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ipsogen[®] JAK2 MutaQuant[®] Handbook

 12 (catalog no. 673512)

 24 (catalog no. 673513)

For JAK2 V617F/G1849T quantification

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

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

REF 673512, 673513



QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden,
GERMANY

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Contents

Intended Use	4
Principle of the Procedure	4
Materials Provided	7
Kit contents	7
Materials Required but Not Provided	8
Warnings and Precautions	9
General precautions	9
Reagent Storage and Handling	10
Procedure	11
Sample DNA preparation	11
Protocols	
■ qPCR on Rotor-Gene Q 5plex HRM instruments with 72-tube rotor	12
■ qPCR on ABI PRISM 7900HT SDS, Applied Biosystems 7500 Real-Time PCR System, and LightCycler 480 instrument	17
■ qPCR on LightCycler 1.2 instrument	23
Results	27
Troubleshooting guide	31
Quality Control	34
References	34
Symbols	35
Contact Information	36
Ordering Information	37

Intended Use

The *ipsogen* JAK2 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

Several different techniques have been proposed to quantitatively determine the proportion of single nucleotide polymorphisms (SNPs) in DNA samples. Of these, methods based on real-time quantitative polymerase chain reaction (qPCR) are preferred because of their higher sensitivity allowing monitoring of allele burden in a longitudinal fashion. Many of these techniques have a moderate sensitivity of 1–10%, for example, TaqMan® allelic discrimination, Pyrosequencing®, melting curve assay, and direct sequencing. Some, such as melting curve and sequencing, are only semiquantitative while others, such as Pyrosequencing, require post-PCR processing or need instrumentation that is not readily available or has prohibitively high setup costs for routine laboratory testing. A highly sensitive approach with a sensitivity <0.1% requires the use of a SNP specific primer that permits the selective amplification of the mutant or wild type allele that is easily detectable on a real-time qPCR instrument. The *ipsogen* JAK2 MutaQuant Kit is based on this technique.

The use of 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. 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 1). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

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

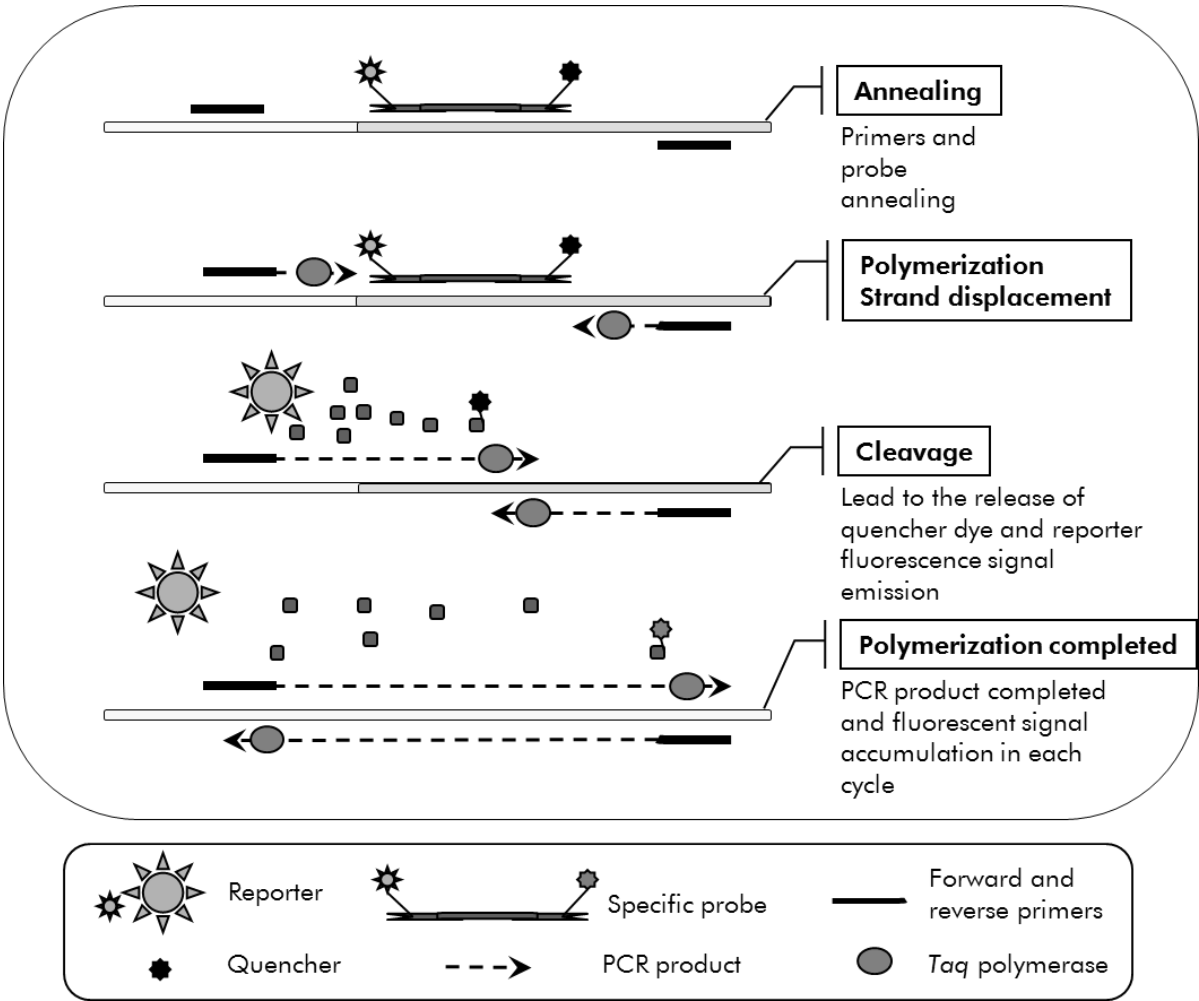


Figure 1. Reaction principle.

The quantitative allele specific PCR technology used in this assay kit allows a sensitive and accurate detection of SNPs. This technique is based on the use of specific forward primers, for the wild-type and the V617F allele (1). Only perfect

match between primer and target DNA allows extension and amplification in the PCR (see Figure 2).

