

March 2015

ipsogen[®] NPM1 mutB&D *MutaQuant*[®] Handbook

 24 (type B)

 24 (type D)

For detection of NPM1 mutations type B and type D

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

For use with Rotor-Gene[®] Q, Applied Biosystems[®], ABI PRISM[®],
and LightCycler[®] instruments

REF 677613



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Intended Use

The *ipsogen* NPM1 mutB&D MutaQuant 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

NPM1 mutations type B and type D correspond to an insertion-duplication of 4 nucleotides [CATG or CCTG respectively] at position nt960. The *ipsogen* NPM1 mutB&D MutaQuant Kit allows quantification of NPM1 mutB and mutD transcripts by real-time quantitative PCR (qPCR), depending on the already-characterized NPM1 genotype of the sample to be analyzed. The kit includes primers and probe spanning NPM1 exons 11 and 12 to detect exclusively NPM1 mutations in cDNA (see Figure 1), specific ABL primers and probe, and plasmids specific for ABL and NPM1 mutB or mutD to determine and normalize NPM1 copy numbers in samples.

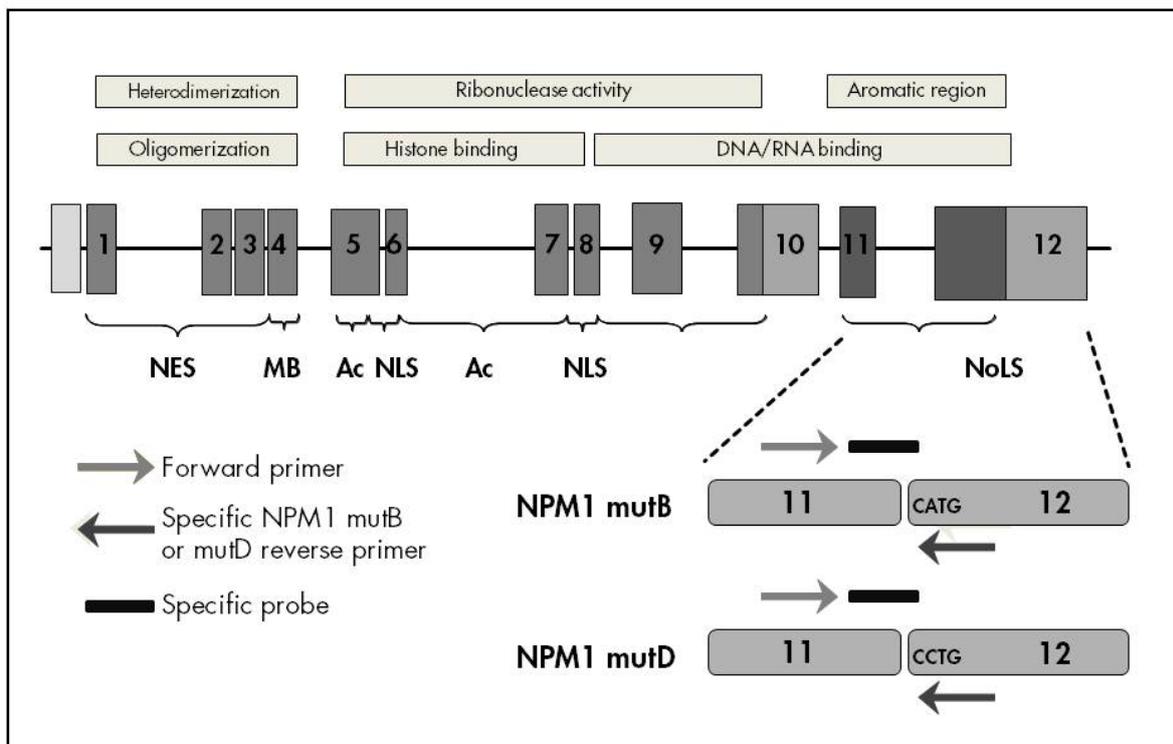


Figure 1. Human NPM1 gene showing the position of the mutB and mutD point mutations in exon 12.

Analysis by qPCR permits the accurate quantification of PCR products during the exponential phase of the PCR amplification process. Quantitative PCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during and/or subsequent to PCR cycling, thereby drastically reducing the risk of PCR product contamination. At present, 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 product. 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.

