January 2013

ipsogen® BAALC Profile*Quant*® Handbook

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For research use only. Not for use in diagnostic procedures

For use with Rotor-Gene® Q 5plex HRM®, ABI PRISM® 7700 and 7900HT SDS, LightCycler®, and SmartCycler® instruments

REF 676613

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GERMANY

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Contents

Intended Use

The *ipsogen* BAALC Profile*Quant* Kit is 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 of recombinant DNA experiments, or to other applicable guidelines.

Principle of the Procedure

The use of qPCR permits the quantification of PCR products during the exponential phase of the PCR amplification process. Quantitative PCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during and/or subsequent to PCR cycling, thereby reducing the risk of PCR product contamination. At present, 3 main types of qPCR techniques are available: qPCR analysis using SYBR® Green I Dye, qPCR analysis using hydrolysis probes, and qPCR analysis using hybridization probes.

This assay exploits the qPCR double-dye oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. A double-dye oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream, 3' quencher dye, hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes exploits the 5'→3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reported 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 reported 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 1). This process occurs every cycle and does not interfere with the exponential accumulation of the 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, increase in fluorescence is directly proportional to the target amplification during PCR.

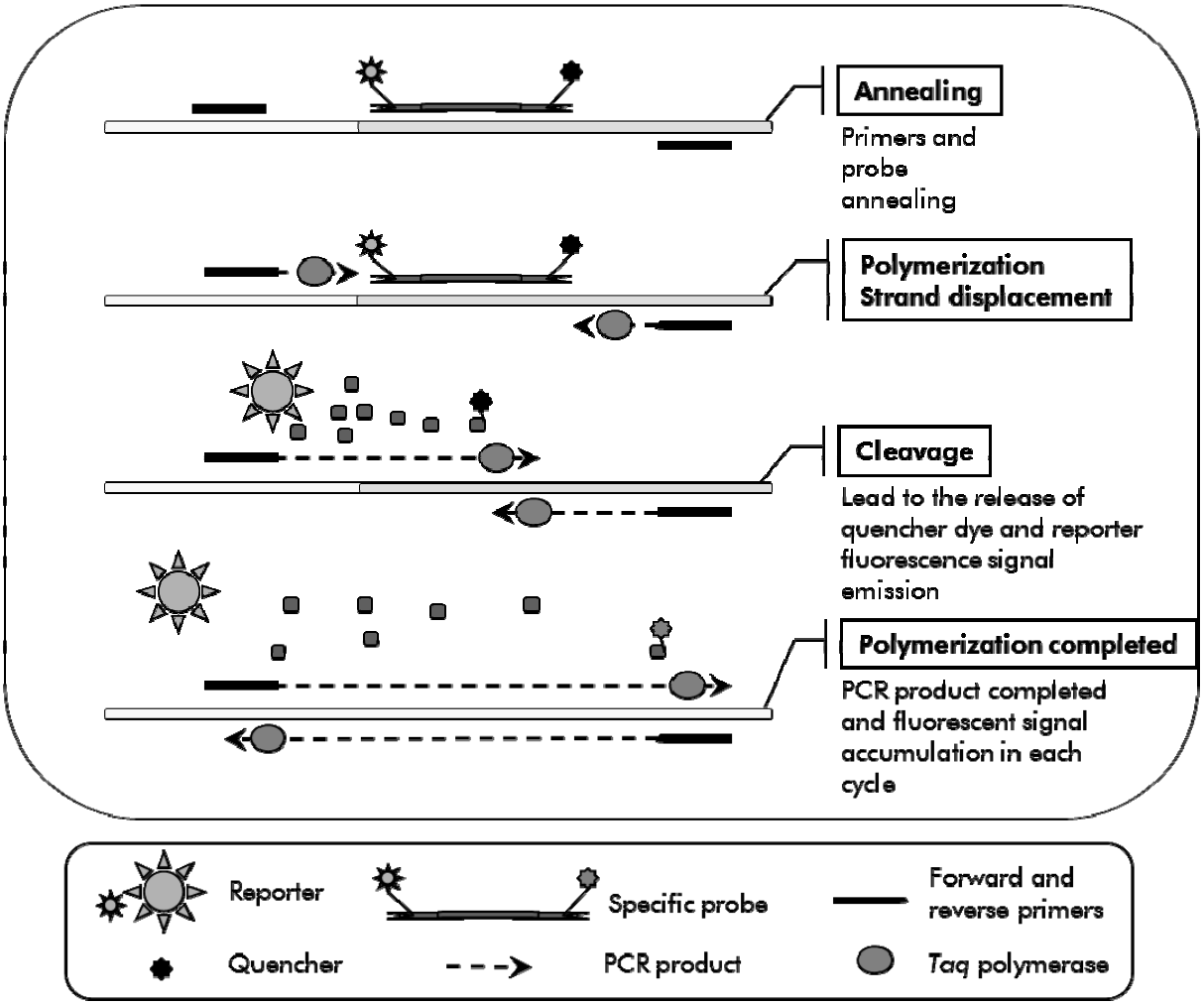

Figure 1. Reaction principle.