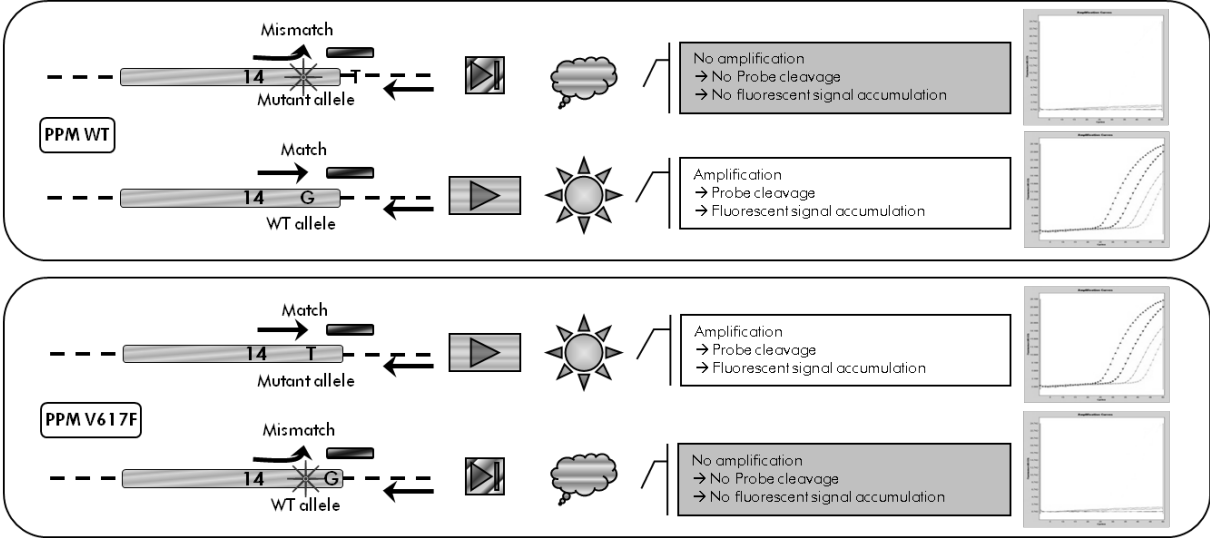


Figure 2. Allele specific PCR. Use of wild-type or the V617F primers and probe mix allows the specific detection of the wild-type or mutated allele in two separate reactions conducted using the same sample. Results can be expressed as percentage of VF copies among total JAK2 copies.

Materials Provided

Kit contents

ipsogen JAK2 MutaQuant Kit		(12)	(24)
Catalog no.		673512	673513
Number of reactions		12	24
V617F positive control (100% V617F allele)	PC-VF-JAK2 PC-VF-JAK2 Mini	40 μ l	60 μ l
V617F negative control (100% wild-type allele)	NC-VF-JAK2 NC-VF-JAK2 Mini	40 μ l	60 μ l
M1-VF Standard Dilution, 50 copies (5 x 10 ¹ V617F copies/5 μ l)	M1-VF M1-VF Mini	20 μ l	30 μ l
M2-VF Standard Dilution, 500 copies (5 x 10 ² V617F copies/5 μ l)	M2-VF M2-VF Mini	20 μ l	30 μ l
M3-VF Standard Dilution, 5000 copies (5 x 10 ³ V617F copies/5 μ l)	M3-VF M3-VF Mini	20 μ l	30 μ l
M4-VF Standard Dilution, 50,000 copies (5 x 10 ⁴ V617F copies/5 μ l)	M4-VF M4-VF Mini	20 μ l	30 μ l
WT-1 Standard Dilution, 50 copies (5 x 10 ¹ wild-type copies/5 μ l)	WT-1 WT-1 Mini	20 μ l	30 μ l
WT-2 Standard Dilution, 500 copies (5 x 10 ² wild-type copies/5 μ l)	WT-2 WT-2 Mini	20 μ l	30 μ l
WT-3 Standard Dilution, 5000 copies (5 x 10 ³ wild-type copies/5 μ l)	WT-3 WT-3 Mini	20 μ l	30 μ l
WT-4 Standard Dilution, 50,000 copies (5 x 10 ⁴ wild-type copies/5 μ l)	WT-4 WT-4 Mini	20 μ l	30 μ l
Primers and Probe Mix JAK2 WT*	PPM-JAK2 WT 25x PPM-JAK2 WT Mini 25x	52 μ l	95 μ l
Primers and Probe Mix JAK2 V617F [†]	PPM-JAK2 V617F 25x PPM-JAK2 V617F Mini 25x	52 μ l	95 μ l
ipsogen JAK2 MutaQuant Handbook		1	1

* Mix of specific reverse and forward primers for the wild-type JAK2 control gene plus a specific FAM™–TAMRA™ probe.

† Mix of specific reverse and forward primers for the JAK2 V617F mutation 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
- 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) or LightCycler FastStart DNA Master^{PLUS} HybProbe[®] (Master Mix 5x) (Roche, cat. No. 03515567001)

Consumables

- Nuclease-free aerosol-resistant sterile PCR pipet tips with hydrophobic filters
- 0.5 ml or 1.5 ml nuclease-free PCR tubes
- Ice

Equipment

- Microliter pipet* dedicated for PCR (1–10 μ l; 10–100 μ l; 100–1000 μ l)
- Benchtop centrifuge* with rotor for 0.5 ml/1.5 ml reaction tubes and microplates (capable of attaining 13,000–14,000 rpm)
- Real-time PCR instrument:* Rotor-Gene Q 5plex HRM or other Rotor-Gene; LightCycler 1.2, or 480; ABI PRISM 7900HT SDS; Applied Biosystems 7500 Real-Time PCR System; 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 laboratory coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

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

General precautions

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

This kit is intended for research use. Reagents and instructions supplied in this kit have been tested for optimal performance. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data. PPM-WT and PPM-VF 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.

Use extreme caution to prevent:

- DNase contamination that might cause degradation of the template DNA
- DNA 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 (DNA, plasmid, or PCR products) are introduced. Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).

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 (PPM-WT and PPM-VF 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 DNA preparation

Genomic DNA should be obtained either from whole blood, purified peripheral blood lymphocytes of whole blood, polynuclear cells, or granulocytes. For comparable results it is recommended that the same cellular fraction and DNA extraction method are used. DNA extraction can be performed using a home-brew method or a commercially available kit.

DNA quantity should be determined by measuring the optical density (OD) of the sample at 260 nm and DNA quality can be determined either by spectrophotometry or gel* electrophoresis.

- The OD_{260}/OD_{280} ratio should be 1.7–1.9 and smaller ratios than this may indicate protein contamination or the presence of organic chemicals.
- Electrophoretic analysis on a 0.8–1.0% agarose gel* should allow the visualization of the isolated DNA as a distinct band of approximately 20 kb (a slight smear will give acceptable results).

The resultant DNA will need to be diluted to a concentration of 5 ng/ μ l in 1x TE buffer* at pH 8.0 and then stored at +4 to +8°C for 1 week or at –20°C if longer term storage is required.