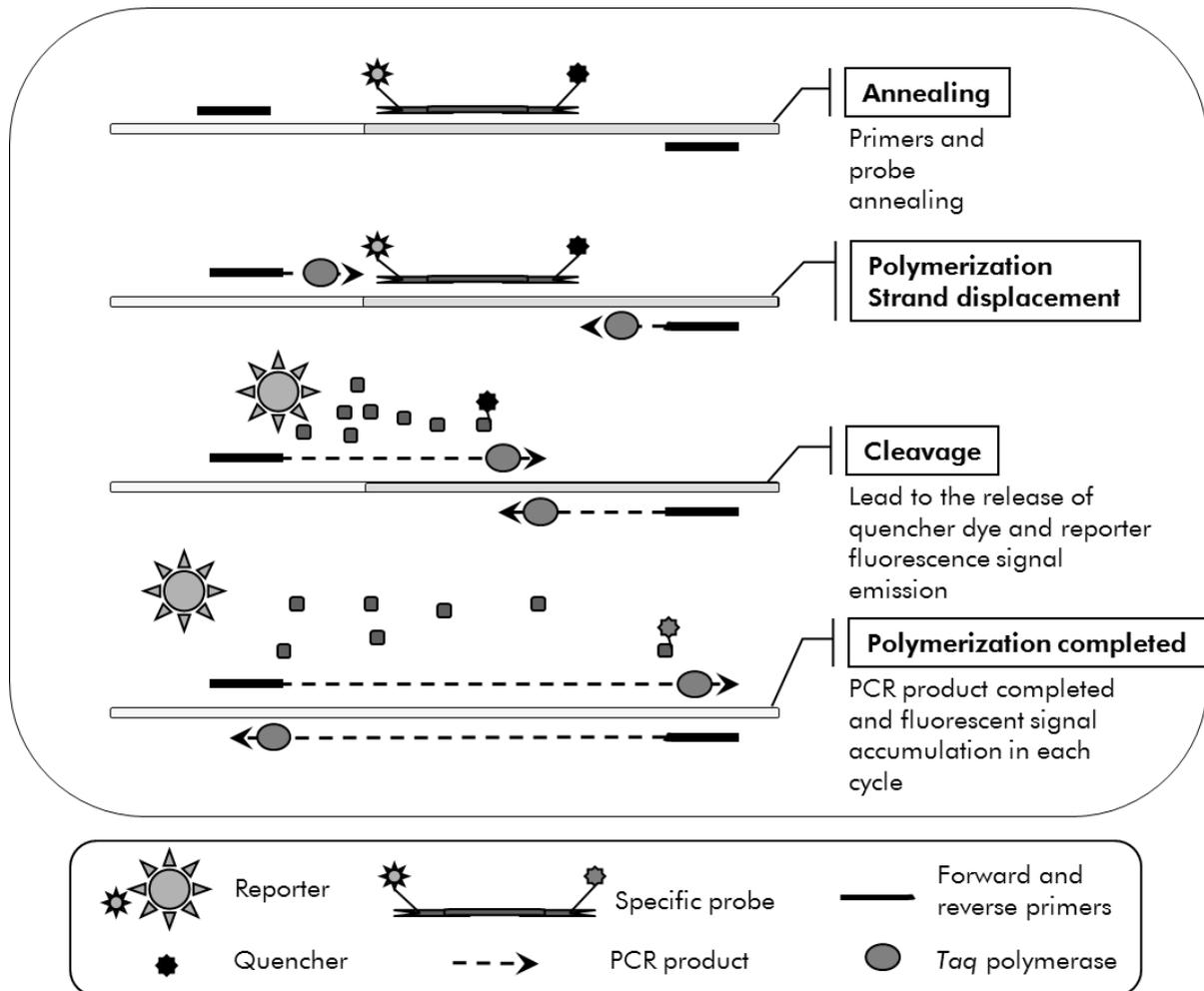


Figure 2. Reaction principle. Total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–TAMRA™). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* DNA polymerase extends 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. In the *ipsogen* NPM1 mutB&D MutaQuant Kit, an endogenous control (transcript from ABL) is amplified from the sample together with the NPM1 mutB or mutD transcripts. Standard curves of known amounts of the endogenous ABL control and NPM1 mutB or mutD cDNAs allow the calculation of the ratios of NPM1 mutB or mutD signals to endogenous ABL signal in each sample. Specific primers and probe mixes and standard serial dilutions of the control ABL gene and NPM1 mutB and mutD plasmids are provided for the quantification of the ABL control and NPM1 mutation type B or type D transcripts.

The allele specific qPCR technology used in this assay allows a sensitive, accurate, and highly reproducible detection of single nucleotide polymorphisms (SNPs). This technique is based on the use of specific reverse primers for the NPM1 mutB or mutD alleles. Only a perfect match between primer and target DNA allows extension and amplification in the PCR reaction (see Figure 3).

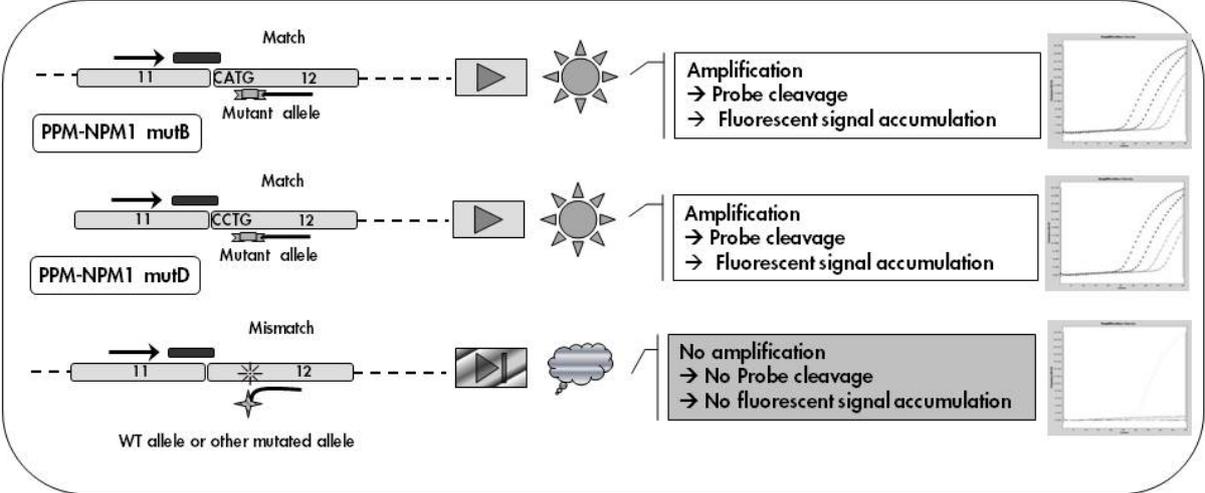


Figure 3. Allele specific PCR. Use of specific reverse primers for NPM1 mutB or mutD in the NPM1 mutB or mutD primers and probe mixes (PPM-NPM1 mutB and PPM-NPM1 mutD) allows the specific detection of NPM1 mutation type B or type D transcripts in an individual reaction. Results can be expressed as normalized copies of NPM1 mutB or mutD relative to ABL.