Materials Provided

Kit contents

***ipsogen* BAALC Profile *Quant* Kit**		**(24)**
Catalog no.		**676613**
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
BAALC Profile Standard Dilution (10^1 copies/5 µl)	P1-BAALC	50 µl
BAALC Profile Standard Dilution (10^2 copies/5 µl)	P2-BAALC	50 µl
BAALC Profile Standard Dilution (10^3 copies/5 µl)	P3-BAALC	50 µl
BAALC Profile Standard Dilution (10^5 copies/5 µl)	P4-BAALC	50 µl
BAALC Profile Standard Dilution (10^6 copies/5 µl)	P5-BAALC	50 µl
Primers and Probe Mix ABL*	PPC-ABL 25x	90 µl
Primers and Probe Mix BAALC†	PPP-BAALC 25x	110 µl
ipsogen *BAALC Profile*Quant *important note*		1

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

† Mix of specific reverse and forward primers for the *BAALC* gene plus a specific FAM–TAMRA probe.

Note: Vortex and 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 lab 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 nonamer
- $MgCl_2$
- 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 nuclease-free PCR tubes
- 0.1 ml strip tubes and caps if using the Rotor-Gene Q 5plex HRM instrument
- 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 (capable of attaining 13,000 to 14,000 rpm)
- Real-time PCR instrument:* Rotor-Gene Q 5plex HRM; LightCycler; ABI PRISM 7700 or 7900HT SDS; or SmartCycler instrument; and associated specific material
- Biophotometer

* 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 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.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

General precautions

Use of qPCR tests require good laboratory practices, including maintenance of equipment, that are dedicated to molecular biology and are 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 and PPP reagents may be altered if exposed to light. All reagents are formulated specifically for use with this kit. 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 (pipes, 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

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.

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 lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available for the product supplier.

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 nonamer (100 µM)	5.0	25 µM
Superscript II or Superscript Reverse Transcriptase (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
Heated sample RNA (1 µg)	1–4
Nuclease-free PCR grade water	0–3
Final volume	20

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.**

Protocol: qPCR on Rotor-Gene Q 5plex HRM Instruments

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 with 0.1 ml strip tubes and caps

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 BAALC primers and probe mix (PPP-BAALC)	
n cDNA samples	n x 2 reactions
BAALC 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 with 0.1 ml strip tubes and caps

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. Each *ipsogen* BAALC Profile*Quant* Kit provides enough reagents to perform an 8-sample experiment 3 times using the 72-tube rotor.