The qPCR reaction is optimized for DNA samples containing 25 ng purified genomic DNA.

* 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.

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 1.

Table 1. Number of reactions for Rotor-Gene Q instruments with 72-tube rotor

Samples	Reactions
With the JAK2 V617F primers and probe mix (PPM-VF)	
4 M-VF standards	8 reactions, each one tested in duplicate
n DNA samples	n x 2 reactions
2 DNA controls	4 reactions: positive control (PC-VF) and negative control (NC-VF), each one tested in duplicate
Water control	2 reactions
With the JAK2 wild-type primers and probe mix (PPM-WT)	
4 wild-type standards	8 reactions, each one tested in duplicate
n DNA samples	n x 2 reactions
2 DNA controls	4 reactions: PC-VF and NC-VF, 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 DNA samples with the 24 reaction kit (catalog no. 673513) and at least 6 DNA samples with the 12 reaction kit (catalog no. 673512) in the same experiment to optimize the use of the standards and the primers and probe mixes.

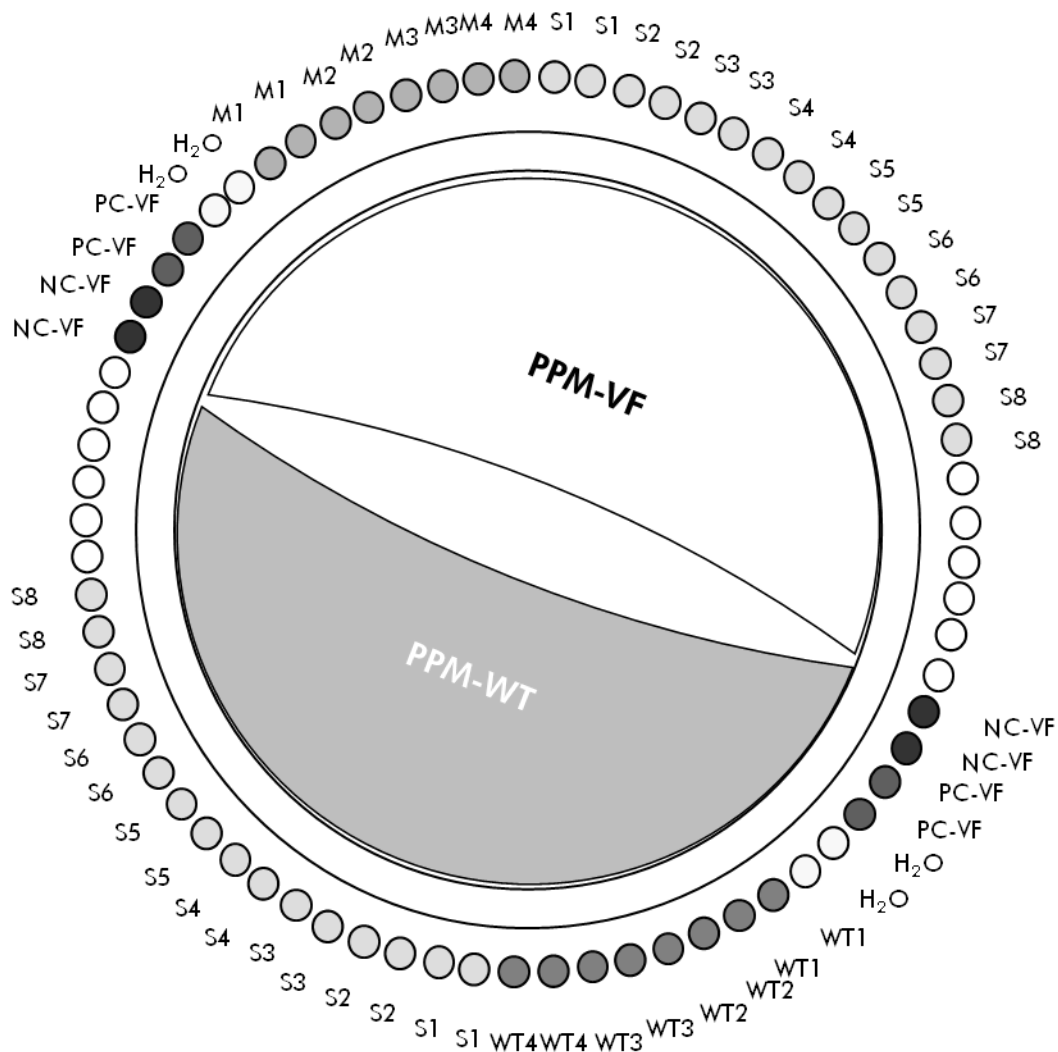


Figure 3. Suggested rotor setup for each experiment with the *ipsogen 24* sample JAK2 MutaQuant Kit. PC-VF: V617F positive control; NC-VF: V617F negative control; M-VF: V617F standards; M-WT: wild-type standards; S: DNA sample; H₂O: water control.

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. Prepare the following qPCR mix according to the number of samples being processed.

All concentrations are for the final volume of the reaction.

Tables 2 and 3 describe 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 primer and probe mix (either PPM-VF or PPM-WT). Extra volumes are included to compensate for pipetting error.

Table 2. Preparation of qPCR mix

Component	1 reaction (μl)	V617F pre-mix 30 + 1 reactions (μl)	Final concentration
TaqMan Universal PCR Master Mix, 2x	12.5	387.5	1x
Primers and probe mix, PPM-VF 25x	1.0	31	1x
Nuclease-free PCR grade water	6.5	201.5	–
Sample (to be added at step 4)	5.0	5 each	–
Total volume	25.0	25 each	–

Table 3. Preparation of qPCR mix

Component	1 reaction (μl)	WT pre-mix 30 + 1 reactions (μl)	Final concentration
TaqMan Universal PCR Master Mix, 2x	12.5	387.5	1x
Primers and probe mix, PPM-WT 25x	1.0	31	1x
Nuclease-free PCR grade water	6.5	201.5	–
Sample (to be added at step 4)	5.0	5 each	–
Total volume	25.0	25 each	–

- 3. Dispense 20 μ l of the qPCR pre-mix (VF or WT) per tube.**
- 4. Add 5 μ l of the material to be quantified (25 ng sample genomic DNA or control) in the corresponding tube (total volume 25 μ l).**
- 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 4.**

Table 4. Temperature profile

Mode of analysis	Quantitation
Hold	Temperature: 50 deg Time: 2 mins
Hold 2	Temperature: 95°C Time: 10 mins
Cycling	50 times 95°C for 15 secs 62°C for 1 min with acquisition of FAM fluorescence in channel Green: Single

- 8. Start the thermal cycling program, as indicated in Table 4.**
- 9. For Rotor-Gene Q instruments, select “Slope Correct” for the analysis. We recommend setting the threshold at 0.03.**

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 5.