Materials Provided

Kit contents

<i>ipsogen</i> NPM1 mutB&D MutaQuant Kit		(24)
Catalog no.		677613
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
NPM1 mutB Gene Standard Dilution (10 ¹ copies/5 µl)	M1-NPM1-MutB	50 µl
NPM1 mutB Gene Standard Dilution (10 ² copies/5 µl)	M2-NPM1-MutB	50 µl
NPM1 mutB Gene Standard Dilution (10 ³ copies/5 µl)	M3-NPM1-MutB	50 µl
NPM1 mutB Gene Standard Dilution (10 ⁵ copies/5 µl)	M4-NPM1-MutB	50 µl
NPM1 mutB Gene Standard Dilution (10 ⁶ copies/5 µl)	M5-NPM1-MutB	50 µl
Primers and Probe Mix ABL*	PPC-ABL 25x	90 µl
Primers and Probe Mix NPM1 mutB Gene [†]	PPM-NPM1 mutB 25x	110 µl

* 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 NPM1 mutB cDNA plus a specific FAM-TAMRA probe.

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

Kit contents

ipsogen NPM1 mutB&D MutaQuant Kit		(24)
Catalog no.		677613
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
NPM1 mutD Gene Standard Dilution (10 ¹ copies/5 µl)	M1-NPM1-MutD	50 µl
NPM1 mutD Gene Standard Dilution (10 ² copies/5 µl)	M2-NPM1-MutD	50 µl
NPM1 mutD Gene Standard Dilution (10 ³ copies/5 µl)	M3-NPM1-MutD	50 µl
NPM1 mutD Gene Standard Dilution (10 ⁵ copies/5 µl)	M4-NPM1-MutD	50 µl
NPM1 mutD Gene Standard Dilution (10 ⁶ copies/5 µl)	M5-NPM1-MutD	50 µl
Primers and Probe Mix ABL*	PPC-ABL 25x	90 µl
Primers and Probe Mix NPM1 mutD Gene†	PPM-NPM1 mutD 25x	110 µl

* 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 NPM1 mutD cDNA 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 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 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
- MgCl₂
- 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 (capable of attaining 10,000 rpm)
- Real-time PCR instrument:* Rotor-Gene Q 5plex HRM or other Rotor-Gene instrument; LightCycler 1.2, 1.5, and 2.0, or 480; ABI PRISM 7000, 7700, or 7900HT SDS; or Applied Biosystems 7500 Real-Time PCR System; and associated specific material
- Thermal cycler* or water bath* (reverse transcription step)
- Spectrophotometer*

* 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.

General precautions

Use of qPCR tests requires good laboratory practices, including equipment maintenance, that are dedicated to molecular biology and 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 PPM 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, plasmid, or PCR products) 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 M1–5) 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 PPM 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.

Sample Storage and Handling

Whole blood samples should be anti-coagulated with potassium EDTA and stored at $2-8^{\circ}\text{C}$ for no more than 5 days before RNA extraction.

Procedure

Sample RNA preparation

RNA extraction should be performed with a recommended procedure (QIAGEN RNeasy® Mini Kit, cat. no. 74104 or RNeasy Midi Kit, cat. no. 75144; or Life Technologies TRIzol®, cat. nos. 15596-026 and 15596-018).

The performance of an assay is dependent on the concentration and quality of input RNA. We therefore recommend qualifying the purified RNA, prior to downstream analysis, by agarose* gel electrophoresis, Agilent® Bioanalyzer®, or spectrophotometry.

Note: This kit does not allow the synthesis of cDNA from purified RNA.

Protocol: 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.

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/ μ l)	0.5	1 U/ μ l
Random nonamer (100 μ M)	5.0	25 μ M
Superscript II Reverse Transcriptase (200 U/ μ l)	0.5	5 U/ μ l
Heated RNA sample (to be added in step 5)	1.0–4.0	50 ng/ μ l
Nuclease-free PCR grade water (to be added in step 5)	0.0–3.0	–
Final volume	20.0	–

- 5. Pipet 16 μ l of RT mix into each PCR tube. Then add 1–4 μ l (1 μ g) RNA (from step 3), and adjust the volume to 20 μ l 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 μ g)	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 spin (approximately 5 seconds, to collect the liquid in the bottom of the tube). Then keep on ice.
11. Dilute the final cDNA with 30 μ l of nuclease-free PCR grade water so that the final volume is 50 μ l.
12. Carry out PCR according to the following protocols, according to your qPCR instrument.

Protocol: qPCR on 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 control gene primers and probe mix (PPC)	
n cDNA samples	n x 2 reactions
Control gene standard	2 x 3 reactions (3 dilutions, each one tested in duplicate)
Water control	2 reactions
With the NPM1 mutB or mutD gene primers and probe mix (PPM)	
n cDNA samples	n x 2 reactions
NPM1 mutB or mutD gene 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 at least 8 cDNA samples in the same experiment to optimize the use of the standards and the primers and probe mixes. The *ipsogen* NPM1 mutB&D MutaQuant Kit provides enough reagents to perform an 8-sample experiment 3 times for each mutation using the 72-tube rotor.