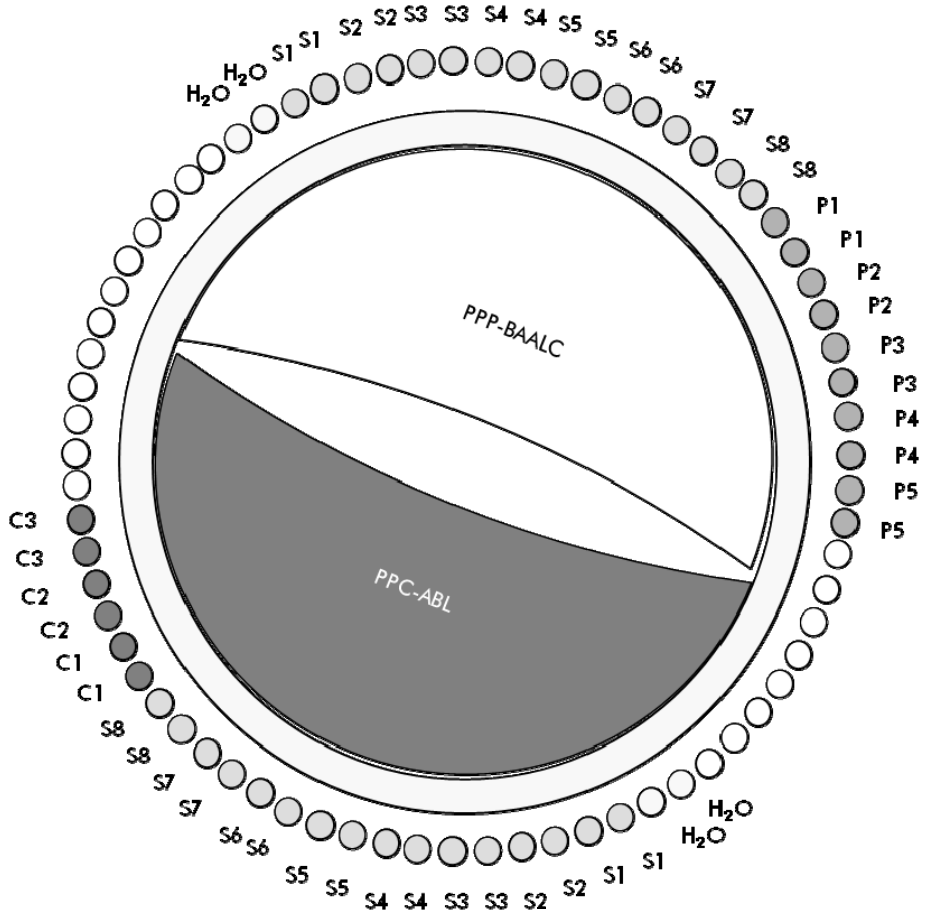

Figure 2. Suggested rotor setup for each experiment with the *ipsogen* BAALC Profile*Quant* Kit. P1–5: BAALC standards; **C1–3**: ABL standards; **H_2O**: water control; **S**: cDNA sample.

Note: Take care to always place a sample to be tested in position 1 of the rotor. Otherwise, during the calibration step, the instrument will not perform calibration, and incorrect fluorescence data will be acquired.

Fill all other positions with empty tubes.

qPCR on Rotor-Gene Q instruments with 72-tube rotor with 0.1 ml strip tubes and caps

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-BAALC). Extra volumes are included to compensate for pipetting error.

Table 4. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 24+1 reactions (µl)	BAALC: 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	25.0	29.0	1x
Nuclease-free PCR grade water	6.5	162.5	188.5	–
Sample (to be added at step 4)	5	5 each	5 each	–
Total volume	25	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 10) 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 1	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 7700 and 7900HT SDS Instruments

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

Sample processing on ABI PRISM 7700 and 7900HT SDS 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 3 shows an example of such an experiment.

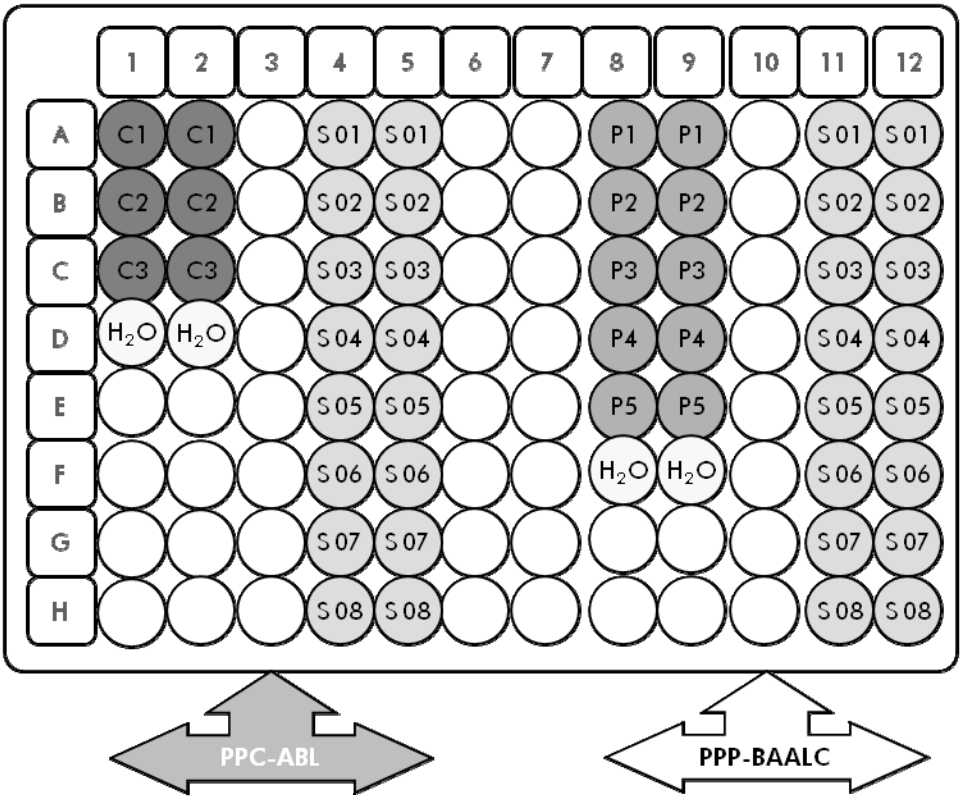

Figure 3. Suggested plate setup for one experiment. P1–5: BAALC standards; **C1–3**: ABL standards; **H_2O**: water control; **S**: cDNA sample.

qPCR on ABI PRISM 7700 and 7900HT SDS 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. If using 96-well-plate qPCR equipment, we recommend performing all measurements in duplicate.**

   All concentrations are for the final volume of the reaction.