Table 5. Number of reactions using 96-well-plate qPCR equipment

Samples	Reactions
With the JAK2 V617F primers and probe mix (PPM-VF)	
4 M-VF standards	8 reactions, each one tested in duplicate
n DNA samples	n x 2 reactions
2 DNA controls	4 reactions: PC-VF and NC-VF, each one tested in duplicate
Water control	2 reactions
With the JAK2 wild-type primers and probe mix (PPM-WT)	
4 wild-type standards	8 reactions, each one tested in duplicate
n DNA samples	n x 2 reactions
2 DNA controls	4 reactions: PC-VF and NC-VF, 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 instrument

We recommend testing 8 DNA samples with the 24 reaction kit (catalog no. 673513) and at least 6 DNA samples with the 12 reaction kit (catalog no. 673512) in the same experiment to optimize the use of the standards and the primers and probe mixes.

The plate scheme in Figure 4 shows an example of such an experiment using the 24 reaction kit (catalog no. 673513), and Figure 5 shows an example of such an experiment using the 12 reaction kit (catalog no. 673512).

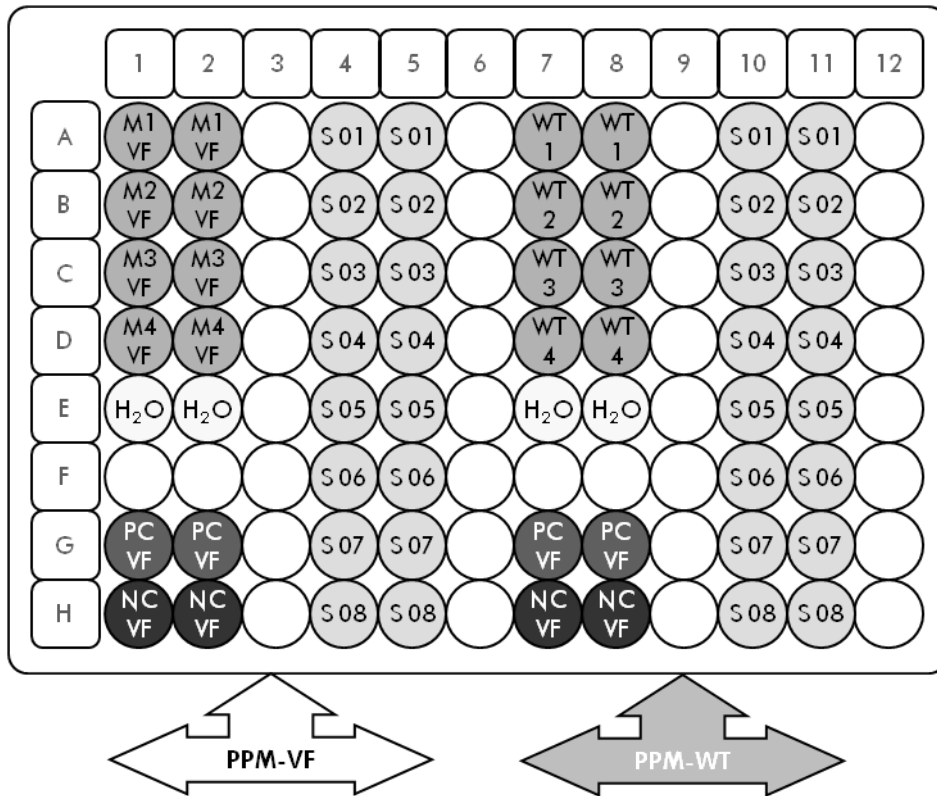


Figure 4. Suggested plate setup for one experiment using the 24 reaction kit (catalog no. 673513). PC-VF: V617F positive control; NC-VF: V617F negative control; M-VF: V617F standards; M-WT: wild-type standards; S: DNA sample; H₂O: water control

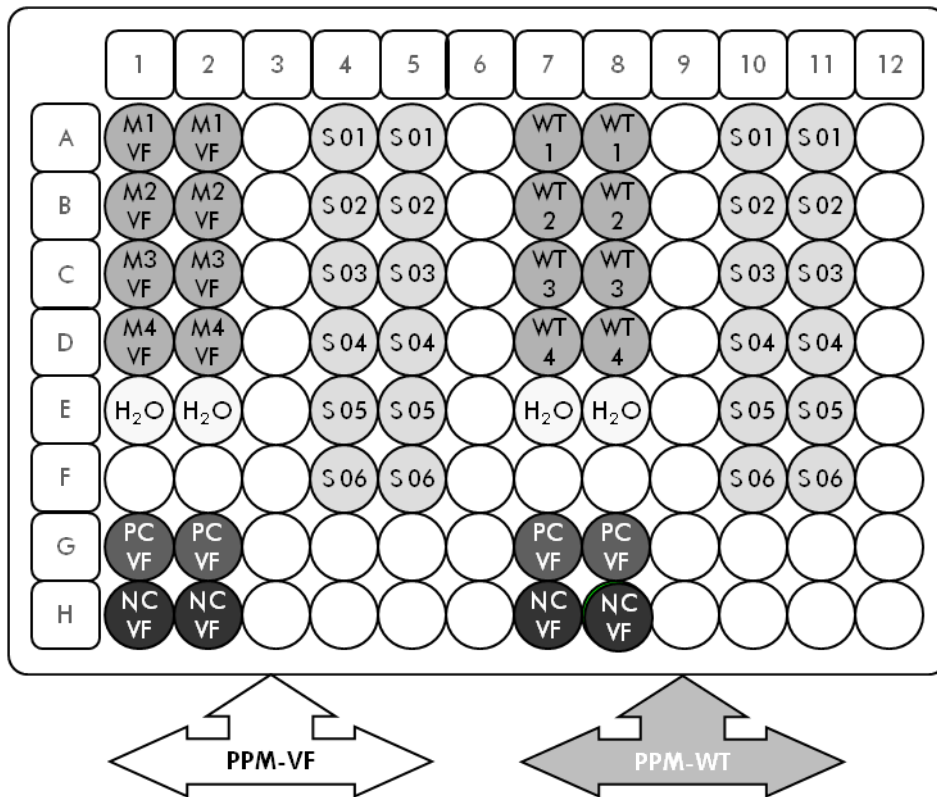


Figure 5. Suggested plate setup for one experiment using the 12 reaction kit (catalog no. 673512). PC-VF: V617F positive control; NC-VF: V617F negative control; M-VF: V617F standards; M-WT: wild-type standards; S: DNA sample; H₂O: water control

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

Tables 6 and 7 describe 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 primer and probe mix (either PPM-VF or PPM-WT). Extra volumes are included to compensate for pipetting error.

