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

Σ 24

Version 1

IVD

Quantitative in vitro diagnostics

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

CE

REF 676923

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ELN European LeukemiaNet

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Intended Use

The *ipsogen* WT1 Profile*Quant* Kit is intended for the quantification of Wilm's tumor (WT) gene transcripts in total RNA isolated from patients with acute myeloid leukemia (AML). The results obtained are intended to aid monitoring of early treatment response and for minimal residual disease (MRD).

Summary and Explanation

Current treatment protocols for acute myeloid leukemia (AML) are based on prognostic factors, which contribute to therapy stratification (1, 2). Key prognostic factors so far identified include pre-treatment characteristics such as age and white blood cell (WBC) count as well as patient karyotype and the presence of specific genomic mutations such as FLT3 and NPM1 (3, 4). The morphological response to induction chemotherapy provides a further predictive factor, which has been incorporated into current risk-stratification schemes used to inform decisions regarding consolidation therapy particularly allogeneic transplant (5). While these parameters distinguish groups of patients at broadly different risks of relapse, there is a pressing need to refine risk-stratification to identify more reliably those patients most (or least) likely to benefit from transplant. A number of studies have highlighted the potential of MRD monitoring by real-time quantitative polymerase chain reaction (qPCR) to detect leukemia-specific targets, i.e., fusion gene (FG) transcripts such as PML-RARA, CBFB-MYH11, AML1-ETO (RUNX1-RUNX1T1), or mutations in specific genes like NPM1. This allows the identification of patients at highest risk of relapse and therefore indicates candidates for early treatment intervention (6).

Approximately half of AML patients lack a suitable leukemia-specific target, and there has been considerable interest in developing alternative approaches that would allow MRD monitoring to be applicable to a much larger proportion of patients. One strategy involves use of flow cytometry to identify and monitor leukemia associated aberrant phenotypes, but, while this strategy has wide applicability, it is technically demanding (6). Another approach involves the use of qPCR to detect transcripts that are highly over-expressed in AML blasts relative to normal blood and marrow, with most attention focused on the *WT1* gene (6).

The *WT1* gene is located on chromosome 11p13, encodes a zinc-finger transcription factor, and was originally identified for its involvement in the pathogenesis of Wilms' tumor (7). The *WT1* gene has been shown to be highly expressed in several hematopoietic tumors including AML (7, 8). Although the mechanisms leading to WT1 overexpression remain poorly understood, this phenomenon can be exploited as a marker that indicates the presence, persistence or reappearance of leukemic hematopoiesis.

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.

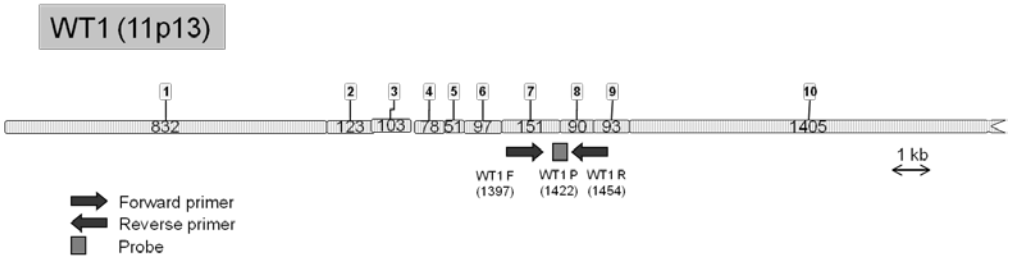

Figure 1. Schematic diagram of the WT1 transcript covered by the ELN qPCR primers and probe set: WT1-ELN F–WT1-ELN P–WT1-ELN R. The number under the primers and probe refers to their nucleotide position in the normal gene transcript. Exon 5 may be alternatively spliced.

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.

