products during the exponential phase of the PCR amplification process. Data from qPCR 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.

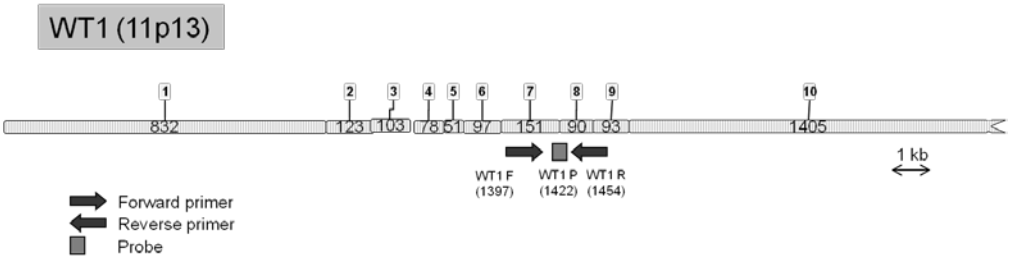

Figure 1. Schematic diagram of the WT1 transcript covered by the ELN qPCR primers and probe set: WT1-ELN F–WT1-ELN P–WT1-ELN R. The number under the primers and probe refers to their nucleotide position in the normal gene transcript. Exon 5 may be alternatively spliced.

This assay exploits the qPCR double-dye oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. A double-dye oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream, 3'quencher dye, hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes exploits the 5'→3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer.

During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'→3' exonuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3'

end of the probe is blocked to prevent extension of the probe during PCR (Figure 2). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

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

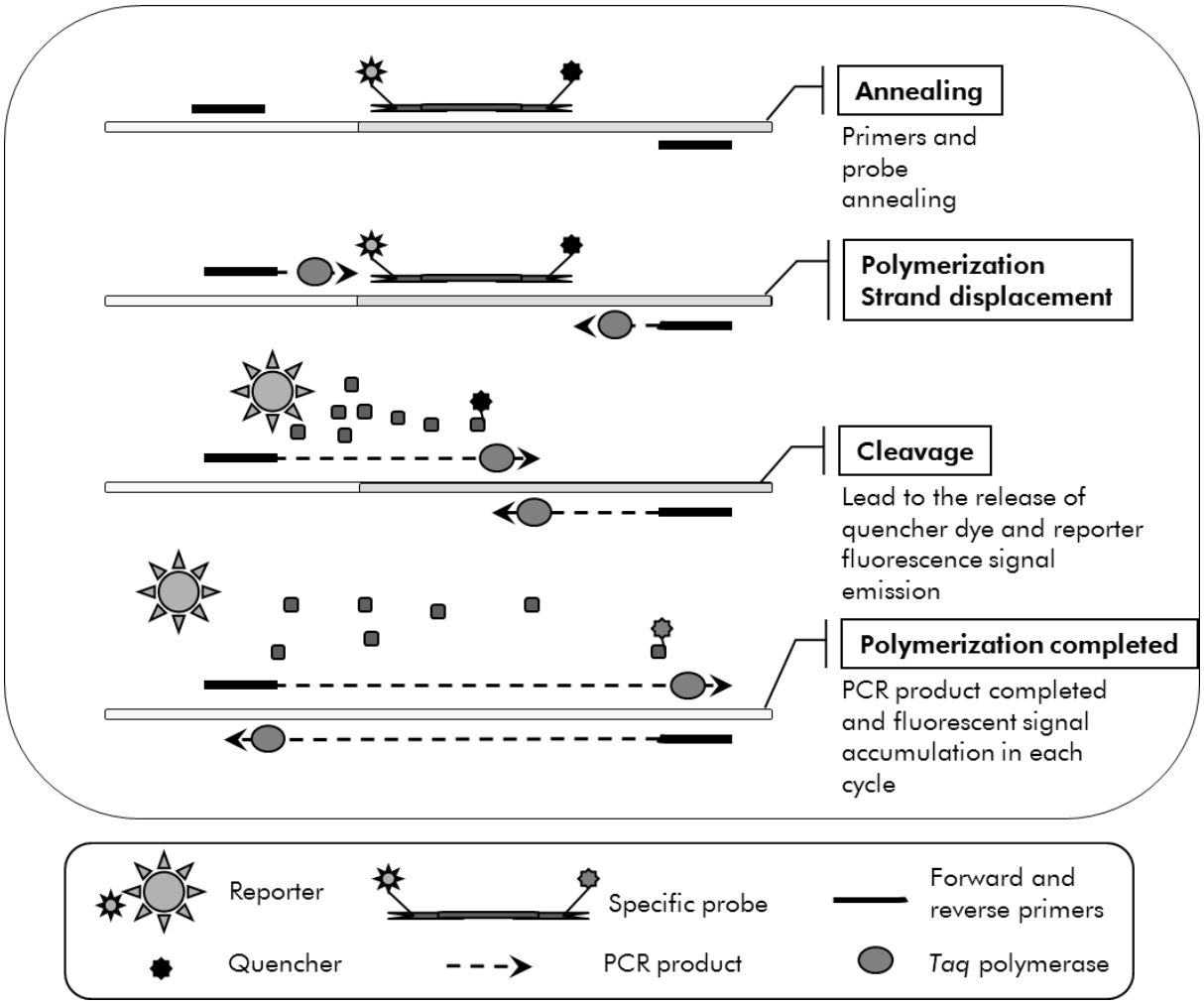

Figure 2. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–TAMRA™). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'→3' exonuclease activity of the *Taq* DNA polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them and leading to an increase in fluorescence from the FAM and a decrease in fluorescence from the TAMRA.