Table 6. Preparation of qPCR mix

Component	V617F pre-mix			Final concentration
	1 reaction (μ l)	26 + 1 reactions (μ l)	30 + 1 reactions (μ l)	
TaqMan Universal PCR Master Mix, 2x	12.5	337.5	387.5	1x
Primers and probe mix, PPM-VF 25x	1.0	27	31	1x
Nuclease-free PCR grade water	6.5	175.5	201.5	–
Sample (to be added at step 4)	5.0	5 each	5 each	–
Total volume	25.0	25 each	25 each	–

Table 7. Preparation of qPCR mix

Component	WT pre-mix			Final concentration
	1 reaction (μl)	26 + 1 reactions (μl)	30 + 1 reactions (μl)	
TaqMan Universal PCR Master Mix, 2x	12.5	337.5	387.5	1x
Primers and probe mix, PPM-WT 25x	1.0	27	31	1x
Nuclease-free PCR grade water	6.5	175.5	201.5	–
Sample (to be added at step 4)	5.0	5 each	5 each	–
Total volume	25.0	25 each	25 each	–

- 3. Dispense 20 μ l of the qPCR pre-mix (VF or WT) per well.**
- 4. Add 5 μ l of the material to be quantified (25 ng sample genomic DNA or control) in the corresponding well (total volume 25 μ l).**
- 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 and set the instrument for the acquisition of dual labeled FAM fluorescent probe as indicated in Table 8 for ABI PRISM 7900HT SDS and Applied Biosystems 7500 Real-Time PCR System, or Table 9 for the LightCycler 480 instrument.**

Table 8. Temperature profile for ABI PRISM 7900HT SDS and 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 63°C for 1 minute 30 seconds 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 63°C for 1 minute 30 seconds 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 ABI PRISM 7900HT and Applied Biosystems 7500 Real-Time PCR System, follow step 9a. For the LightCycler 480 instrument, follow step 9b.

9a. ABI PRISM 7900HT and Applied Biosystems 7500 Real-Time PCR System: We recommend a threshold set at 0.1. Start the cycling program, as indicated in Table 8.

9b. 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 JAK2 V617F primers and probe mix (PPM-VF)	
4 M-VF standards	4 reactions, each tested once
n DNA samples	n x 2 reactions
2 DNA controls	2 reactions: PC-VF and NC-VF, each tested once
Water control	1 reaction
With the JAK2 wild-type primers and probe mix (PPM-WT)	
4 wild-type standards	4 reactions, each one tested once
n DNA samples	n x 2 reactions
2 DNA controls	2 reactions: PC-VF and NC-VF, each one tested once
Water control	1 reaction

Sample processing on LightCycler 1.2 instrument

We recommend testing 4 DNA 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.

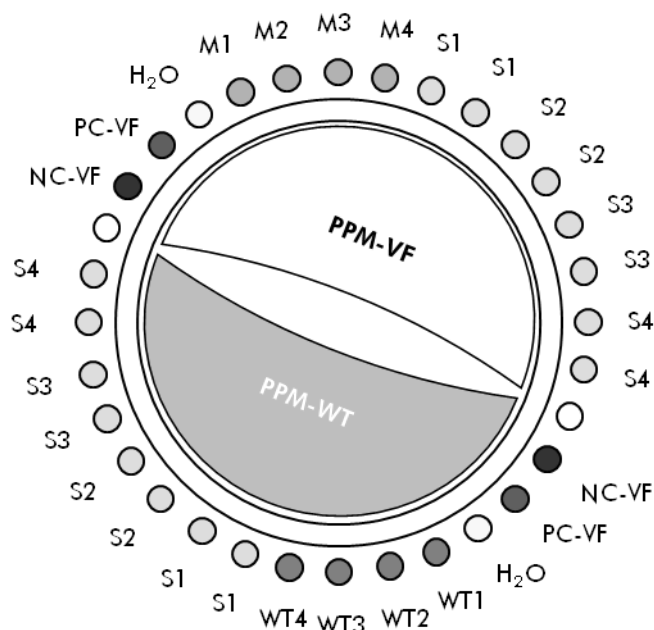


Figure 6. Suggested rotor setup for each experiment with the ipsogen JAK2 MutaQuant Kit. PC-VF: V617F positive control; NC-VF: V617F negative control; M-VF: V617F standards; M-WT: wild-type standards; S: DNA sample; H₂O: water control.

qPCR on LightCycler 1.2 instrument

Note: Because of particular technological requirements, LightCycler experiments must be performed using specific reagents. We recommend the use of LightCycler FastStart DNA Master^{PLUS} HybProbe and following 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.

Tables 11 and 12 describe the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 20 μ l. A pre-mix can be prepared, according to the number of reactions, using the same primer and probe mix (either PPM-VF or PPM-WT). Extra volumes are included to compensate for pipetting error.

Table 11. Preparation of qPCR mix

Component	1 reaction (μl)	V617F pre-mix 15 + 1 reactions (μl)	Final concentration
Freshly prepared LightCycler FastStart DNA Master ^{PLUS} HybProbe Mix, 5x	4.0	64.0	1x
Primers and probe mix, PPM-VF 25x	0.8	12.8	1x
Nuclease-free PCR grade water	10.2	163.2	–
Sample (to be added at step 4)	5.0	5 each	–
Total volume	20.0	20 each	–

Table 12. Preparation of qPCR mix

Component	1 reaction (μl)	WT pre-mix 15 + 1 reactions (μl)	Final concentration
Freshly prepared LightCycler FastStart DNA Master ^{PLUS} HybProbe Mix, 5x	4.0	64.0	1x
Primers and probe mix, PPM-WT 25x	0.8	12.8	1x
Nuclease-free PCR grade water	10.2	163.2	–
Sample (to be added at step 4)	5.0	5 each	–
Total volume	20.0	20 each	–

3. Dispense 15 μ l of the qPCR pre-mix (VF or WT) per capillary.
4. Add 5 μ l of the material to be quantified (25 ng sample genomic DNA or control) in the corresponding tube (total volume 20 μ l).
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 13.