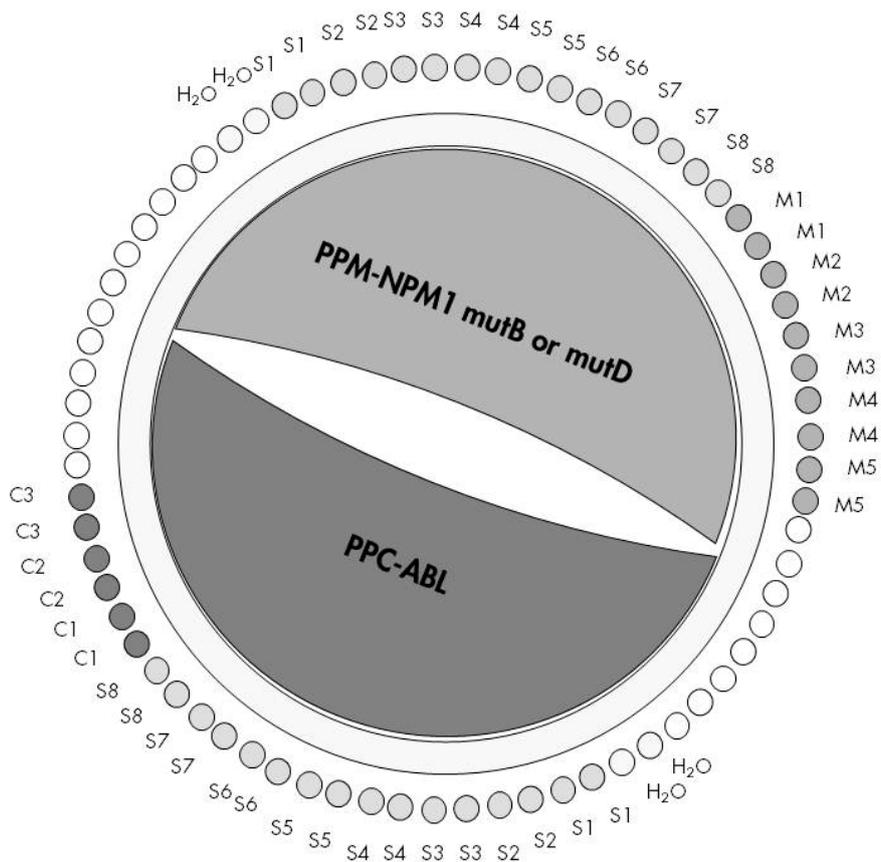


Figure 4. Suggested rotor setup for one experiment with the *ipsogen* NPM1 mutB&D MutaQuant Kit. M1–5: NPM1 gene standards: mutation type B or type D; C1–3: Control gene standards; S: cDNA sample; H₂O: water control.

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 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. Briefly centrifuge (approximately 10 seconds, 10,000 rpm, to collect the liquid in the bottom of the tube). Then keep on ice.
3. 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 or PPM). Extra volumes are included to compensate for pipetting error.

Table 4. Preparation of qPCR mix

Component	1 reaction (μl)	Control: 24+1 reactions (μl)	MutB or D: 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	29	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	–

Note: Steps 4-6 to be performed on the cooling block (provided with the Rotor-Gene Q 5plex HRM).

4. **Dispense 20 μl of the qPCR pre-mix per tube.**
5. **Add 5 μl of the RT product (cDNA, 100 ng RNA equivalent) obtained in the reverse transcription (see “Protocol: Reverse transcription”, page 13) in the corresponding tube (total volume 25 μl).**
6. **Mix gently, by pipetting up and down.**
7. **Place the tubes in the thermal cycler according to the manufacturer’s recommendations.**
8. **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 63 deg for 1 min with acquisition of FAM fluorescence in channel Green: Single

9. 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 Applied Biosystems 7500, ABI PRISM 7000, 7700, and 7900HT SDS instruments, 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 control gene primers and probe mix (PPC)	
n cDNA samples	n x 2 reactions
Control gene standard	2 x 3 reactions (3 dilutions, each one tested in duplicate)
Water control	2 reactions
With the NPM1 mutB or mutD gene primers and probe mix (PPM)	
n cDNA samples	n x 2 reactions
NPM1 mutB or mutD gene standard	2 x 5 reactions (5 dilutions, each one tested in duplicate)
Water control	2 reactions

Sample processing on Applied Biosystems 7500, ABI PRISM 7000, 7700, and 7900 SDS, 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 *ipsogen* NPM1 mutB&D MutaQuant Kit provides enough reagents to perform an 8-sample experiment 3 times for each mutation.