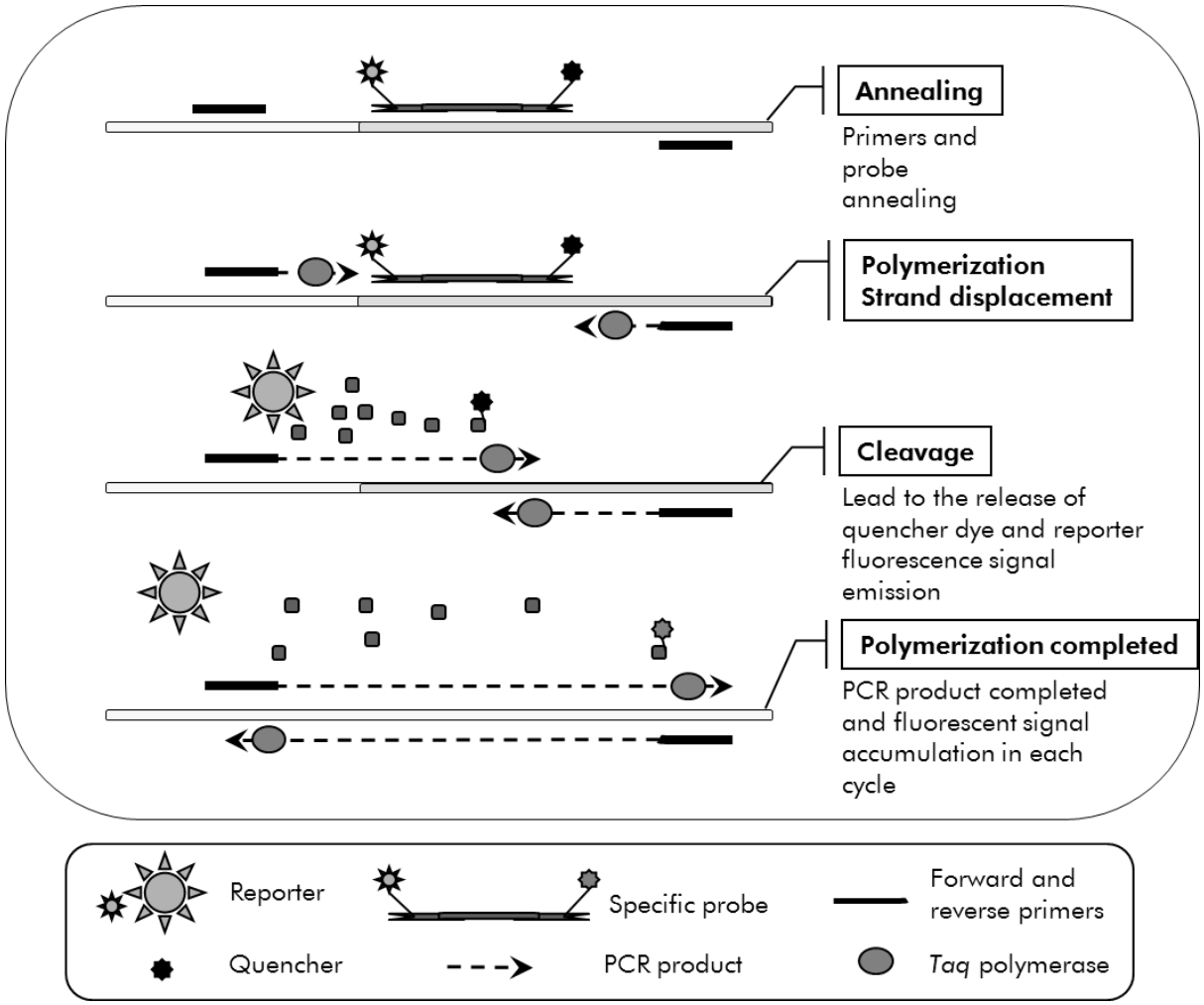

Figure 2. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–TAMRA™). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer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