When fluorescence is plotted against cycle number, the accumulation of PCR product is shown in Figure 3. This amplification curve is composed successively of an early background phase (below the detection level of the instrument), an exponential phase (or logarithmic phase), and a plateau. The most accurate quantitative determination can only be made during the exponential phase. The

first cycle at which the instrument can distinguish the amplification generated fluorescence as being above the background signal is called the threshold cycle (C_T) or crossing point (C_P). By selecting the threshold within the logarithmic-linear phase, it is possible to calculate the actual amount of initial starting molecules since the fluorescence intensity is directly proportional to the amount of PCR product in the exponential phase.

During the plateau phase, no significant increase in PCR product amount takes place. This is mainly due to depletion of PCR components and re-annealing of PCR product strands caused by the high concentration of end-products, which prevent further primer annealing.

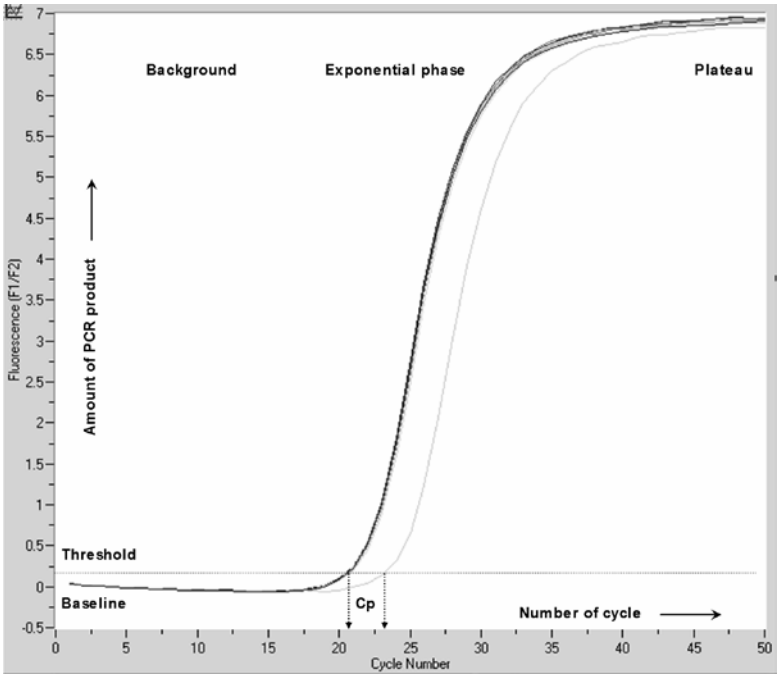

Figure 3. Fluorescence acquisition during cycling and successive phases of amplification.

The most direct and precise approach for analyzing quantitative data is to use a standard curve that is prepared from a dilution series of control template of known concentration. This is known as "standard curve" or "absolute" quantification. Following amplification of the standard dilution series, the standard curve is generated by plotting the log of the initial template copy number against the C_P generated for each dilution. Plotting these points generates a standard curve. Using the equation of this standard curve allows the determination of initial copy number of the samples to be quantified.

The WT1 Profile*Quant* Kit (ELN) includes specific plasmids and primers and probe mixes for WT1 and ABL. These components have been tested together in the context of a collaborative study led by a group of experts from the European Leukemia Net consortium. The assay previously published by Van Dijk and coworkers consistently out-performed the other assays and is less prone to mutations in AML due to its configuration (1). Consequently, it was selected as

the ELN WT1 assay. The *ipsogen* WT1 Profile*Quant* Kit is based on this technique. In this kit, an endogenous control (ABL transcript) is amplified from the sample as well as the WT1 transcript. Standard serial dilutions of control and WT1 cDNA are provided and the standard curves generated allow the accurate calculation of the copy number of WT1 transcripts and ABL in each sample.

Materials Provided

Kit contents

***ipsogen* WT1 ProfileQuant Kit**		**(24)**
Catalog no.		**676913**
Number of reactions		**24**
ABL Control Gene Standard Dilution (10^3 copies/5 µl)	C1-ABL	50 µl
ABL Control Gene Standard Dilution (10^4 copies/5 µl)	C2-ABL	50 µl
ABL Control Gene Standard Dilution (10^5 copies/5 µl)	C3-ABL	50 µl
WT1 Profile Gene Standard Dilution (10^1 copies/5 µl)	P1-WT1	50 µl
WT1 Profile Gene Standard Dilution (10^2 copies/5 µl)	P2-WT1	50 µl
WT1 Profile Gene Standard Dilution (10^3 copies/5 µl)	P3-WT1	50 µl
WT1 Profile Gene Standard Dilution (10^5 copies/5 µl)	P4-WT1	50 µl
WT1 Profile Gene Standard Dilution (10^6 copies/5 µl)	P5-WT1	50 µl
Primers and Probe Mix ABL*	PPC-ABL 25x	90 µl
Primers and Probe Mix PPP-WT1 (ELN)†	PPP-WT1 (ELN) 25x	110 µl
ipsogen *WT1 Profile*Quant *Handbook*		1

* Mix of specific reverse and forward primers for the ABL control gene (CG) plus a specific FAM–TAMRA probe.

† Mix of specific reverse and forward primers for the WT1 (exon 1-2) gene plus a specific FAM–TAMRA probe.

Note: Briefly centrifuge the standard dilutions and the primers and probe mixes before use.