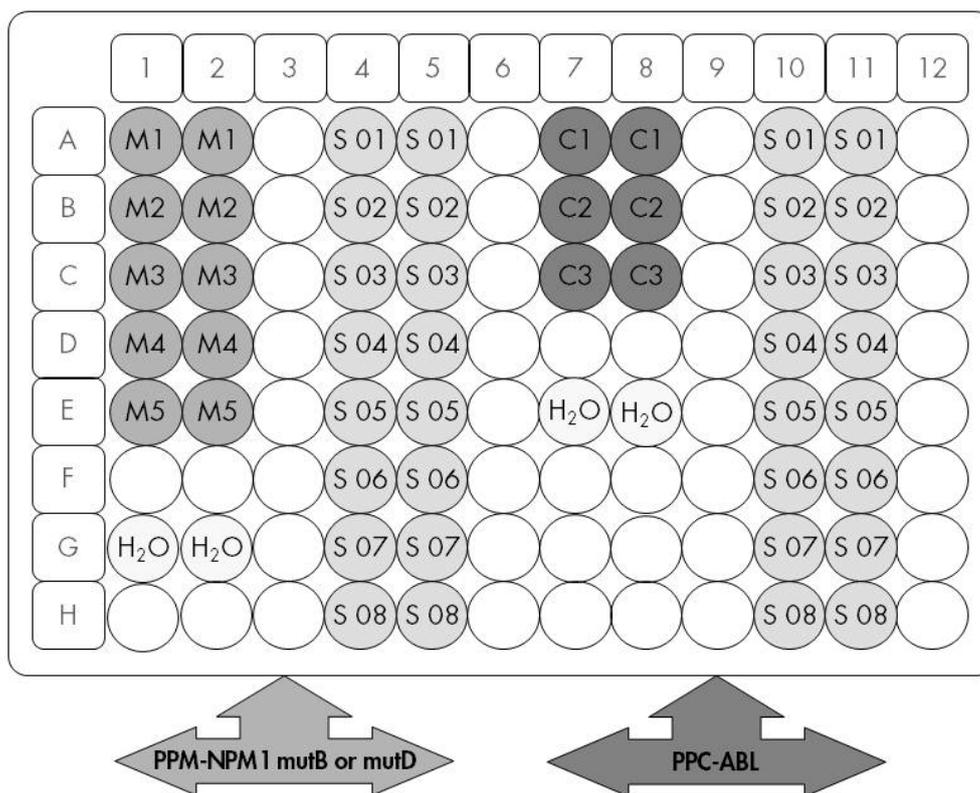


Figure 5. Suggested plate setup for one experiment with the *ipsogen* NPM1 mutB&D MutaQuant Kit. S: cDNA sample; M1–5: NPM1 gene standards: mutation type B or type D; C1–3: Control gene standards; H₂O: water control.

qPCR on Applied Biosystems 7500, ABI PRISM 7000, 7700, and 7900 SDS, and LightCycler 480 instruments

Note: Perform all steps on ice.

Procedure

1. Thaw all necessary components and place them on ice.
2. Briefly centrifuge (approximately 10 seconds, 10,000 rpm, to collect the liquid in the bottom of the tube). Then keep on ice.
3. 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 μ l. A pre-mix can be prepared, according to the number of reactions, using the same primers and probe mix (either PPC or PPM). Extra volumes are included to compensate for pipetting error.

Table 7. Preparation of qPCR mix

Component	1 reaction (μl)	Control: 24+1 reactions (μl)	MutB or D: 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	29	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	–

4. Dispense 20 μ l of the qPCR pre-mix per well.
5. Add 5 μ l of the RT product (cDNA, 100 ng RNA equivalent) obtained in the reverse transcription (see “Protocol: Reverse transcription”, page 13) in the corresponding well (total volume 25 μ l).
6. Mix gently, by pipetting up and down.
7. Close the plate and briefly centrifuge (300 x g, approximately 10 seconds).
8. Place the plate in the thermal cycler according to the manufacturer’s recommendations. Program the thermal cycler with the thermal cycling program as indicated in Table 8 for Applied Biosystems 7500, ABI PRISM 7000, 7700, and 7900HT SDS, or Table 9 for the LightCycler 480 instrument.

Table 8. Temperature profile for Applied Biosystems 7500, ABI PRISM 7000, 7700, and 7900HT SDS

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 63°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 1	Temperature: 50°C Time: 2 minutes
Hold 2	Temperature: 95°C Time: 10 minutes
Cycling	50 times 95°C for 15 seconds 63°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

- 9. For the Applied Biosystems 7500, ABI PRISM 7000, 7700, and 7900HT SDS, follow step 9a. For the LightCycler 480 instrument, follow step 9b.**
- 9a. Applied Biosystems 7500, ABI PRISM 7000, 7700. and 7900HT SDS: We recommend a threshold set at 0.1 in the analysis step on the ABI PRISM SDS and a baseline set between cycles 3 and 15. Start the cycling program, as indicated in Table 8.**
- 9b. LightCycler 480 instrument: 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, 1.5, and 2.0 instruments

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, 1.5, and 2.0 instruments

Samples	Reactions
With the control gene primers and probe mix (PPC)	
n cDNA samples	n x 2 reactions
Control gene standard	1 x 3 reactions (3 standard dilutions, each one tested once)
Water control	1 reaction
With the NPM1 mutB or mutD gene primers and probe mix (PPM)	
n cDNA samples	n x 2 reactions
NPM1 mutB or mutD gene standard	1 x 5 reactions (5 standard dilutions, each one tested once)
Water control	1 reaction

Sample processing on LightCycler 1.2, 1.5, and 2.0 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 *ipsogen* NPM1 mutB&D MutaQuant kit provides enough reagents to perform a 5-sample experiment 6 times for each mutation.