   Table 7 describes the pipetting scheme for the preparation of one reagent mix, 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-BAALC). Extra volumes are included to compensate for pipetting error.

Table 7. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 24+1 reactions (µl)	BAALC: 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	25.0	29.0	1x
Nuclease-free PCR grade water	6.5	162.5	188.5	–
Sample (to be added at step 4)	5	5 each	5 each	–
Total volume	25	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 10) 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.**
8. **Program the thermal cycler with the thermal cycling program as indicated in Table 8 for ABI PRISM 7700 and 7900HT SDS instruments.**

Table 8. Temperature profile for ABI PRISM 7700 and 7900HT SDS instruments

Mode of analysis	Standard Curve — Absolute Quantitation
Hold 1	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

9. **We recommend a threshold set at 0.1 as described in the EAC protocol in the analysis step on the ABI PRISM 7700 and 7900HT SDS instruments, and a baseline set between cycles 3 and 15. Start the cycling program, as indicated in Table 8.**

Protocol: qPCR on LightCycler Instruments

Using capillary instruments, we recommend measuring samples in duplicate and controls only once, as indicated in Table 9.

Table 9. Number of reactions for LightCycler instruments

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 BAALC primers and probe mix (PPP-BAALC)	
n cDNA samples	n x 2 reactions
BAALC standard	1 x 5 reactions (5 standard dilutions, each one tested once)
Water control	1 reaction

Sample processing on LightCycler instruments

We recommend testing at least 5 cDNA samples in the same experiment to optimize the use of the standards and primers and probe mixes. The capillary scheme in Figure 4 shows an example of an experiment.

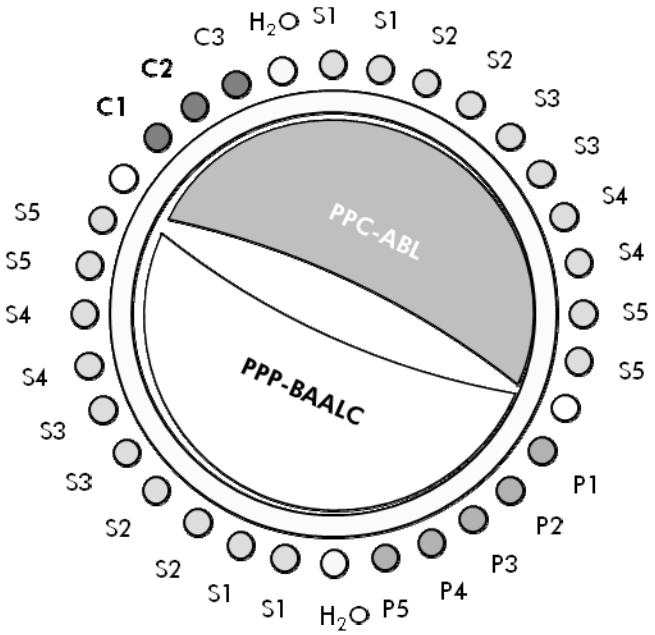

Figure 4. Suggested rotor setup for each experiment with the *ipsogen* BAALC Profile*Quant* Kit. P1–5: BAALC standards; **C1–3**: ABL standards; **H_2O**: water control; **S**: unknown cDNA sample to be analyzed.

qPCR on LightCycler instruments

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 10 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-BAALC). Extra volumes are included to compensate for pipetting error.

Table 10. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 14+1 reactions (µl)	BAALC: 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	5.0 each	–
Total volume	20.0	20 each	20.0 each	–

4. **Dispense 15 µl of the qPCR pre-mix per capillary.**
5. **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 10) in the corresponding tube (total volume 20 µl).**
6. **Mix gently, by pipetting up and down.**
7. **Place the capillaries in the adapters provided with the instrument, and briefly centrifuge (700 x *g*, approximately 10 seconds).**
8. **Load the capillaries into the thermal cycler according to the manufacturer recommendations.**
9. **Program the LightCycler instrument with the thermal cycling program as indicated in Table 11.**

Table 11. Temperature profile

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

10. For the LightCycler 1.2, follow step 9a. For the LightCycler 2.0, follow step 9b.

10a. LightCycler 1.2: The F1/F2 and "2nd derivative analysis" mode is recommended. Start the thermal cycling program, as indicated in Table 11.

10b. LightCycler 2.0: We recommend using Automated (F''max) analysis on LightCycler 2.0 Software version 4.0 to obtain reproducible results. Start the thermal cycling program, as indicated in Table 11.

Protocol: qPCR on the SmartCycler Instrument

Using this instrument, we recommend measuring samples in duplicate and controls only once, as indicated in Table 12.