Materials Required but Not Provided

When working with chemicals, always wear a suitable laboratory coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Reagents

- Nuclease-free PCR grade water
- Reagents for reverse transcription: The recommended reagent is SuperScript® II (or SuperScript) Reverse Transcriptase, includes 5x first-strand buffer, 100 mM DTT (Life Technologies, cat. no. 18064-022)
- RNase inhibitor: The recommended reagent is RNaseOUT™ (Life Technologies, cat. no. 10777-019)
- Set of dNTPs, PCR grade
- Random hexamer
- $MgCl_2$
- Buffer and *Taq* DNA polymerase: The recommended reagents are TaqMan® Universal PCR Master Mix (Master Mix PCR 2x) (Life Technologies, cat. no. 4304437) and LightCycler TaqMan Master (Master Mix PCR 5x) (Roche, cat. no. 04535286001)

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 pipet* 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 and a maximum speed of 13,000-14,000 rpm
- Real-time PCR instrument:* Rotor-Gene Q 5plex HRM or other Rotor-Gene instrument; LightCycler 1.2, or 480; or ABI PRISM 7900HT SDS; Applied Biosystems 7500 Real-Time PCR System; 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

When working with chemicals, always wear a suitable laboratory coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

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

General precautions

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

This kit is intended for research use. Reagents and instructions supplied in this kit have been tested for optimal performance. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data. PPC-ABL and PPP-WT1 reagents may be altered if exposed to light. All reagents are formulated specifically for use with this test. For optimal performance of the kit, 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 standard dilutions (C1–3 and P1–5) in a separate room.

Reagent Storage and Handling

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

- Minimize exposure to light of the primers and probe mixes (PPC and PPP 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.

Procedure

Sample RNA preparation

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

Protocol: Recommended standardized EAC reverse transcription

Things to do before starting

- Prepare dNTPs, 10 mM each. Store at –20°C in aliquots.
- Prepare random hexamer, 50 mM. Store at –20°C in aliquots.
- Prepare $MgCl_2$, 50 mM. Store at –20°C in aliquots.

Procedure

1. **Thaw all necessary components and place them on ice.**
2. **Incubate 1 µg of RNA (1–4 µl) for 10 minutes at 70°C and immediately cool on ice for 5 minutes.**
3. **Centrifuge briefly (approximately 10 seconds, 10,000 rpm) to collect the liquid in the bottom of the tube. Then keep on ice.**
4. **Prepare the following RT mix according to the number of samples being processed (Table 1).**

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

Table 1. Preparation of RT mix

Component	Volume per sample (µl)	Final concentration
First-Strand Buffer (supplied with SuperScript II Reverse Transcriptase), 5x	4.0	1x
$MgCl_2$ (50 mM)	2.0	5 mM
dNTPs (10 mM each, to be prepared previously and stored at –20°C in aliquots)	2.0	1 mM
DTT (100 mM, supplied with SuperScript II Reverse Transcriptase)	2.0	10 mM
RNase inhibitor (40 U/µl)	0.5	1 U/µl
Random hexamer (100 µM)	5.0	25 µM
SuperScript II (200 U/µl)	0.5	5 U/µl
Heated RNA sample (to be added in step 5)	1.0–4.0	50 ng/µl
Nuclease-free PCR grade water (to be added in step 5)	0.0–3.0	–
Final volume	20.0	–

5. Pipet 16 µl of RT mix into each PCR tube. Then add 1–4 µl (1 µg) RNA (from step 3), and adjust the volume to 20 µl with nuclease-free PCR grade water (see Table 2).

Table 2. Preparation of reverse transcription reaction

Component	Volume (µl)
RT mix	16.0
Heated sample RNA (1 µg)	1.0–4.0
Nuclease-free PCR grade water	0.0–3.0
Final volume	20.0

6. **Mix well and centrifuge briefly (approximately 10 seconds, 10,000 rpm) to collect the liquid in the bottom of the tube.**
7. **Incubate at 20°C for 10 minutes.**
8. **Incubate at 42°C on a thermal cycler for 45 minutes, then immediately at 99°C for 3 minutes.**
9. **Cool on ice (to stop the reaction) for 5 minutes.**
10. **Briefly centrifuge (approximately 10 seconds, 10,000 rpm) to collect the liquid in the bottom of the tube. Then keep on ice.**
11. **Dilute the final cDNA with 30 µl of nuclease-free PCR grade water so that the final volume is 50 µl.**
12. **Carry out PCR according to the following protocols, according to your qPCR instrument.**

Note: This reverse transcription protocol was derived from the "Europe Against Cancer" (EAC) studies (2).

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

Using this instrument, we recommend performing all measurements in duplicate, as indicated in Table 3.

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)	
n cDNA samples	n x 2 reactions
ABL standard	2 x 3 reactions (3 dilutions, each one tested in duplicate)
Water control	2 reactions
With the WT1 primers and probe mix (PPP-WT1)	
n cDNA samples	n x 2 reactions
WT1 standard	2 x 5 reactions (5 dilutions, each one tested in duplicate)
Water control	2 reactions

Sample processing on Rotor-Gene Q instruments with 72-tube rotor

We recommend testing 8 cDNA samples in the same experiment to optimize the use of the standards and the primers and probe mixes.