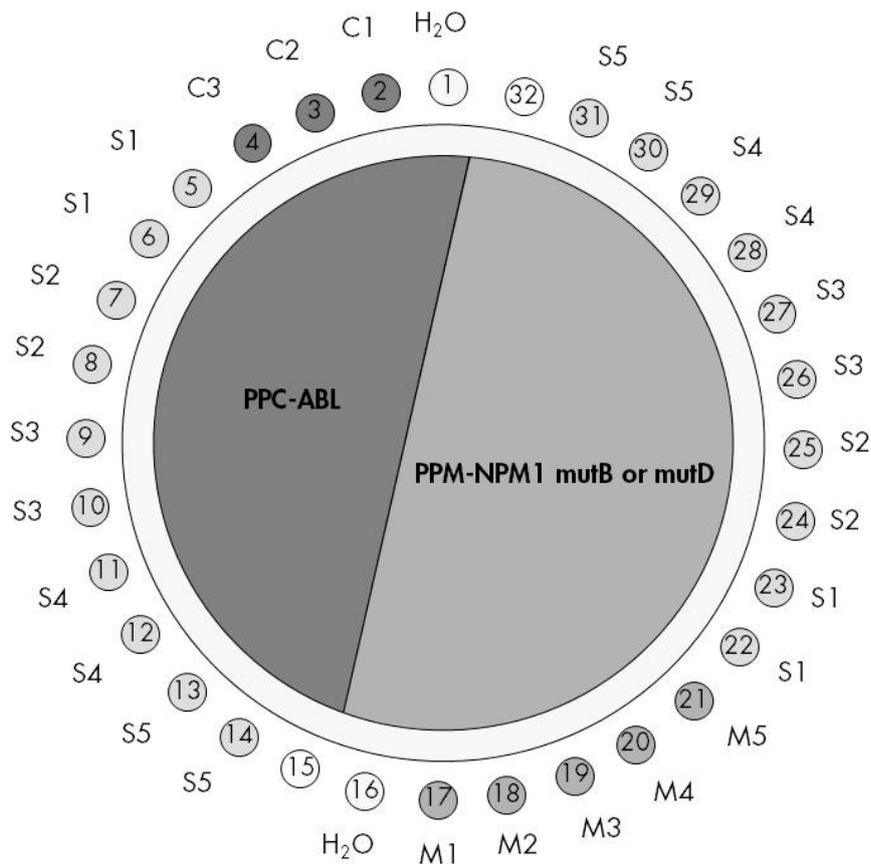


Figure 6. Suggested rotor setup for one experiment with the *ipsogen* NPM1 mutB&D MutaQuant Kit. M1–5: NPM1 gene standards: mutation type B or type D; C1–3: Control gene standards; S: cDNA sample; H₂O: water control.

qPCR on LightCycler 1.2, 1.5, and 2.0 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. Briefly centrifuge (approximately 10 seconds, 10,000 rpm, to collect the liquid in the bottom of the tube). Then keep on ice.
3. 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 or PPM). Extra volumes are included to compensate for pipetting error.

Table 11. Preparation of qPCR mix

Component	1 reaction (μl)	Control: 14+1 reactions (μl)	MutB or D: 16+1 reactions (μl)	Final concentration
Freshly prepared LightCycler TaqMan Master Mix, 5x	4.0	60	68.0	1x
Primers and probe mix, 25x	0.8	12	13.6	1x
Nuclease-free PCR grade water	10.2	153	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 μ l of the qPCR pre-mix per capillary.
5. Add 5 μ l of the RT product (cDNA, 100 ng RNA equivalent) obtained in the reverse transcription (see “Protocol: Reverse transcription”, page 13) in the corresponding tube (total volume 20 μ l).
6. Mix gently, by pipetting up and down.
7. Place the capillaries in the adapters provided with the apparatus, and briefly centrifuge (700 x g, approximately 10 seconds).
8. Load the capillaries into the thermal cycler according to the manufacturer’s recommendations.
9. Program the LightCycler 1.2, 1.5, or 2.0 instruments with the thermal cycling program as indicated in Table 12.

Table 12. Temperature profile

Mode of analysis	Quantification
Hold 1	Temperature: 50°C Time: 2 minutes
Hold 2	Temperature: 95°C Time: 10 minutes
Cycling	50 times 95°C for 15 seconds 63°C for 1 minute; with acquisition of FAM fluorescence: Single

10. For the LightCycler 1.2 and 1.5, follow step 10a. For the LightCycler 2.0, follow step 10b.

10a. LightCycler 1.2 and 1.5: The F1/F2 and “2nd derivative analysis” mode is recommended. Start the thermal cycling program, as indicated in Table 12.

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 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; we use 3 plasmid standard dilutions for the control gene, and 5 standard dilutions for the mutated gene, in order to ensure accurate standard curves. Figures 7 and 8 show examples of TaqMan amplification curves obtained with the *ipsogen* NPM1 mutB&D MutaQuant Kit (cat. no.677613).

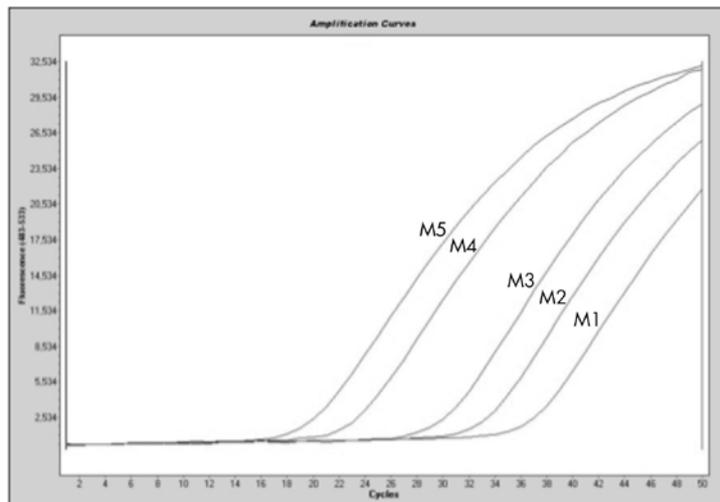


Figure 7. Detection of NPM1 mutB or mutD gene standards (M1–M5). 10^1 , 10^2 , 10^3 , 10^5 , 10^6 copies/ $5 \mu\text{l}$.