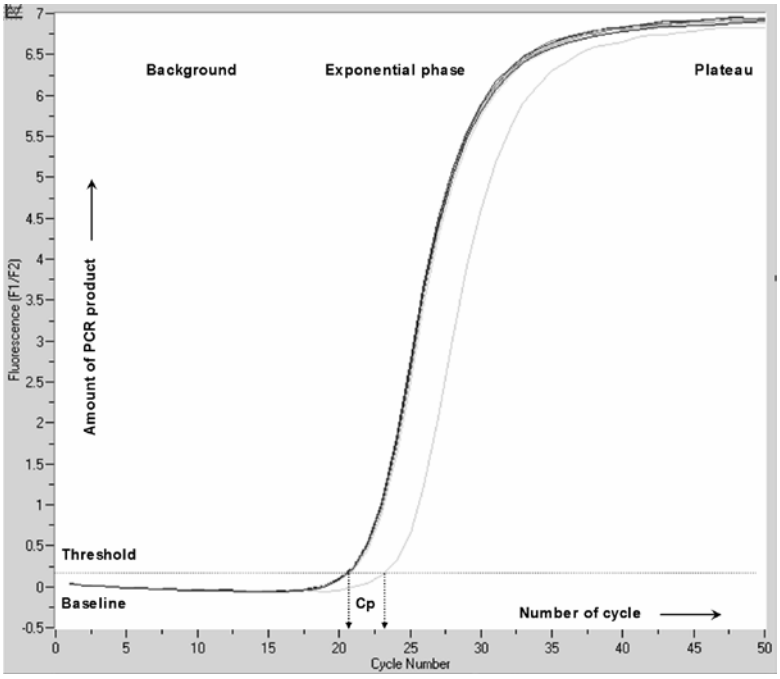

Figure 3. Fluorescence acquisition during cycling and successive phases of amplification.

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 validated 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 (9). 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.		**676923**
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 *Kit Handbook (English)*		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 validated 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 validated reagent is RNaseOUT™ (Life Technologies, cat. no. 10777-019)
- Set of dNTPs, PCR grade
- Random hexamer
- $MgCl_2$
- Buffer and *Taq* DNA polymerase: The validated 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)

Note: Ensure that the thermal cycler or water bath have been checked and calibrated according to the manufacturer's recommendations.

Warnings and Precautions

For in vitro diagnostic use

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

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

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 in vitro diagnostic use. Reagents and instructions supplied in this kit have been validated for optimal performance. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data. PPC-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 test, no substitutions should be made.

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

Use extreme caution to prevent:

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

We therefore recommend the following.

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Prepare pre-PCR master mix with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrixes (cDNA, DNA, plasmid) are introduced. Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).
- Handle the 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 patient samples (blood or bone marrow) must have been performed using 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 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 (10, 11).

Protocol: qPCR on Rotor-Gene Q MDx 5plex HRM or Rotor-Gene Q 5plex HRM instruments with 72-tube rotor

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

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.