Table 13. Temperature profile

Mode of analysis	Quantification
Hold 1	Temperature: 55°C Time: 2 minutes Ramp: 20
Hold 2	Temperature: 95°C Time: 10 minutes Ramp: 20
Cycling	50 times 95°C for 15 seconds; ramp: 20 66°C for 1 minute; ramp: 20; with acquisition of FAM fluorescence: Single

9. For the LightCycler 1.2, the F1/F2 and “2nd derivative analysis” mode is recommended. Start the thermal cycling program, as indicated in Table 13.

Results

Data analysis principle

Data for the threshold cycle (C_T) and crossing point (C_p) values can be exported from the qPCR instrument and pasted into an Excel[®] file for analysis. These values can then be used to calculate the mean value for C_p and C_T and the standard mean C_T values can be plotted to obtain a standard curve for both the wild-type and V617F standards using the following equation and Table 14.

$y = \text{Mean } C_p; x = \log_{10} \text{ CN}$ where CN = gene copy number in the 5 μl sample

Table 14. Quantitative data for the wild-type and V617F standards

Standard	Copy number (CN)	$\log_{10} \text{ CN}$
M1-VF	$5 \times 10^1 \text{ VF}$	1.7
M2-VF	$5 \times 10^2 \text{ VF}$	2.7
M3-VF	$5 \times 10^3 \text{ VF}$	3.7
M4-VF	$5 \times 10^4 \text{ VF}$	4.7
WT-1	$5 \times 10^1 \text{ WT}$	1.7
WT-2	$5 \times 10^2 \text{ WT}$	2.7
WT-3	$5 \times 10^3 \text{ WT}$	3.7
WT-4	$5 \times 10^4 \text{ WT}$	4.7

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

Standard curve and quality criteria

Figures 7 and 9 show examples of results obtained with *ipsogen* JAK2 MutaQuant Kit and Figures 8 and 10 show an example of the theoretical curve calculated on 4 standard dilutions.

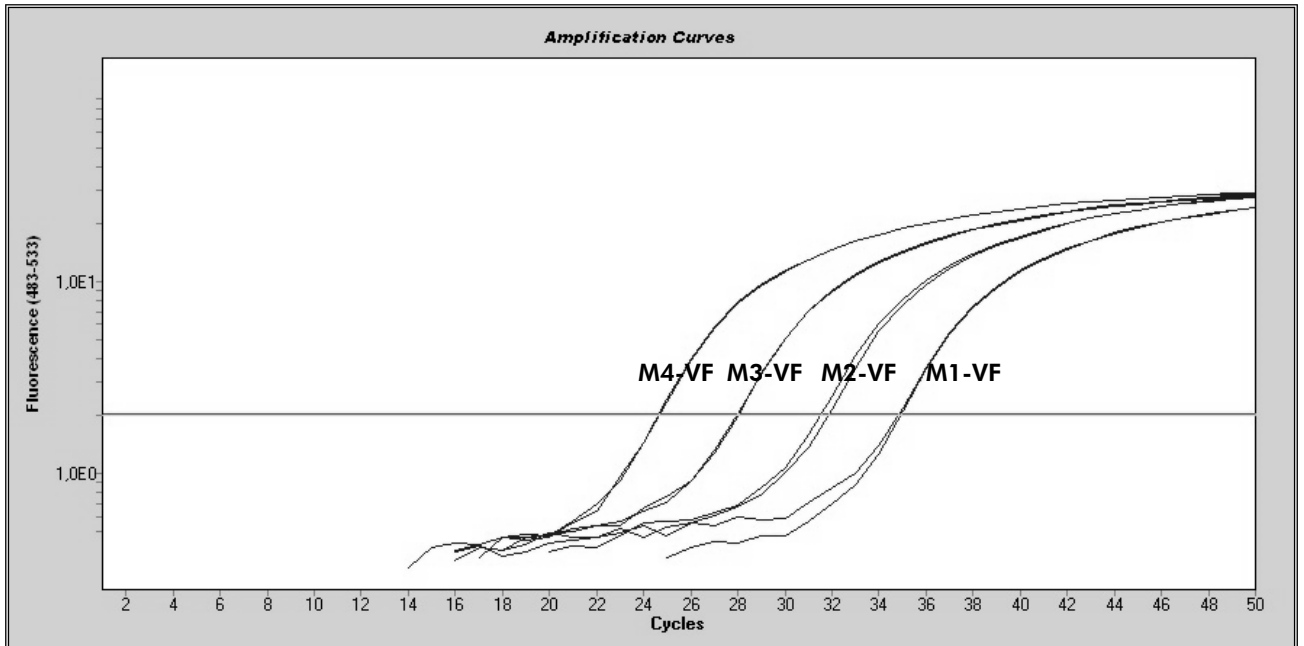


Figure 7. Amplification plot of 5×10^1 , 5×10^2 , 5×10^3 , and 5×10^4 copies of the JAK2 V617F plasmid (controls M1-VF, M2-VF, M3-VF, M4-VF, respectively).

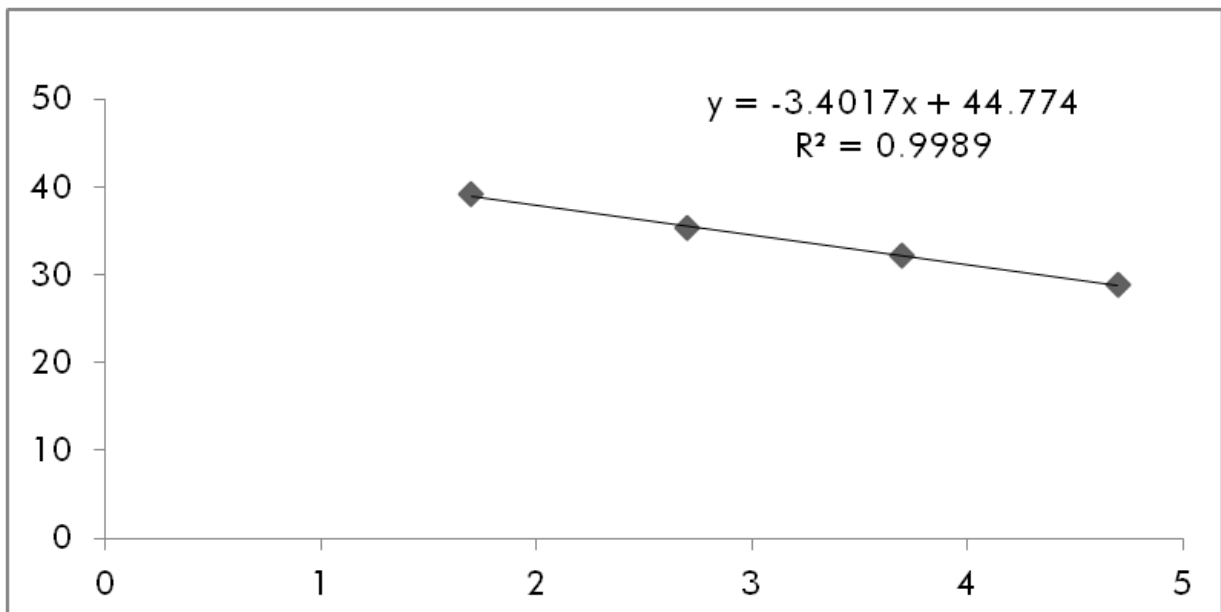


Figure 8. Standard curve for JAK2 V617F.