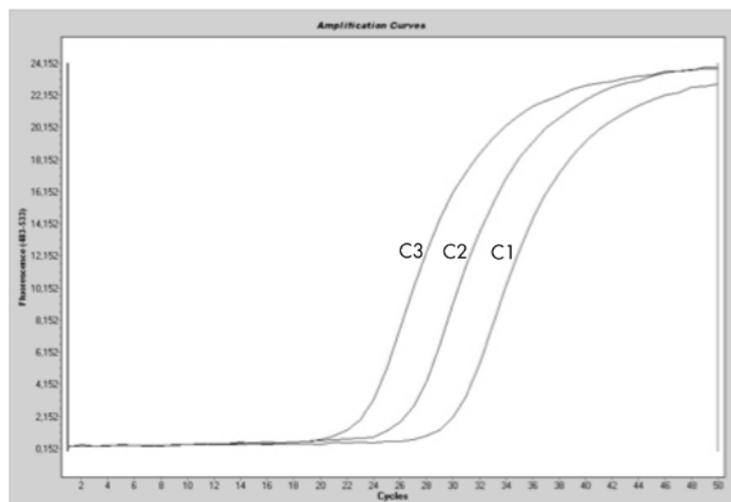


Figure 8. Detection of ABL control gene standards (C1, C2, C3). 10^3 , 10^4 , and 10^5 copies/ $5 \mu\text{l}$.

Standard curve and quality criteria

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

For each gene (control gene and mutated gene), 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 M1, M2, M3, M4, and M5). Figure 9 shows an example of the theoretical curve calculated on 5 standard dilutions. Figure 10 shows an example of the standard curve calculated on 3 dilutions of the ABL gene.

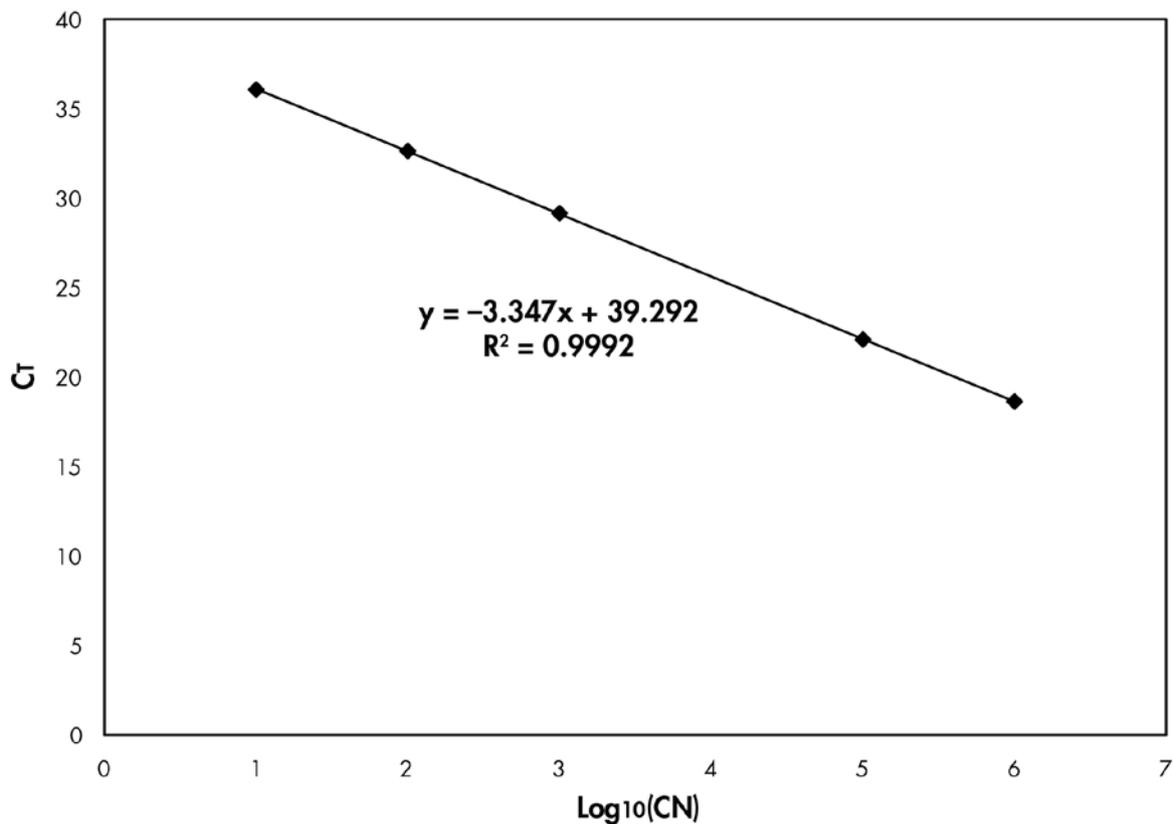


Figure 9. Theoretical curve calculated from the 5 standard dilutions. A linear regression curve ($y = ax + b$) is calculated where a is the slope of the line and b is the y -intercept, which is the y -coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination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