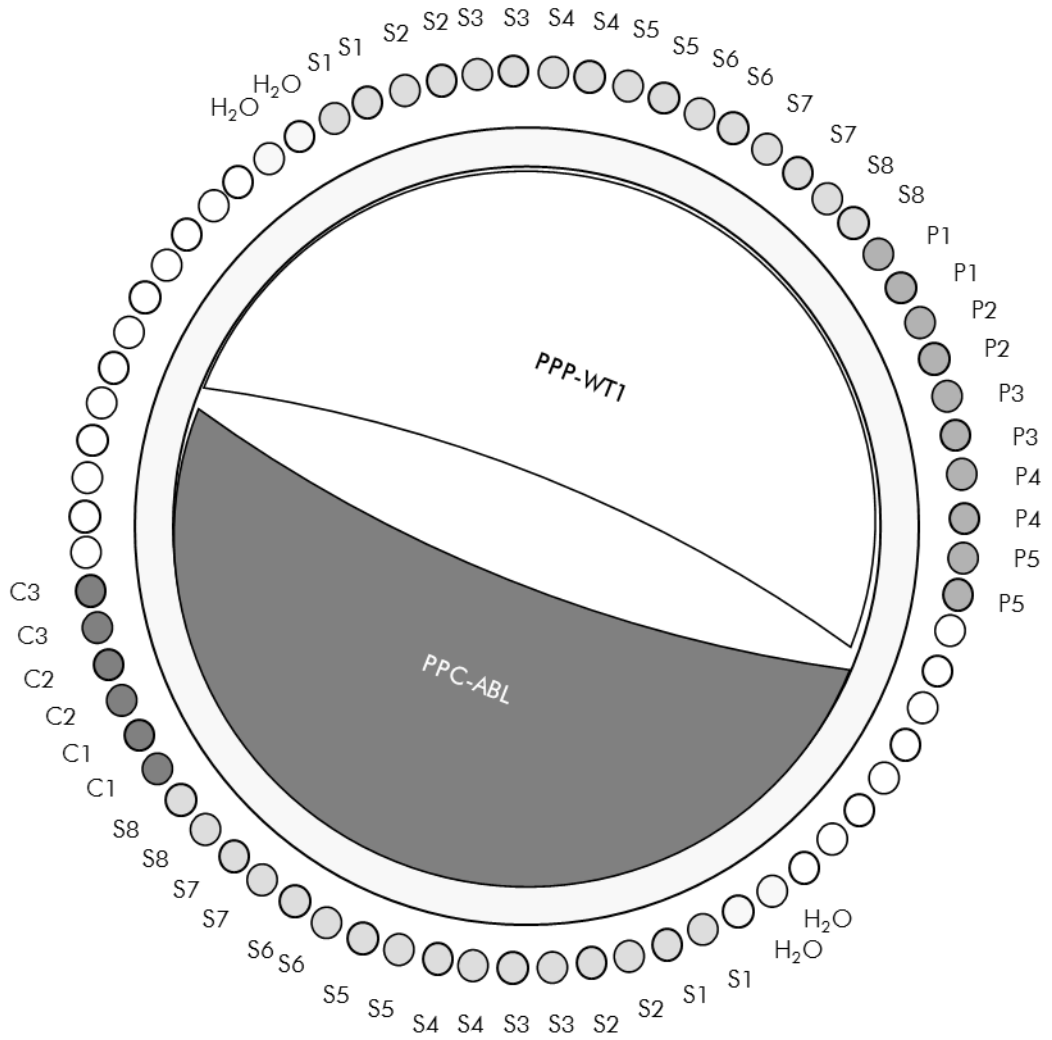

Figure 4. Suggested rotor setup for each experiment with the *ipsogen* WT1 ProfileQuant Kit. P1–5: WT1 standards; **C1–3**: ABL standards; **S**: cDNA sample; **H_2O**: water control.

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.

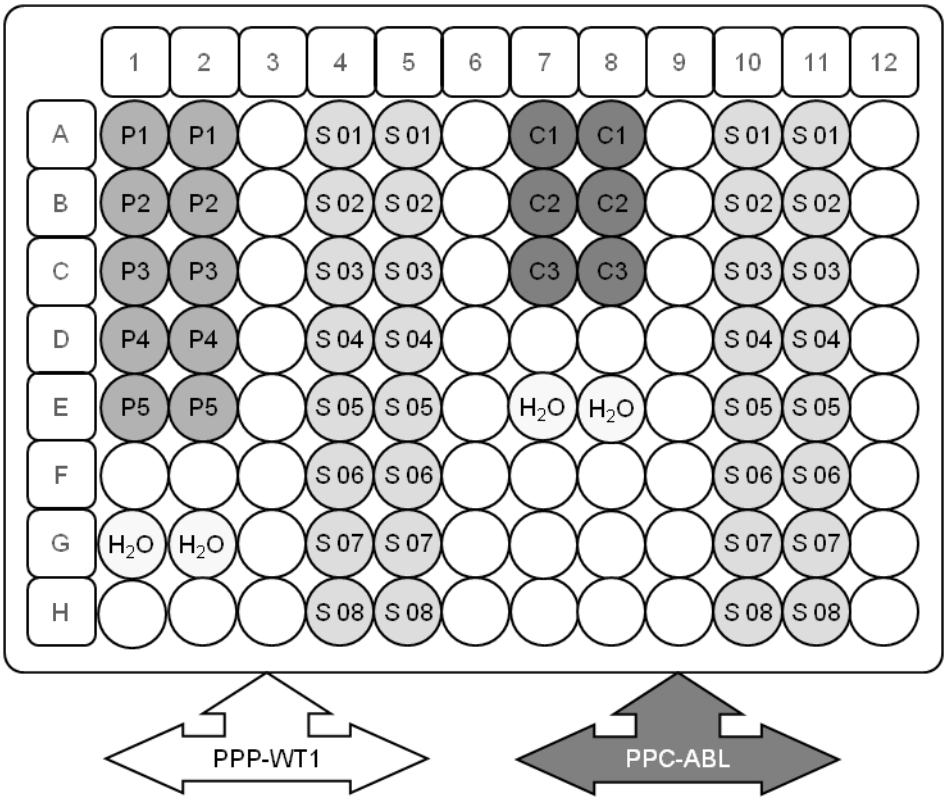

Figure 5. Suggested plate setup for one experiment. S: cDNA sample; **P1–5**: WT1 standards; **C1–3**: ABL standards; **H_2O**: water control.