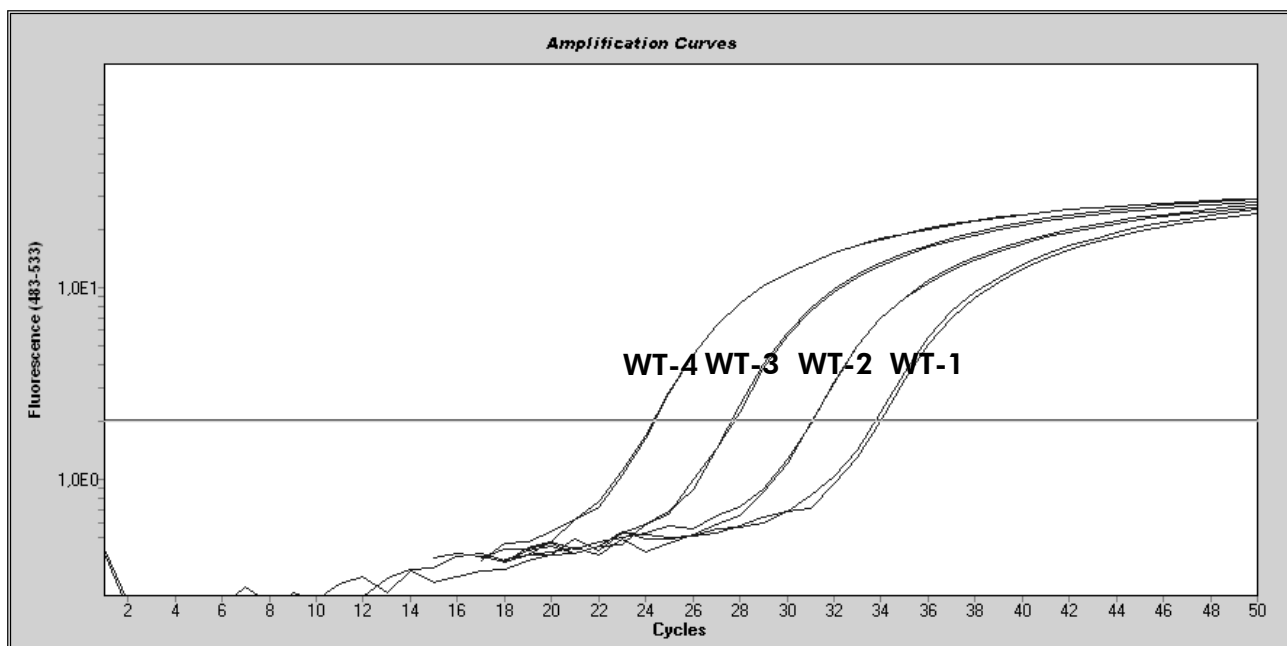


Figure 9. Amplification plot of 5×10^1 , 5×10^2 , 5×10^3 , and 5×10^4 copies of the JAK2 wild-type plasmid (controls WT-1, WT-2, WT-3, and WT-4, respectively).

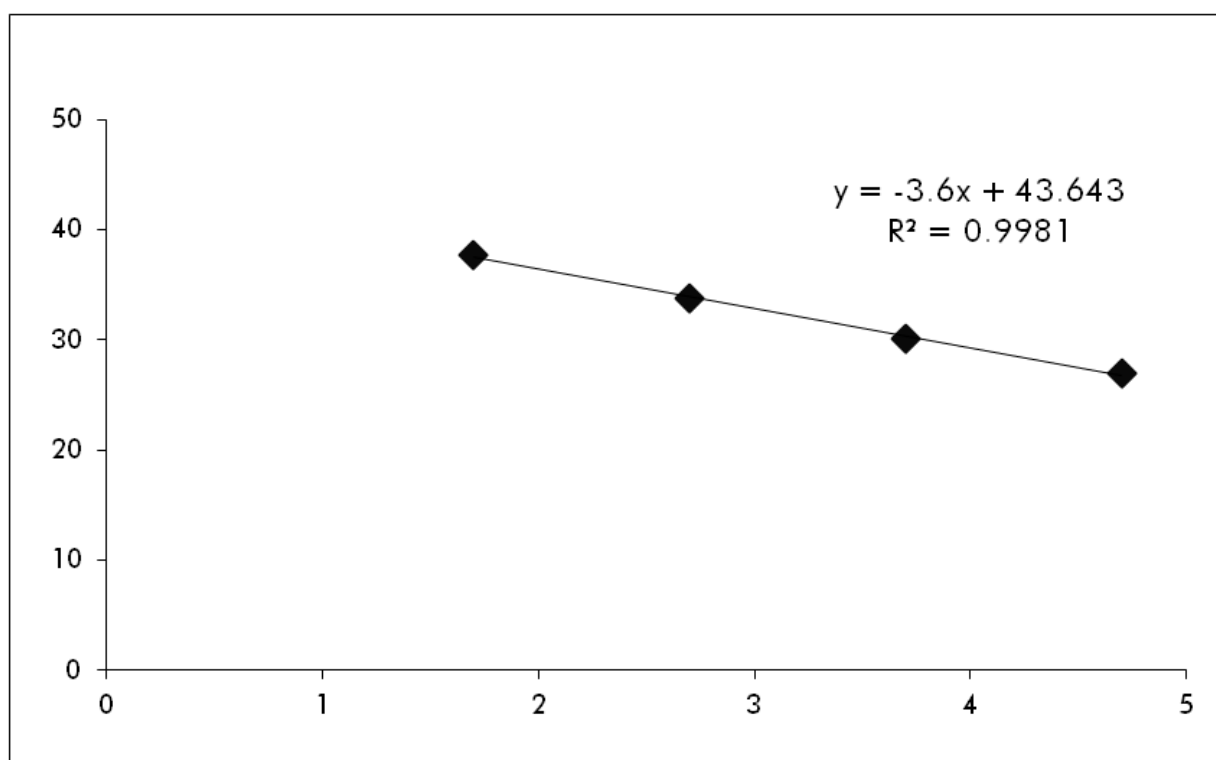


Figure 10. Standard curve for JAK2 wild-type.

