

September 2017

ipsogen[®] JAK2 RGQ PCR Kit Handbook



For use with Rotor-Gene[®] Q MDx 5plex HRM instrument

Version 1

Quantitative in vitro diagnostics

IVD

CE

REF

673623



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Intended Use

The *ipsogen* JAK2 RGQ PCR Kit is an in vitro quantitative test intended for the detection of the JAK2 V617F/G1849T allele in genomic DNA extracted from whole blood. The test is intended to be an aid in the diagnosis of myeloproliferative neoplasm (MPN) in conjunction with other clinicopathological factors.

Summary and Explanation

A recurrent somatic mutation, V617F, affecting the Janus tyrosine kinase 2 (JAK2) gene, was identified in 2005 (1–4), leading to a major breakthrough in the understanding, classification and diagnosis of MPN. JAK2 is a critical intracellular signaling molecule for a number of cytokines, including erythropoietin.

The JAK2 V617F mutation is detected in >95% of patients with polycythemia vera (PV), 50–60% of patients with essential thrombocythemia (ET) and in 50% of patients with primary myelofibrosis (PMF). JAK2 V617F has also been detected in some rare cases of chronic myelomonocytic leukemia, myelodysplastic syndrome (MDS), systemic mastocytosis and chronic neutrophilic leukemia, but in 0% of chronic myeloid leukemia (CML) (5).

The mutation corresponds to a single nucleotide change of JAK2 nucleotide 1849 in exon 14, resulting in a unique valine (V) to phenylalanine (F) substitution at position 617 of the protein (JH2 domain). The mutation leads to constitutive activation of JAK2, hematopoietic transformation in vitro and erythropoietin-independent erythroid colony (EEC) growth in all patients with PV and a large proportion of ET and PMF patients (6). JAK2 V617F represents a key driver in the transformation of hematopoietic cells in MPN, but the exact pathological mechanisms, with the same unique mutation, leading to such different clinical and biological entities has not yet been fully elucidated.

Traditionally, the diagnosis of MPNs was based on clinical, bone marrow histology and cytogenetic criteria. The discovery of a disease-specific molecular marker resulted in both simplification of the process and increased diagnostic accuracy. Detection of the JAK2 V617F mutation is now part of the reference World Health Organization (WHO) 2008 criteria for the diagnosis of BCR-ABL negative MPN (Table 1, next page), and presence of this mutation is a major criterion for diagnostic confirmation.

Table 1. WHO criteria for the diagnosis of MPN (adapted from reference 7)

Criteria for a diagnosis of PV	
Major	1. Hemoglobin (Hgb) >18.5 g.dl ⁻¹ (men) or >16.5 g.dl ⁻¹ (women) or Hgb or hematocrit >99 th percentile of reference range for age, sex or altitude of residence or Hgb >17 g.dl ⁻¹ (men) or >15 g.dl ⁻¹ (women) if associated with sustained increase of ≥2 g.dl ⁻¹ from baseline that cannot be attributed to correction of iron deficiency or Elevated red cell mass >25% above mean normal predicted value 2. Presence of JAK2V617F or similar mutation
Minor	1. Bone marrow trilineage myeloproliferation 2. Subnormal serum erythropoietin level 3. EEC growth
Criteria for a diagnosis of ET	
Major	1. Platelet count ≥450 × 10 ⁹ l ⁻¹ 2. Megakaryocyte proliferation with large and mature morphology. No or little granulocyte or erythroid proliferation 3. Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm 4. Demonstration of JAK2V617F or other clonal marker or No evidence of reactive thrombocytosis
Minor	–
Criteria for a diagnosis of PMF	
Major	1. Megakaryocyte proliferation and atypia accompanied by either reticulin and/or collagen fibrosis or In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e., prefibrotic PMF) 2. Not meeting WHO criteria for CML, PV, MDS or other myeloid neoplasm 3. Demonstration of JAK2V617F or other clonal marker or No evidence of reactive marrow fibrosis
Minor	1. Leukoerythroblastosis 2. Increased serum lactate dehydrogenase 3. Anemia 4. Palpable splenomegaly

CML: Chronic myeloid leukemia; EEC: endogenous erythroid colony; ET: essential thrombocythemia; Hgb: hemoglobin; MDS: myelodysplastic syndrome; PMF: primary myelofibrosis; PV: polycythemia vera; WHO: World Health Organization.

Since 2006, several methods essentially based on PCR techniques or sequencing are available, as laboratory-developed tests to detect the presence and potentially quantify JAK2V617F. These tests have different analytical performance, especially with regard to precision and level of sensitivity. This difference may impact the need for bone marrow analysis, time required to establish a final diagnosis and, potentially, on diagnostic performance.