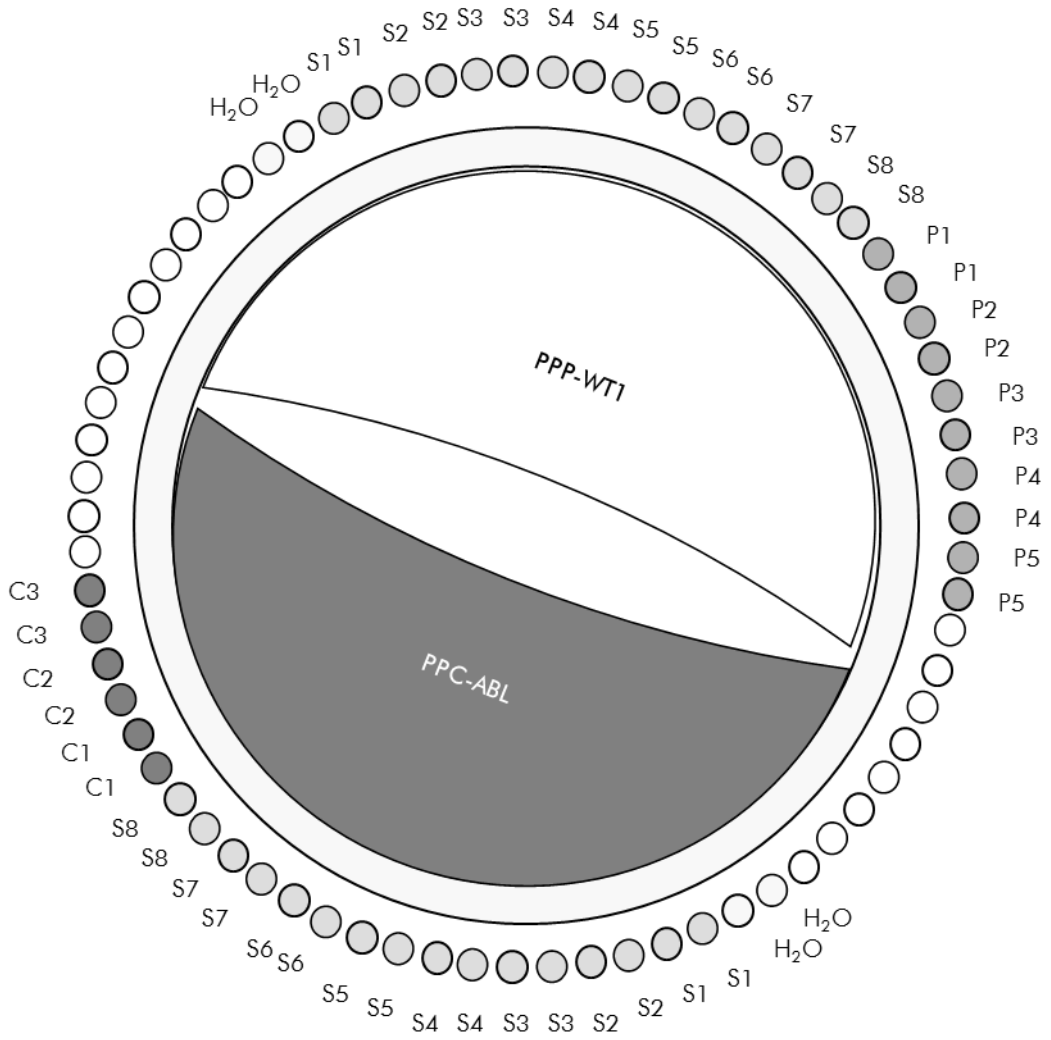

Figure 4. Suggested rotor setup for each experiment with the *ipsogen* WT1 ProfileQ*uant* Kit. P1–5: WT1 standards; **C1–3**: ABL standards; **S**: cDNA sample; **H_2O**: water control.

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 the 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. **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 PPP-WT1). Extra volumes are included to compensate for pipetting error.

Table 4. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 24 + 1 reactions (µl)	WT1: 28 +1 reactions (µl)	Final concentration
TaqMan Universal PCR Master Mix, 2x	12.5	312.5	362.5	1x
Primers and probe mix, 25x	1.0	25.0	29.0	1x
Nuclease-free PCR grade water	6.5	162.5	188.5	–
Sample (to be added at step 4)	5.0	5 each	5 each	–
Total volume	25.0	25 each	25 each	–

3. **Dispense 20 µl of the qPCR pre-mix per tube.**
4. **Add 5 µl of the RT product (cDNA, 100 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Recommended standardized EAC reverse transcription", page 12) in the corresponding tube (total volume 25 µl).**
5. **Mix gently, by pipetting up and down.**
6. **Place the tubes in the thermal cycler according to the manufacturer recommendations.**
7. **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	Temperature: 50 deg Time: 2 mins
Hold 2	Temperature: 95 deg Time: 10 mins
Cycling	50 times 95 deg for 15 secs 60 deg for 1 min with acquisition of FAM fluorescence in channel Green: Single

8. **For Rotor-Gene Q instruments, select "Slope Correct" for the analysis. We recommend setting the threshold at 0.03. Start the thermal cycling program, as indicated in Table 5.**

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

Using 96-well-plate qPCR equipment, we recommend performing all measurements in duplicate, as indicated in Table 6.

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

Samples	Reactions
With the ABL primers and probe mix (PPC-ABL)	
n cDNA samples	n x 2 reactions
ABL standard	2 x 3 reactions (3 dilutions, each one tested in duplicate)
Water control	2 reactions
With the WT1 primers and probe mix (PPP-WT1)	
n cDNA samples	n x 2 reactions
WT1 standard	2 x 5 reactions (5 dilutions, each one tested in duplicate)
Water control	2 reactions

Sample processing on ABI PRISM 7900HT SDS, Applied Biosystems 7500 Real-Time PCR System, and LightCycler 480 instruments

We recommend testing at least 8 cDNA samples in the same experiment to optimize the use of the standards and the primers and probe mixes. The plate scheme in Figure 5 shows an example of such an experiment.