Table 12. Number of reactions for the SmartCycler 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 BAALC primers and probe mix (PPP-BAALC)	
n cDNA samples	n x 2 reactions
BAALC standard	1 x 5 reactions (5 standard dilutions, each one tested once)
Water control	1 reaction

Sample processing on the SmartCycler instrument

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

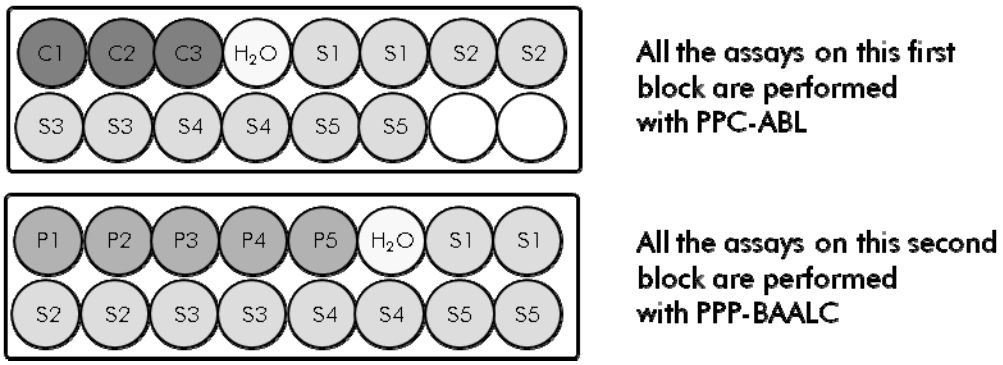

Figure 5. Suggested plate setup for one experiment. P1–5: BAALC standards; **C1–3**: ABL standards; **H_2O**: water control; **S**: cDNA sample.

qPCR on the SmartCycler instrument

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 13 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-BAALC). Extra volumes are included to compensate for pipetting error.

Table 13. Preparation of qPCR mix

Component	1 reaction (µl)	ABL: 14+1 reactions (µl)	BAALC: 16+1 reactions (µl)	Final concentration
TaqMan Universal PCR Master Mix, 2x	12.5	187.5	212.5	1x
Primers and probe mix, 25x	1	15.0	17.0	1x
Nuclease-free PCR grade water	6.5	97.5	110.5	–
Sample (to be added at step 4)	5	5 each	5 each	–
Total volume	25	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 10) in the corresponding well (total volume 25 µl).**
5. **Mix gently, by pipetting up and down.**
6. **Load the samples into the thermal cycler according to the manufacturer's recommendations.**
7. **Program the SmartCycler instrument with the thermal cycling program as indicated in Table 14.**

Table 14. Temperature profile

Hold 1	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: Single

9. **We recommend a threshold set at 30. Start the thermal cycling program, as indicated in Table 14.**

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, a standard curve can be established and the precise amount of target present in the test sample determined. The theoretical slope of the standard curve is -3.32 for PCR with an optimal efficiency. Figures 6 and 7 show an example of TaqMan amplification curves obtained with the *ipsogen* BAALC Profile*Quant* Kit.

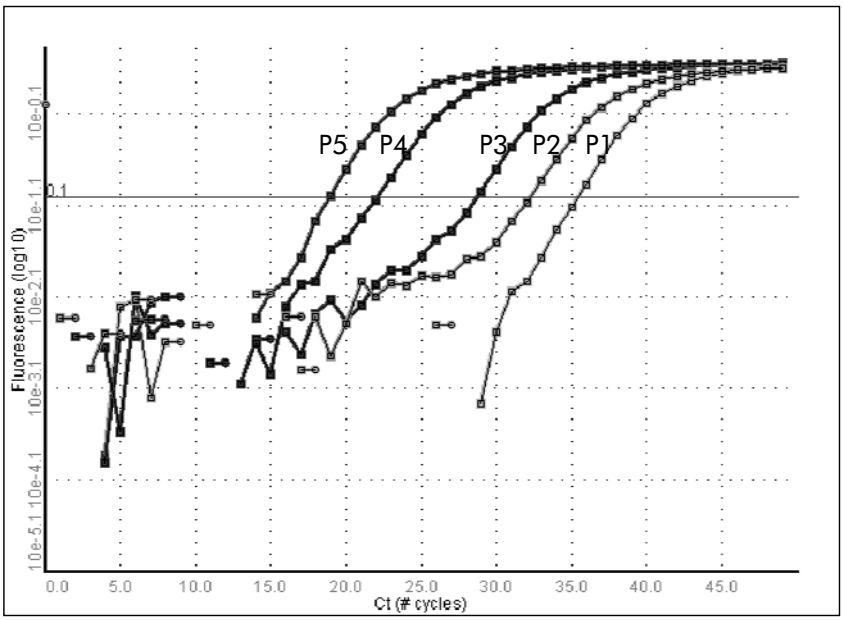

Figure 6. Amplification plot of BAALC standards (P1–P5). 10^1, 10^2, 10^3, 10^5, and 10^6 copies/5 µl.