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.

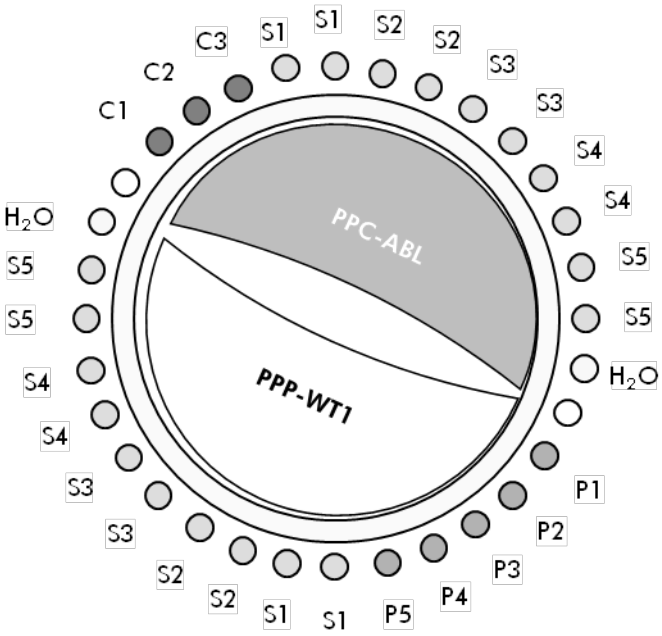

Figure 6. Suggested rotor setup for each experiment with the *ipsogen* WT1 Profile*Quant* Kit. P1–5: WT1 standards; **C1–3**: ABL standards; **S**: unknown DNA sample to be analyzed; **H_2O**: water control.

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.**

Interpretation of Results

Data analysis principle

Using TaqMan technology, the number of PCR cycles necessary to detect a signal above the threshold is called the threshold cycle (C_T) and is directly proportional to the amount of target present at the beginning of the reaction.

Using standards with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample. The *ipsogen* standard curves are plasmid-based 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.

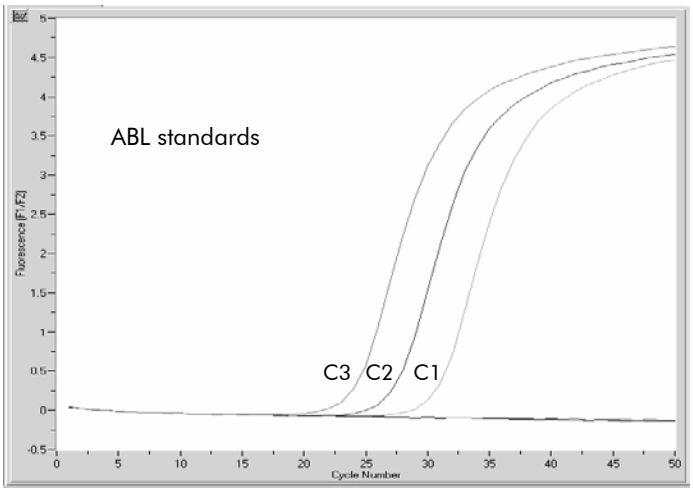

Figure 7. Detection of ABL standards (C1, C2, C3). 10^3, 10^4, and 10^5 copies/5 µl.