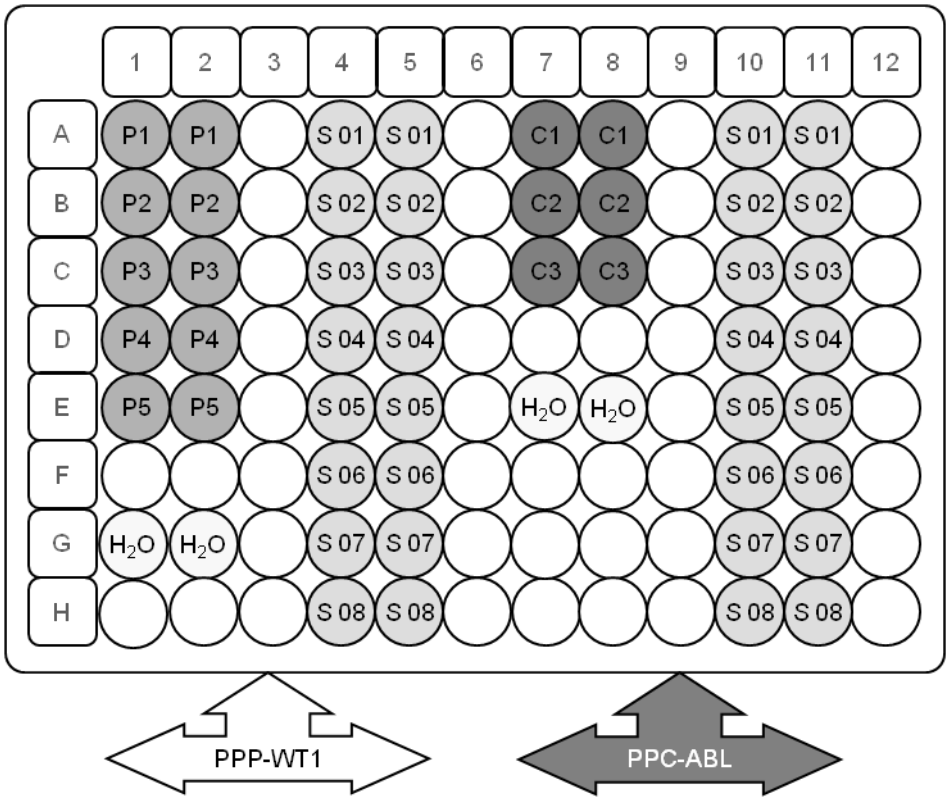

Figure 5. Suggested plate setup for one experiment. S: cDNA sample; **P1–5**: WT1 standards; **C1–3**: ABL standards; **H_2O**: water control.

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

Note: Perform all steps on ice.

Procedure

1. **Thaw all necessary components and place them on ice.**
2. **Prepare the following qPCR mix according to the number of samples being processed.**

   All concentrations are for the final volume of the reaction.

   Table 7 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 PPP-WT1). Extra volumes are included to compensate for pipetting error.

Table 7. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 24 + 1 reactions (µl)	WT1: 28 + 1 reactions (µl)	Final concentration
TaqMan Universal PCR Master Mix, 2x	12.5	312.5	362.5	1x
Primers and probe mix, 25x	1.0	25.0	29.0	1x
Nuclease-free PCR grade water	6.5	162.5	188.5	–
Sample (to be added at step 4)	5.0	5 each	5 each	–
Total volume	25.0	25 each	25 each	–

3. **Dispense 20 µl of the qPCR pre-mix per well.**
4. **Add 5 µl of the RT product (cDNA, 100 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Recommended standardized EAC reverse transcription", page 12) in the corresponding well (total volume 25 µl).**
5. **Mix gently, by pipetting up and down.**
6. **Close the plate and briefly centrifuge (300 x *g*, approximately 10 seconds).**
7. **Place the plate in the thermal cycler according to the manufacturer recommendations. Program the thermal cycler with the thermal cycling program as indicated in Table 8 for ABI PRISM 7900HT SDS or Applied Biosystems 7500 Real-Time PCR System, or Table 9 for the LightCycler 480 instrument.**

Table 8. Temperature profile for ABI PRISM 7900HT SDS or Applied Biosystems 7500 Real-Time PCR System

Mode of analysis	Standard Curve — Absolute Quantitation
Hold	Temperature: 50°C Time: 2 minutes
Hold 2	Temperature: 95°C Time: 10 minutes
Cycling	50 times 95°C for 15 seconds 60°C for 1 minute with acquisition of FAM fluorescence; quencher: TAMRA

Table 9. Temperature profile for LightCycler 480 instrument

Mode of analysis	Absolute Quantification ("Abs Quant")
Detection formats	Select "Simple Probe" in the Detection formats window
Hold	Temperature: 50°C Time: 2 minutes
Hold 2	Temperature: 95°C Time: 10 minutes
Cycling	50 times 95°C for 15 seconds 60°C for 1 minute with acquisition of FAM fluorescence corresponding to (483–533 nm) for LC version 01 and (465–510 nm) for LC version 02

8. For the ABI PRISM 7900HT SDS and Applied Biosystems 7500 Real-Time PCR System, follow step 8a. For the LightCycler 480 instrument, follow step 8b.