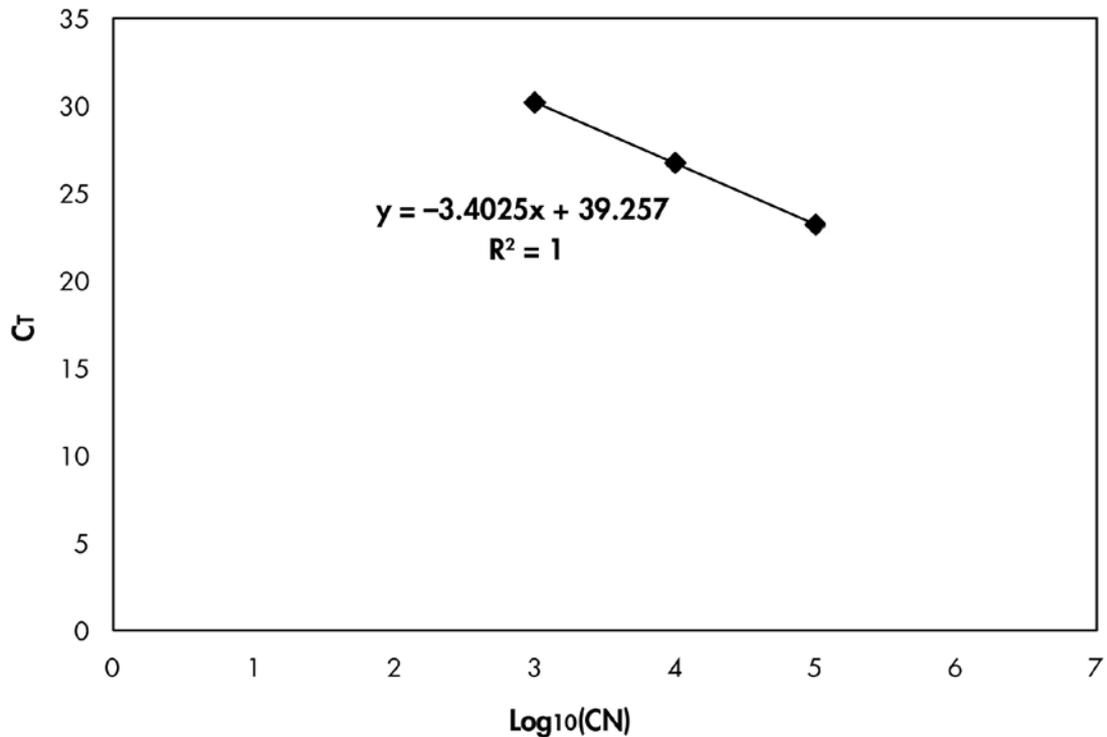


Figure 10. Theoretical curve calculated from the 3 dilutions of the control ABL gene. A linear regression curve ($y = ax + b$) is calculated where a is the slope of the line and b is the y -intercept, which is the y -coordinate of the point where the line crosses the y axis. Its equation and coefficient of determination (R^2) are printed on the graph.

As standards are tenfold dilutions, the theoretical slope of the curve is -3.3 . A slope between -3.0 and -3.9 is acceptable as long as R^2 is >0.95 .

Normalized copy number (NCN)

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

The mutated gene standard curve equation should be used to transform raw C_T values (obtained with PPM) for the unknown samples, into mutated gene copy numbers ($NPM1\ mutB_{CN}$ or $NPM1\ mutD_{CN}$).

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

$$NCN = \frac{NPM1\ mutB/D_{CN}}{ABL_{CN}} \times 100$$

Reproducibility between replicates

The data obtained should be consistent between duplicates.

Water controls

Negative controls should give less than 1 CN.

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 36).

Comments and suggestions

Negative result for the control gene and mutated gene 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 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 <i>ipsogen</i> NPM1 mutB&D MutaQuant Kit at –15 to –30°C and keep primers and probe mixes (PPC and PPM) protected from light. See “Reagent Storage and Handling”, page 12.
Avoid repeated freezing and thawing.
Aliquot reagents for storage. |

Comments and suggestions

Standard curves not linear

- a) Vial inversion; inversion during distribution; cross-contamination Check pipetting scheme and the setup of the reaction.
Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carry-over contamination.
- b) Partial degradation of standards Store the *ipsogen* NPM1 mutB&D MutaQuant Kit at -15 to -30°C and keep primers and probe mixes (PPC and PPM) protected from light. See "Reagent Storage and Handling", page 12.
Avoid repeated freezing and thawing.
- c) Inappropriate storage of kit components Store the *ipsogen* NPM1 mutB&D MutaQuant Kit at -15 to -30°C and keep primers and probe mixes (PPC and PPM) protected from light. See "Reagent Storage and Handling", page 12.
Aliquot reagents.
- d) Problems with PCR programs Check the cycle programs.

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.

Comments and suggestions

- | | |
|--|--|
| 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 <i>ipsogen</i> NPM1 mutB&D MutaQuant Kit at -15 to -30°C and keep primers and probe mixes (PPC and PPM) protected from light. See "Reagent Storage and Handling", page 12.

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. |

Poor reproducibility of duplicates

- | | |
|--|---|
| Pipetting error or cross-contamination | Check pipetting scheme and the setup of the reaction. |
|--|---|

High background

- | | |
|-----------------------|---|
| Fluorophore bleaching | Store primers and probe mixes (PPC and PPM) protected from light. |
|-----------------------|---|

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. |
|--------------------|--|

Comments and suggestions

- | | |
|---|---|
| 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 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

The following symbols may appear on the packaging and labeling:

 Σ	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

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.
<i>ipsogen</i> NPM1 mutB&D MutaQuant Kit (24)	<p>For 24 reactions: ABL Control Gene Standards, NPM1 MutB Gene Standards, Primers and Probe Mix ABL, Primers and Probe Mix NPM1 mutB Mutated Gene</p> <p>For 24 reactions: ABL Control Gene Standards, NPM1 MutD Gene Standards, Primers and Probe Mix ABL, Primers and Probe Mix NPM1 mutD Mutated Gene</p>	677613
<p>Rotor-Gene Q — for outstanding performance in real-time PCR</p>		
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

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