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 (2). However, a value for $R^2 >0.98$ is desirable for precise results (3).

The standard curve equations can then be used to calculate V617F and WT \log_{10} copy numbers in the unknown samples. The V617F standard curve equation should be used to transform raw C_p/C_T value means (obtained with PPM-VF) for the unknown and control samples, into JAK2 V617F copy numbers (CN_{V617F}).

$$\log_{10} CN_{V617F} = \frac{(\text{Mean } C_{pV617F} - \text{Standard curve intercept}_{V617F})}{\text{Standard curve slope}_{V617F}}$$

The wild-type standard curve equation should be used to transform raw mean C_p/C_T value (obtained with PPM-WT) for the unknown and control samples, into JAK2 wild-type copy numbers (CN_{WT}).

$$\log_{10} CN_{WT} = \frac{(\text{Mean } C_{pWT} - \text{Standard curve intercept}_{WT})}{\text{Standard curve slope}_{WT}}$$

Expression of the results

Results are relative to 25 ng of total genomic DNA and should be expressed as the percentage of JAK2 V617F as follows.

$$\text{JAK2 V617F \%} = \frac{CN_{V617F}}{(CN_{V617F} + CN_{WT})} \times 100$$

Do not take into account samples for which $(CN_{V617F} + CN_{WT}) < 10\,000$ copies.

Reproducibility between replicates

The data obtained should be consistent between duplicates.

Positive and negative controls

The positive control or PC-VF should give a JAK2 V617F percentage that is higher than 99.9%.

The negative control or NC-VF should give a JAK2 V617F percentage that is lower than 0.1%.

If these controls fail to function correctly, please see the “Troubleshooting guide”, page 31, to find a solution.

Water controls

Negative controls should give zero CN for both the JAK2 V617F and JAK2 wild-type detection.

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

Standard curve for wild-type or V617F are not linear

Vial inversion, inversion during distribution, cross-contamination, partial degradation of the standard, qPCR reagent, non-specific amplification, or PCR program error

Check pipetting scheme and the setup of the reaction.

Store the *ipsogen* JAK2 MutaQuant Kit at –15 to –30°C and keep primers and probe mixes) protected from light. See “Reagent Storage and Handling”, page 10.

Avoid repeated freezing and thawing.

No or low signal for one standard

Standard not distributed, or use of same PPM mix

Check pipetting scheme and the setup of the reaction.

Repeat the PCR run.

Comments and suggestions

Negative (H₂O) control is positive

Cross-contamination, reagent contamination, instrument error, well or capillary inversion, or probe degradation

Replace all critical reagents.

Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carry over contamination.

Keep primers and probe mixes protected from light.

Check for false positives on fluorescence curves.

No signal, even in standard control

a) Incorrect detection channel has been chosen

Set channel to F1/F2 or 530 nm/640 nm.

b) Pipetting error or omitted reagents

Check pipetting scheme and the setup of the reaction.

Repeat the PCR run.

c) No data acquisition program

Check the cycle program.

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

Inhibitory effects of sample material caused by insufficient purification

Always check the DNA quality (OD₂₆₀/OD₂₈₀) and concentration before starting.

Repeat DNA preparation.

Fluorescence intensity too low

a) Inappropriate storage of kit components

Aliquot reagents for storage.

Store the *ipsogen* JAK2 MutaQuant Kit at -15 to -30°C and keep primers and probe mixes protected from light. See "Reagent Storage and Handling", page 10.

Avoid repeated freezing and thawing.

Comments and suggestions

b) Very low initial amount of target DNA

Check the amount of the sample DNA.

Note: Depending of the chosen method of DNA preparation, inhibitory effects may occur.

Negative controls are positive

Carry over 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.

Fluorescence intensity varies

a) Pipetting error

Vortex and spin all reagents after thawing.

LightCycler 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 plate, tubes, or capillaries, or 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.

Comments and suggestions

Wild-type or V617F positive controls signal using the reciprocal PPM

Cross-contamination, reagent contamination, or well or capillary inversion

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.

Check pipetting scheme and setup of the reaction.

Inverted detection of positive control

Distributed inversion of PPM in well or capillary or in premix

Check pipetting scheme and setup of the reaction.

No signal for one positive control or both

PPM or control DNA omitted

Check pipetting scheme and setup of the reaction.

High background

Fluorophore bleaching

Store and handle probe protected from light.

Poor reproducibility for the duplicate samples

Pipetting error or cross-contamination

Check pipetting scheme and the setup of the reaction.

Quality Control

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

References

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










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

Cited references

1. Lippert, E. et al. (2006) The JAK2-V617F mutation is frequently present at diagnosis in patients with essential thrombocythemia and polycythemia vera. *Blood* **108**, 1865.
2. van der Velden, V.H. et al. (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* **17**, 1013.
3. Branford, S. et al. (2006) Rationale for the recommendations for harmonizing current methodology for detecting BCR-ABL transcripts in patients with chronic myeloid leukaemia. *Leukemia* **20**, 1925.

Symbols

The following symbols may appear on the packaging and labeling:

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

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Ordering Information

Product	Contents	Cat. no.
<i>ipsogen</i> JAK2 MutaQuant Kit (12)	For 12 reactions: Wild-type JAK2 Gene Control, JAK2 V617F Control Gene, Primers and Probe Mix PPM-WT, Primers and Probe Mix PPM-VF, JAK2 WT Standard dilutions, JAK2 M-VF Standard dilutions	673512
<i>ipsogen</i> JAK2 MutaQuant Kit (24)	For 24 reactions: Wild-type JAK2 Gene Control, JAK2 V617F Control Gene, Primers and Probe Mix PPM-WT, Primers and Probe Mix PPM-VF, JAK2 WT Standard dilutions, JAK2 M-VF Standard dilutions	673513
Rotor-Gene Q — for outstanding performance in real-time PCR		
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cyclers 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 cyclers 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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Australia ■ techservice-au@qiagen.com

Austria ■ techservice-at@qiagen.com

Belgium ■ techservice-bnl@qiagen.com

Brazil ■ suportetecnico.brasil@qiagen.com

Canada ■ techservice-ca@qiagen.com

China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

Ireland ■ techservice-uk@qiagen.com

Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

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

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

UK ■ techservice-uk@qiagen.com

USA ■ techservice-us@qiagen.com