8a. ABI PRISM 7900HT SDS and Applied Biosystems 7500 Real-Time PCR System: We recommend a threshold set at 0.1 as described in the EAC protocol in the analysis step and a baseline set between cycles 3 and 15. Start the cycling program, as indicated in Table 8.

8b. LightCycler 480: 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 9.

Protocol: qPCR on LightCycler 1.2 instrument

Using capillary instruments, we recommend measuring samples in duplicate and controls only once, as indicated in Table 10.

Table 10. Number of reactions for LightCycler 1.2 instrument

Samples	Reactions
With the ABL primers and probe mix (PPC-ABL)	
n cDNA samples	n x 2 reactions
ABL standard	1 x 3 reactions (3 standard dilutions, each one tested once)
Water control	1 reaction
With the WT1 primers and probe mix (PPP-WT1)	
n cDNA samples	n x 2 reactions
WT1 standard	1 x 5 reactions (5 standard dilutions, each one tested once)
Water control	1 reaction

Sample processing on LightCycler 1.2 instrument

We recommend testing 5 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.

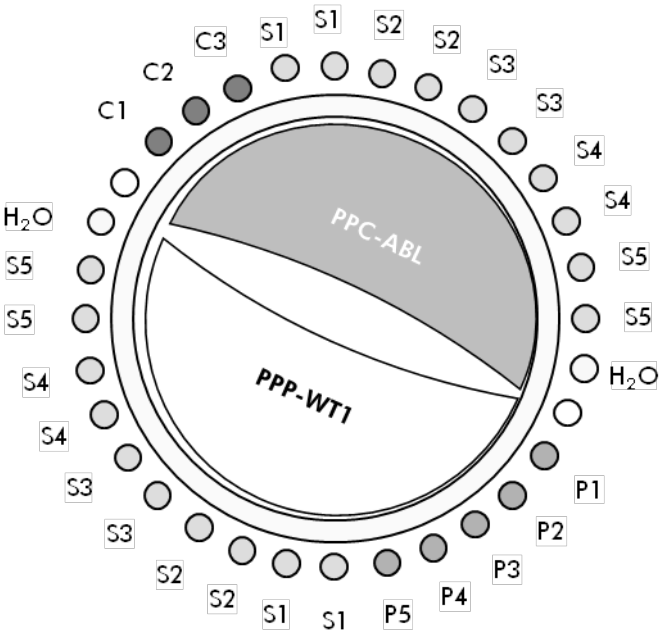

Figure 6. Suggested rotor setup for each experiment with the *ipsogen* WT1 ProfileQuant Kit. P1–5: WT1 standards; **C1–3**: ABL standards; **S**: unknown DNA sample to be analyzed; **H_2O**: water control.

qPCR on LightCycler 1.2 instrument

Note: Because of particular technological requirements, LightCycler experiments must be performed using specific reagents. We recommend to use the LightCycler TaqMan Master and to follow the manufacturer's instructions to prepare the Master Mix 5x.

Note: Perform all steps on ice.

Procedure

1. **Thaw all necessary components and place them on ice.**
2. **Prepare the following qPCR mix according to the number of samples being processed.**

   All concentrations are for the final volume of the reaction.

   Table 11 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 PPP-WT1). Extra volumes are included to compensate for pipetting error.

Table 11. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 14 + 1 reactions (µl)	WT1: 16 + 1 reactions (µl)	Final concentration
Freshly prepared LightCycler TaqMan Master Mix, 5x	4.0	60.0	68.0	1x
Primers and probe mix, 25x	0.8	12.0	13.6	1x
Nuclease-free PCR grade water	10.2	153.0	173.4	–
Sample (to be added at step 4)	5.0	5 each	5 each	–
Total volume	20.0	20 each	20 each	–

3. **Dispense 15 µl of the qPCR pre-mix per capillary.**
4. **Add 5 µl of the RT product (cDNA, 100 ng RNA equivalent) obtained in the reverse transcription (see "Protocol: Recommended standardized EAC reverse transcription", page 12) in the corresponding tube (total volume 20 µl).**
5. **Mix gently, by pipetting up and down.**
6. **Place the capillaries in the adapters provided with the apparatus, and briefly centrifuge (700 x g, approximately 10 seconds).**
7. **Load the capillaries into the thermal cycler according to the manufacturer recommendations.**
8. **Program the LightCycler 1.2 instrument with the thermal cycling program as indicated in Table 12.**

Table 12. Temperature profile

Mode of analysis	Quantification
Hold	Temperature: 95°C Time: 10 minutes Ramp: 20
Cycling	50 times 95°C for 10 seconds; ramp: 20 60°C for 1 minutes; ramp: 20; with acquisition of FAM fluorescence: Single
Hold 2	45°C for 1 minute; ramp: 20

9. **For the LightCycler 1.2 instrument, the F1/F2 and "2nd derivative analysis" mode is recommended. Start the thermal cycling program, as indicated in Table 12.**

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 and use 3 plasmid standard dilutions for the ABL control gene (CG), and 5 standard dilutions for the WT1 gene to ensure accurate standard curves. Figures 7 and 8 show an example of TaqMan amplification curves obtained with the *ipsogen* WT1 Profile*Quant* Kit.