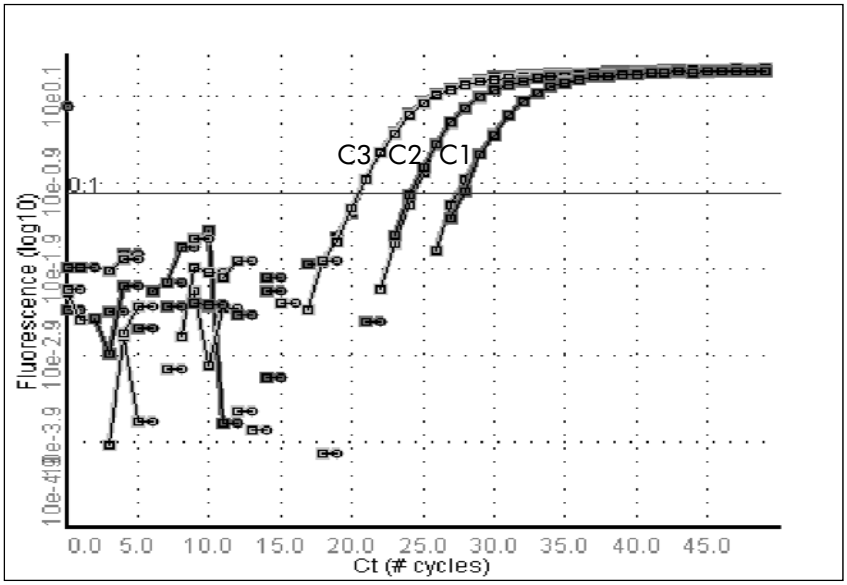

Figure 7. Amplification plot of ABL standards (C1, C2, C3). 10^3, 10^4, and 10^5 copies/5 µl.

Standard curve and quality criteria

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

For each gene (ABL and BAALC), raw C_T values obtained from 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 8 shows an example of the theoretical curve calculated on 5 standard dilutions of the BAALC standards and Figure 9 shows a standard curve for the ABL standards calculated on 3 standard dilutions. A linear regression curve ($y = ax + b$) is calculated for each gene (ABL and BAALC), where a is the slope of the line and b is the y-intercept, which is the y-coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (R^2) are printed on the graph.

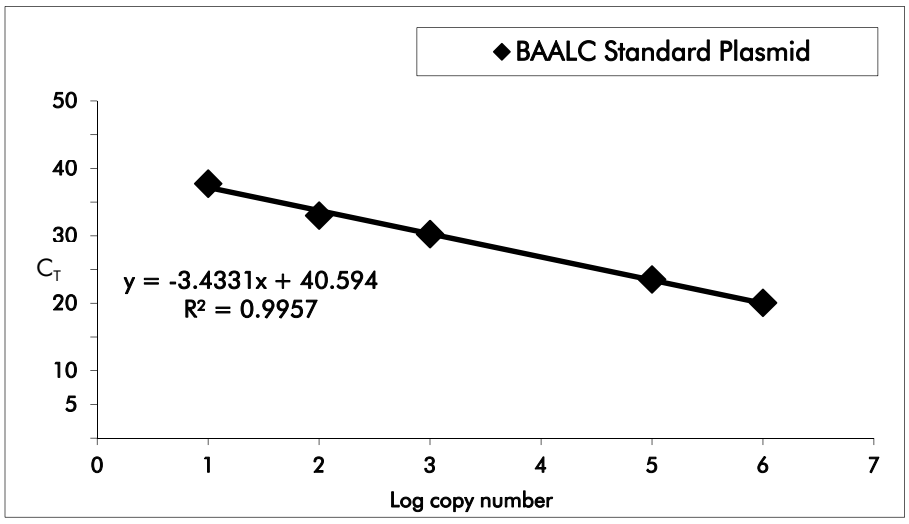

Figure 8. BAALC standard curve calculated from the 5 standard dilutions.