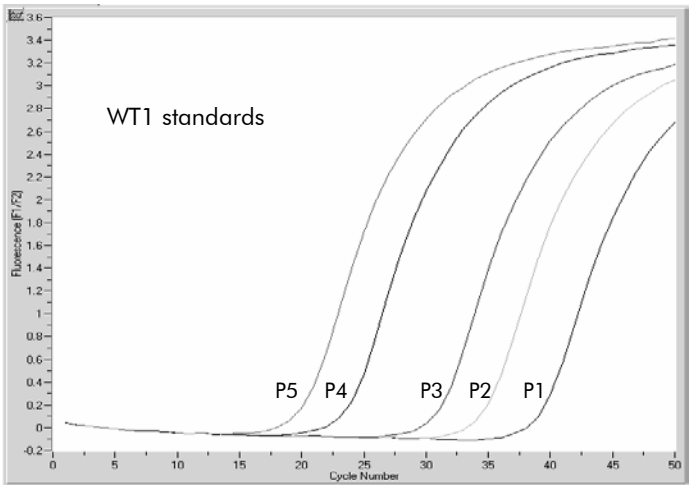

Figure 8. Detection of WT1 standards detection (P1–P5). 10^1, 10^2, 10^3, 10^5, 10^6 copies/5 µl.

Results

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.

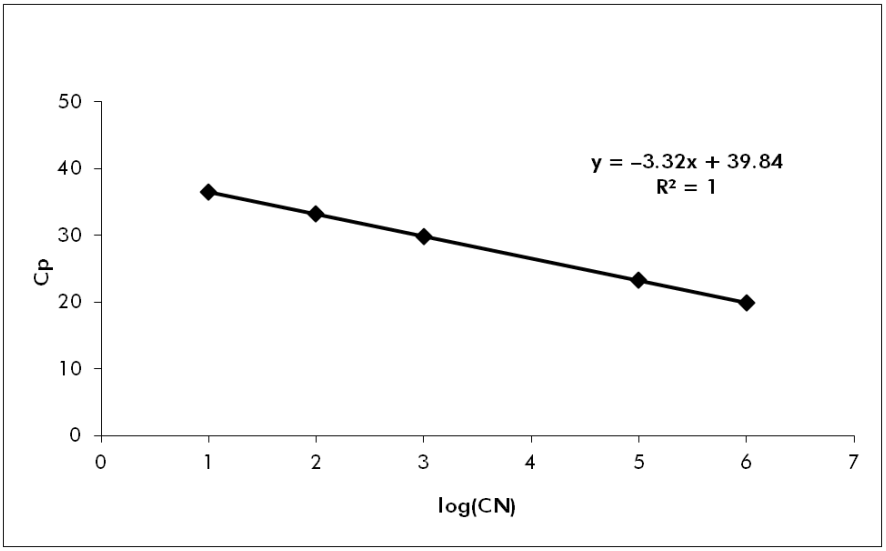

Figure 9. Theoretical curve calculated from the 5 standard dilutions. A linear regression curve (y = ax + b) is calculated for each gene (ABL and WT1), where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and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

As standards are 10-fold 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 (12). However, a value for R^2 >0.98 is desirable for precise results (13).

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 4-fold 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 (12).

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 40).

	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/.

Limitations

The users must be trained and familiar with this technology prior the use of this device. This kit should be used following the instructions given in this manual, in combination with a validated instrument mentioned in "Materials Required but Not Provided", page 9.

Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings. It is the user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by the QIAGEN performance studies.

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

Note: The kit has been designed according to the "European LeukemiaNet" (ELN) studies (10, 11). It should be used following the instructions given in this manual, in combination with validated reagents and instruments. Any off-label

use of this product and/or modification of the components will void QIAGEN's liability.

Performance Characteristics

Nonclinical studies

Materials and methods

Linearity studies were performed on 14 samples; each one obtained from a different mix of RNA extracted from high expression cell line and healthy donors' samples that had a low level of expression for the WT1 gene. Each sample was tested in triplicate. For NCN, values ranged from 2.20 to 3838.11 NCN and this study showed that the *ipsogen* WT1 Profile*Quant* Kit gave linear results over this range of values.

Precision

Precision study was performed on 4 samples; each one obtained from a different mix of RNA extracted from cell lines with high and low WT1 expression. These assays were repeated up to 16 times for each sample. Analytical data are summarized in the following tables.