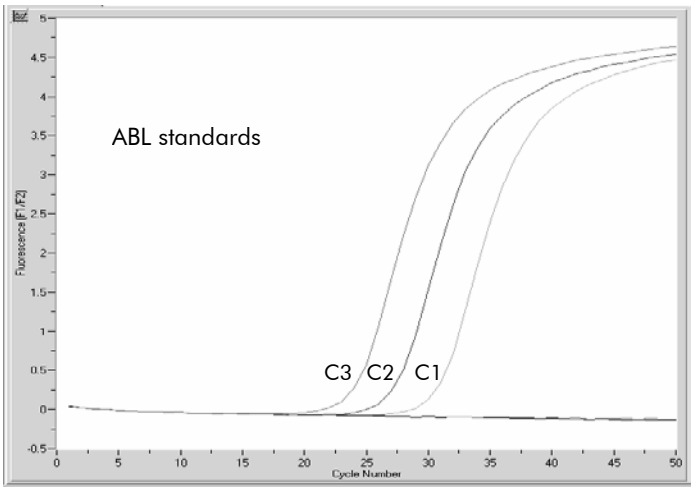

Figure 7. Detection of ABL standards (C1, C2, C3). 10^3, 10^4, and 10^5 copies/5 µl.

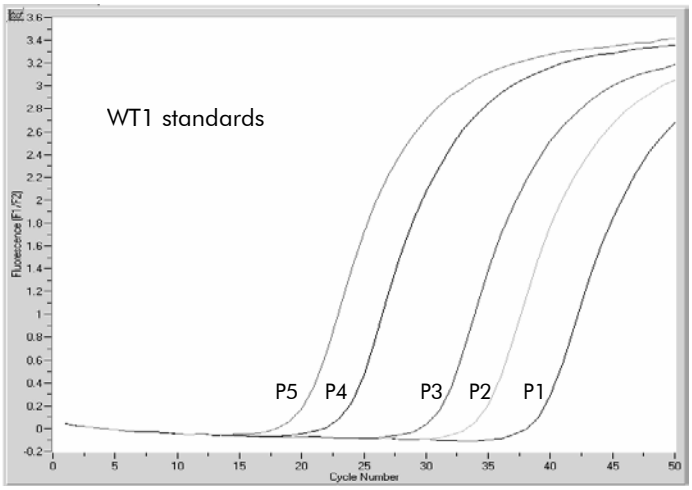

Figure 8. Detection of WT1 standards detection (P1–P5). 10^1, 10^2, 10^3, 10^5, 10^6 copies/5 µl.

Standard curve and quality criteria

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

For each gene (ABL and WT1), raw C_P/C_T 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 P1, P2, P3, P4 and P5). Figure 9 shows an example of the theoretical curve calculated on 5 standard dilutions.

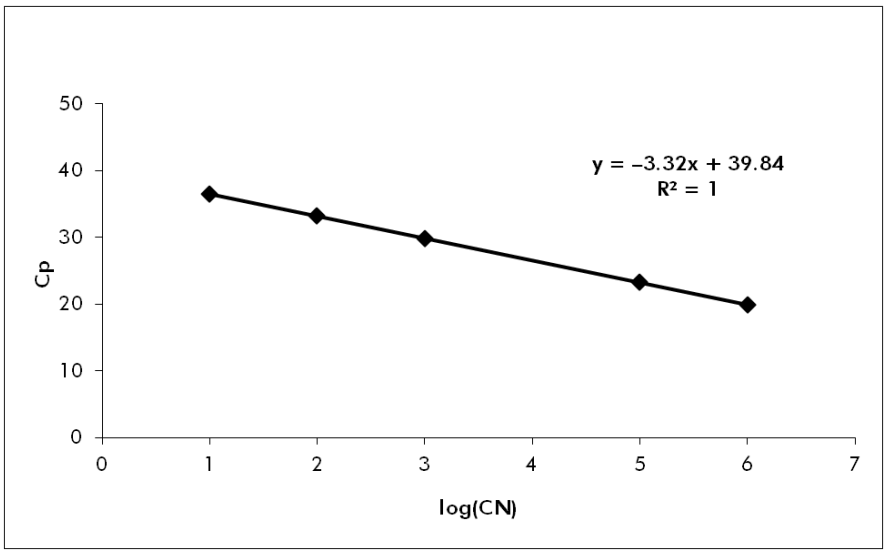

Figure 9. Theoretical curve calculated from the 5 standard dilutions. A linear regression curve (y = ax + b) is calculated for each gene (ABL and WT1), where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (R^2) are printed on the graph.

As standards are tenfold dilutions, the theoretical slope of the curve is –3.32. A slope between –3.0 and –3.9 is acceptable as long as R^2 is >0.95 (3). However, a value for R^2 >0.98 is desirable for precise results (4).

Normalized copy number (NCN)

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

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

The WT1 standard curve equation should be used to transform raw C_P values (obtained with PPP-WT1) for the unknown samples, into WT1 copy numbers ($WT1_{CN}$).

$$\text{Log}_{10}\,\text{sample WT1}_{CN} = \frac{\text{Mean WT1 } C_P - \text{WT1 standard curve intercept}}{\text{WT1 standard curve slope}}$$

The ratio of these CN values gives the normalized copy number (NCN) per 10,000 copies of ABL:

$$NCN = \frac{WT1_{CN}}{ABL_{CN}} \times 10{,}000$$

Quality control on ABL values

Poor quality of the RNA or problems during the qPCR steps result in low ABL_{CN}. We recommend discarding results from samples giving $ABL_{CN} < 4246$.

Reproducibility between replicates

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

Variation in C_P values between replicates is generally <1.5 if the mean C_P value of the replicates is <36 (3).

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

Water controls

Negative controls should give zero CN for both ABL and WT1.

A positive water control results from a cross-contamination. See "Troubleshooting guide", below, to find a solution.