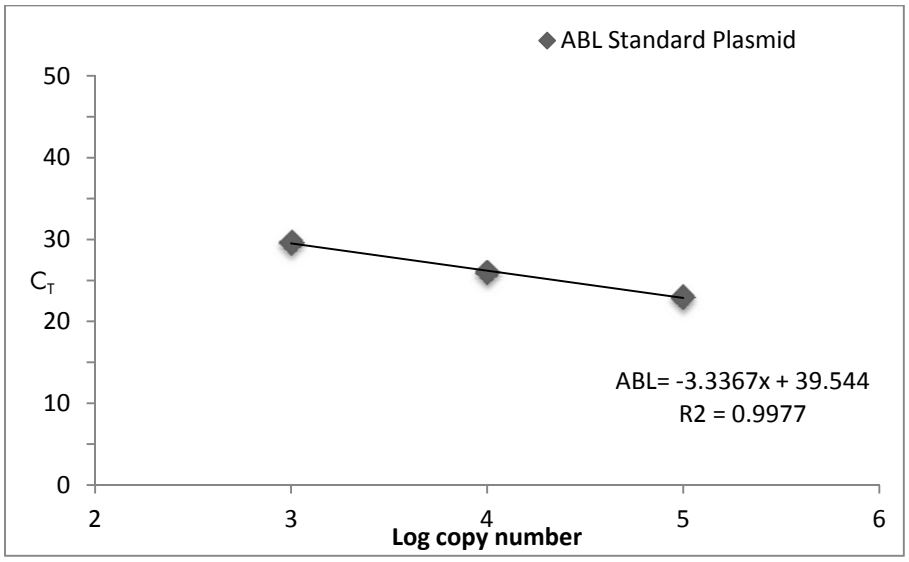

Figure 9. ABL standard curve calculated from the 3 standard dilutions.

PCR efficiency and C_T – copy number correlation

Amplification of different target genes usually gives different results as the efficiency of primer annealing, GC content of the sequence to be amplified, and PCR product size varies. This means that a standard curve needs to be prepared for all target genes. Due to differences in PCR efficiency, the resulting standard curves will not be parallel (and hence have different slopes) and the difference in C_T value will not be constant if different amounts of template are used. In consequence, the C_T value measured for a given copy number could thus be different between different sequences. This difference does not call into question the strict correlation between a C_T value and a number of copies of a particular sequence.

In the case of BAALC standards, the copy number of each dilution was validated using a direct qPCR quantification of the plasmid sequence and comparison with other standards as shown in Table 15 and Figures 10, 11, and 12.

Table 15. Validation of BAALC standards against known standards

	Target gene specific quantification		Plasmid sequence specific quantification	
	BAALC standard	**Mbcr standard**	**BAALC standard**	**Mbcr standard**
10^1 copies	34.9	36.1	35.9	35.4
10^2 copies	32.4	33.7	33.3	32.5
10^3 copies	29.5	29.7	29.9	28.9
10^5 copies	23.0	23.1	23.1	22.3
10^6 copies	19.6	19.5	19.9	18.9
Slope	–3.12	–3.51	–3.26	–3.32
Intercept	38.57	40.57	39.48	38.88

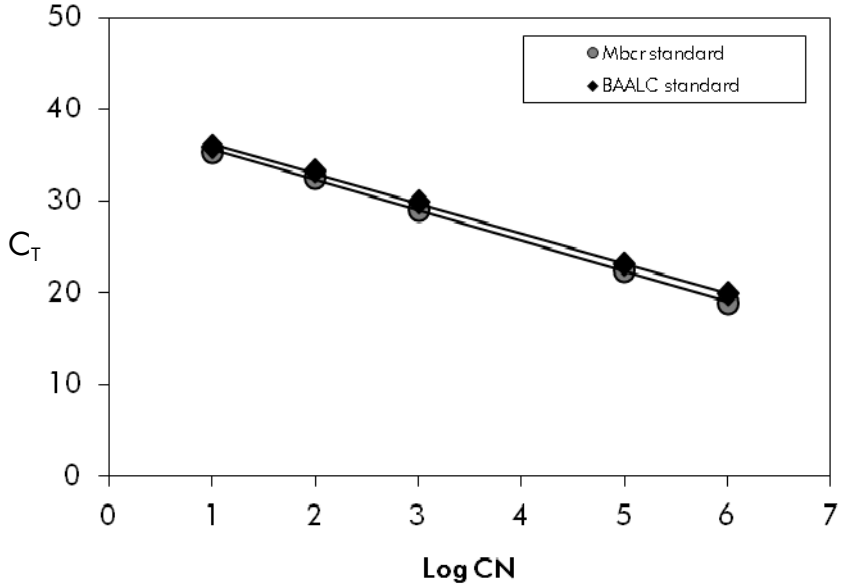

Figure 10. Plasmid sequence specific quantification.