Table 13. Analytical data from the precision study — plasmids

	Dilution	Mean C_T	σ	n	CV (%)
WT1 plasmids	P1: 10^1 copies/5 µl	36.13	0.87	15	2.42
	P2: 10^2 copies/5 µl	32.70	0.40	16	1.21
	P3: 10^3 copies/5 µl	29.39	0.43	16	1.45
	P4: 10^5 copies/5 µl	22.62	0.41	16	1.80
	P5: 10^6 copies/5 µl	19.25	0.38	16	1.98
ABL plasmids	C1: 10^3 copies/5 µl	29.59	0.35	16	1.20
	C2: 10^4 copies/5 µl	26.11	0.40	15	1.52
	C3: 10^5 copies/5 µl	22.77	0.28	16	1.22

Table 14. Analytical data from the precision study — cell lines

	Dilution	Mean NCN	σ	n	CV (%)
Cell line RNA dilution	10%	10,472	5598.76	16	53
	1.5%	1880	747.01	16	40
	0.05%	86	37.79	16	44
	0.0025%	3	1.90	16	57

Limit of blank and limit of detection

The study design was based on recommendations described in the NCCLS document EP17-A Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline. The background level or limit of blank (LOB) was determined on normal blood samples from healthy donors (4 samples, 73 measurements). This was found to be equal to 3.66 WT1 NCN.

The limit of detection (LOD), which indicates analytical sensitivity, was determined on samples with known low expression of WT1 obtained from healthy donors and spiked with high WT1 expression level cells. This ensured that the expected NCN value was 4-fold the LOB. A total of 4 samples and 72 measurements were made and the LOD was found to be equal to 13.08 WT1 NCN.

Clinical studies

As WT1 is expressed in normal hematopoietic cells it is critical to establish the level of expression seen in normal control samples so that a threshold can be defined that distinguishes between residual leukemia and background amplification. Analysis of 204 control samples derived from healthy volunteers using the ELN assay utilized in the *ipsogen* WT1 Profile*Quant* Kit confirmed that very low expression of WT1 is seen in peripheral blood, bone marrow, and peripheral blood stem cells samples. Median values were 19.8 WT1 copies /10^4 ABL copies (range 0–213) in bone marrow, 0.01 (range 0.01–47.6) in peripheral blood and 6.1 (range 0–39) in peripheral blood stem cells (see Figure 10). The expression of WT1 in peripheral blood was significantly lower than in bone marrow ($p<0.0001$). Based on these results, the upper limit of normal was defined as 250 NCN for bone marrow and 50 NCN for peripheral blood.

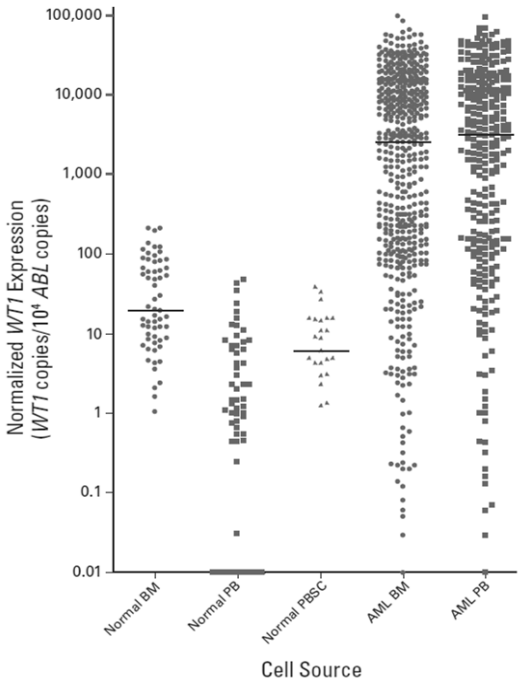

Figure 10. WT1 expression in samples from healthy donors. Acute myeloid leukemia (**AML**); Bone marrow (**BM**); Peripheral blood (**PB**); Peripheral blood stem cells (**PBSC**). (15)

Defining WT1 expression by standardized ELN qPCR assay in pre-treatment AML samples

To evaluate the applicability of the ELN assay utilized in the *ipsogen* WT1 Profile*Quant* Kit to detect MRD, 620 pre-treatment samples (238 from peripheral blood and 382 from bone marrow) were analyzed from 504 patients.

WT1 was overexpressed above background levels (defined as >250 and >50 WT1 copies/10^4 ABL copies in bone marrow and peripheral blood, respectively) in 86% and 91% of bone marrow and peripheral blood diagnostic AML samples (also shown in Figure 10).