Troubleshooting guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol in this handbook or sample and assay technologies (for contact information, see "Contact Information", page 35).

	Comments and suggestions
Negative result for the control gene (ABL) and WT1 in all the samples — standard okay	
a) Poor RNA quality	Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel.
b) Failure of reverse transcription step	Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel.
Negative result for the control gene (ABL) in the samples — standard okay	
a) Poor RNA quality	Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel.
b) Failure of reverse transcription step	Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel.
Standard signal negative	
a) Pipetting error	Check pipetting scheme and the setup of the reaction. Repeat the PCR run.
b) Inappropriate storage of kit components	Store the *ipsogen* WT1 Profile*Quant* Kit at –15 to –30°C and keep primers and probe mixes (PPC and PPP) protected from light. See "Reagent Storage and Handling", page 11. Avoid repeated freezing and thawing. Aliquot reagents for storage.
Negative controls are 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.

	Comments and suggestions
No signal, even in standard controls	
a) Pipetting error or omitted reagents	Check pipetting scheme and the setup of the reaction. Repeat the PCR run.
b) Inhibitory effects of the sample material, caused by insufficient purification	Repeat the RNA preparation.
c) LightCycler: Incorrect detection channel chosen	Set Channel Setting to F1/F2 or 530 nm/640 nm.
d) LightCycler: No data acquisition programmed	Check the cycle programs. Select acquisition mode "single" at the end of each annealing segment of the PCR program.
Absent or low signal in samples but standard controls okay	
a) Poor RNA quality or low concentration	Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel.
b) Failure of reverse transcription step	Always check the RNA quality and concentration before starting. Run a cell line RNA positive control in parallel.
Fluorescence intensity too low	
a) Inappropriate storage of kit components	Store the *ipsogen* WT1 Profile*Quant* Kit at –15 to –30°C and keep primers and probe mixes (PPC and PPP) protected from light. See "Reagent Storage and Handling", page 11. Avoid repeated freezing and thawing. Aliquot reagents for storage.
b) Very low initial amount of target RNA	Increase the amount of sample RNA. **Note**: Depending of the chosen method of RNA preparation, inhibitory effects may occur.

	Comments and suggestions
LightCycler: Fluorescence intensity varies	
a) Pipetting error	Variability caused by so-called "pipetting error" can be reduced by analyzing data in the F1/F2 or 530 nm/640 nm mode.
b) Insufficient centrifugation of the capillaries	The prepared PCR mix may still be in the upper vessel of the capillary, or an air bubble could be trapped in the capillary tip. Always centrifuge capillaries loaded with the reaction mix as described in the specific operating manual of the apparatus.
c) Outer surface of the capillary tip dirty	Always wear gloves when handling the capillaries.
LightCycler: Error of the standard curve	
Pipetting error	Variability caused by so-called "pipetting error" can be reduced by analyzing data in the F1/F2 or 530 nm/640 nm mode.

Quality Control

Quality control of the complete kit has been performed on a LightCycler 480 instrument. This kit is manufactured according to ISO 13485:2003 standard. Certificates of analysis are available on request at www.qiagen.com/support/.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Cited references

1. Van Dijk J.P. et al. (2003) Abnormal WT1 expression in the CD34-negative compartment in myelodysplastic bone marrow. Br. J. Haematol. **118**, 1027.

2. Gabert, J. et al. (2003) Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia — a Europe Against Cancer program. Leukemia **17**, 2318.
3. van der Velden, V.H. et al. (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia **17**, 1013.
4. Branford, S. et al. (2006) Rationale for the recommendations for harmonizing current methodology for detecting BCR-ABL transcripts in patients with chronic myeloid leukaemia. Leukemia **20**, 1925.

Symbols

The following symbols may appear on the packaging and labeling:

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

Contact Information

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

Ordering Information

Product	Contents	Cat. no.
ipsogen WT1 ProfileQ*uant* (24)	For 24 reactions: ABL Control Gene Standards, WT1 (exon 1-2) Gene Standards, Primers and Probe Mix ABL, Primers and Probe Mix PPP-WT1	676913
Rotor-Gene Q — for outstanding performance in real-time PCR		
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9001580
Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9001650

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

Notes

Notes

This product is intended to be used for life science research only. It is not intended for diagnostic use. *ipsogen* products may not be resold, modified for resale or used to manufacture commercial products without written approval of QIAGEN.

Information in this document is subject to change without notice. QIAGEN assumes no responsibility for any errors that may appear in this document. This document is believed to be complete and accurate at the time of publication. In no event shall QIAGEN be liable for incidental, special, multiple, or consequential damages in connection with, or arising from the use of this document.

ipsogen products are warranted to meet their stated specifications. QIAGEN's sole obligation and the customer's sole remedy are limited to replacement of products free of charge in the event products fail to perform as warranted.

The purchase of this product allows the purchaser to use it for the performance of diagnostic services for human in vitro diagnostics. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.

Limited License Agreement for *ipsogen* WT1 ProfileQ*uant* Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see www.qiagen.com.

www.qiagen.com

Australia ■ techservice-au@qiagen.com

Austria ■ techservice-at@qiagen.com

Belgium ■ techservice-bnl@qiagen.com

Brazil ■ suportetecnico.brasil@qiagen.com

Canada ■ techservice-ca@qiagen.com

China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

Ireland ■ techservice-uk@qiagen.com

Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

Korea (South) ■ techservice-kr@qiagen.com

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

UK ■ techservice-uk@qiagen.com

USA ■ techservice-us@qiagen.com

QIAGEN

1094881 03/2015

Sample & Assay Technologies