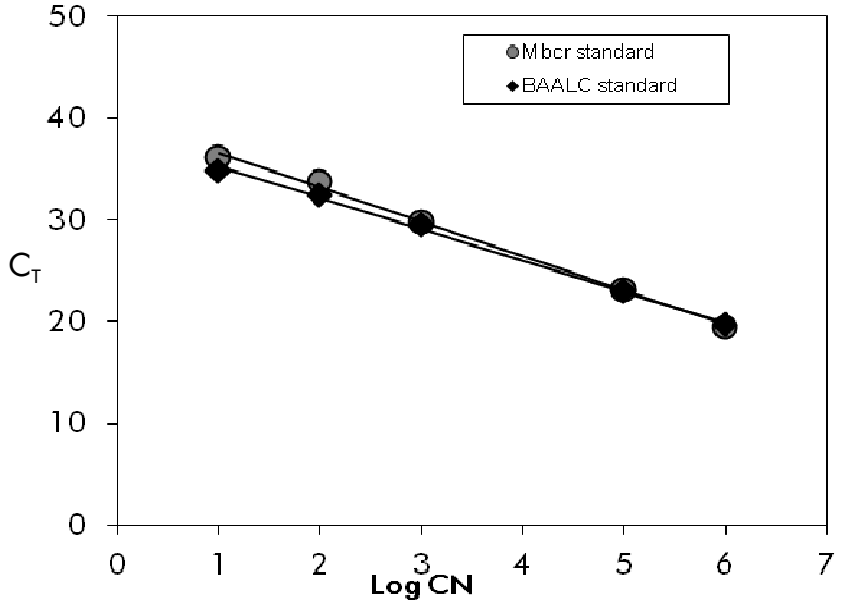

Figure 11. Target gene specific quantification.

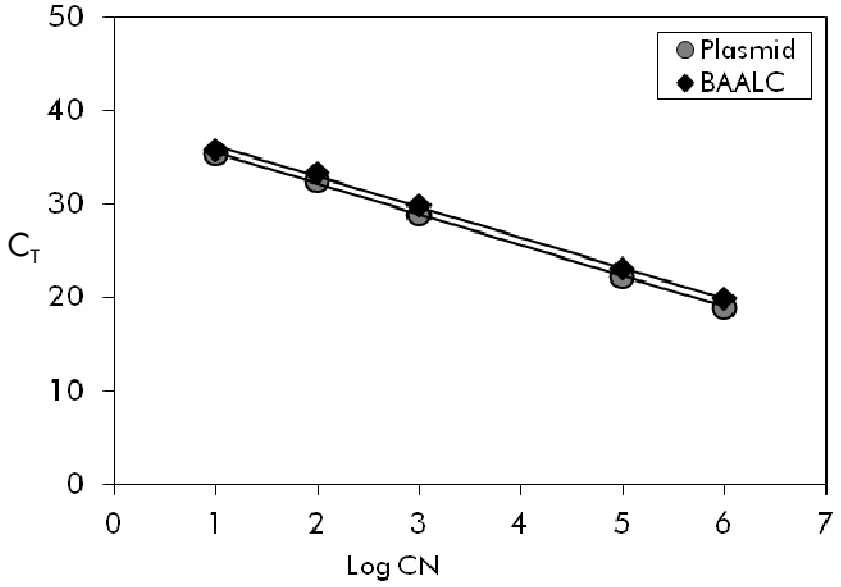

Figure 12. Target gene and plasmid sequence specific quantification for BAALC standards.

The standard curves included in the BAALC Profile*Quant* Kit are plasmid-based. Three plasmid standard dilutions for the control gene, and five standard dilutions for the profile gene are used to ensure accurate standard curves. This methodology has the advantage that probe degradation can be compensated, and data generated on different types of qPCR instruments can be directly compared by data normalization.

The normalized copy number (NCN) is defined as the copy number (CN) of the profile gene (PG) per 10^4 copies of the control gene (CG) transcript: (mean value of PG_{CN} / mean value of CG_{CN}) x 10^4.

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 36).

	Comments and suggestions
Negative result for the control gene (ABL) and BAALC in all the samples — standard okay	
a) Poor RNA quality	Always check the RNA quality and concentration before starting.
b) Failure of reverse transcription step	Always check the RNA quality and concentration before starting.
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.
b) Failure of reverse transcription step	Always check the RNA quality and concentration before starting.
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* BAALC 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 9. Avoid repeated freezing and thawing. Aliquot reagents for storage.

Comments and suggestions	
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.
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.
b) Failure of reverse transcription step	Always check the RNA quality and concentration before starting.
Fluorescence intensity too low	
a) Inappropriate storage of kit components	Store the *ipsogen* BAALC 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 9. Avoid repeated freezing and thawing. Aliquot reagents for storage.

	Comments and suggestions
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.
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 instrument.
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 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.

Symbols

 <N>	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Material number
	Temperature limitation
	Manufacturer
	Consult instructions for use

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 BAALC Profile*Quant* Kit (24)	For 24 reactions: ABL Control Gene Standards, BAALC Standards, Primer and Probe Mix ABL, Primer and Probe Mix BAALC Gene	676613
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

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