The median value of WT1 copies /10^4 ABL copies was 2505, (range 0–7.5×10^5) in bone marrow ($p<0.0001$ vs normal bone marrow) and 3107 (range 0–1.13×10^6) in peripheral blood ($p<0.0001$ vs normal peripheral blood). There was no significant difference in expression between peripheral blood and bone marrow across the whole cohort, as confirmed by results obtained in patients with paired diagnostic peripheral blood and bone marrow samples, see Cilloni D et al., J Clin Oncol, Figure A3 in the Appendix (15).

Variation in normalized WT1 expression level was observed according to cytogenetics (Figure 11, $p<0.001$), with particularly high levels in cases with

inv(16)(p13q22)/t(16;16)(p13;q22) (median 2.31 x 10^4, range 12–3.14 x 10^5). Significantly higher WT1 levels were also detected in AML with NPM1 mutations (NPM1 mutant: median 1.44 x 10^4, range 0–1.13 x 10^6; NPM1 wild type: median 6566, range 0–7.5 x 10^5, p = 0.005).

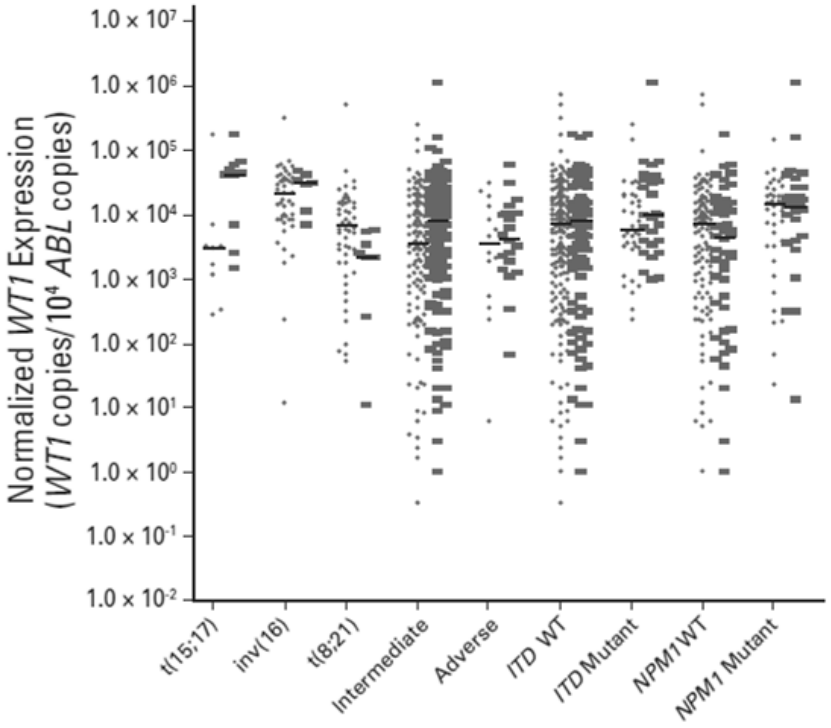

Figure 11. Variation of WT1 expression according to cytogenetics (15).

Reprinted with permission from Cilloni D et al: Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized *WT1* assay to enhance risk stratification in acute myeloid leukemia: A European LeukemiaNet Study: *J Clin Oncol* 27(31):5195-201, 2009. © 2009, American Society of Clinical Oncology, All rights reserved.

The level of WT1 expression as defined by ELN assay in 15 cases harboring mutations in exons 7 and 9 of the WT1 gene were comparable to those with wild type WT1 (p=0.2). However, sequence analysis of a series of 32 cases in which the ELN assay suggested a low level of WT1 transcript expression (<250 copies/10^4 ABL copies), indicated that in 3 cases (9.4%) this low level of expression was associated with mutations that disrupted the forward primer binding site, see Cilloni D et al., J Clin Oncol, Figure A4 in the Appendix (15).

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.

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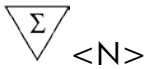
15. Cilloni D. et al., Real-time quantitative polymerase chain reaction detection of minimal residual disease b standardized *WT1* assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet Study. J Clin Oncol **27**, 5195.

Symbols

The following symbols may appear on the packaging and labeling:

	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Consult instructions for use

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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	676923
Rotor-Gene Q MDx — for IVD-validated real-time PCR analysis in clinical applications		
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9002032
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002033

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