Principle of the Procedure

Several different techniques have been proposed to quantitatively determine the proportion of single nucleotide polymorphisms (SNPs) in DNA samples. Some, such as melting curves and sequencing, are only semi-quantitative. Methods based on real-time quantitative polymerase chain reaction (qPCR) are preferred because of their higher sensitivity. The use of a SNP specific primer permits the selective amplification of the mutant (MT) or wild-type (WT) allele that is easily detectable using a real-time qPCR instrument. This allows a sensitivity <0.1%, which is in line with the currently accepted JAK2 cut-off of 1% that is used for clinical positivity. However, it should be noted that some clinical experts consider the presence of any JAK2 load as clinically significant at diagnosis time and hence the need for a sensitive method, such as qPCR (8). The *ipsogen* JAK2 RGQ PCR 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, three 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 oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. Another dye-linked oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream, 3' dye-free quencher, 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 results in suppression of the reporter fluorescence primarily by Förster-type energy transfer.

During PCR, if the target of interest is present, both forward and reverse primers specifically anneal and flank the probe. The 5'→3' exonuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher only if the three oligonucleotides hybridize 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, next page). 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 primers and 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.

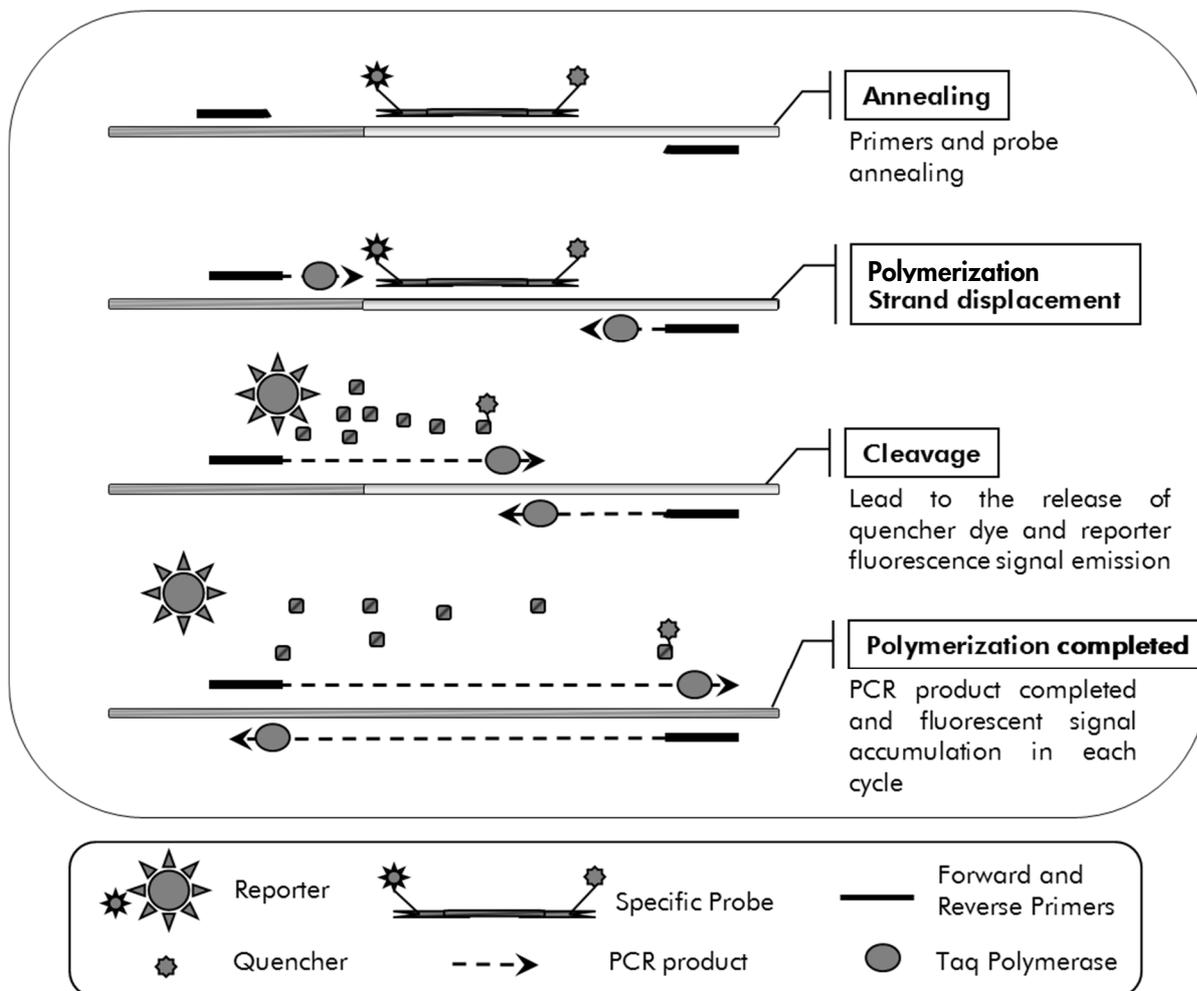


Figure 1. Reaction principle. The quantitative allele-specific PCR technology used in this assay kit allows sensitive, accurate and highly reproducible detection of SNPs. This technique is based on the use of specific reverse primers for the wild-type and the V617F alleles (8). Only a perfect match between primer and target DNA allows extension and amplification in the PCR (Figure 2).

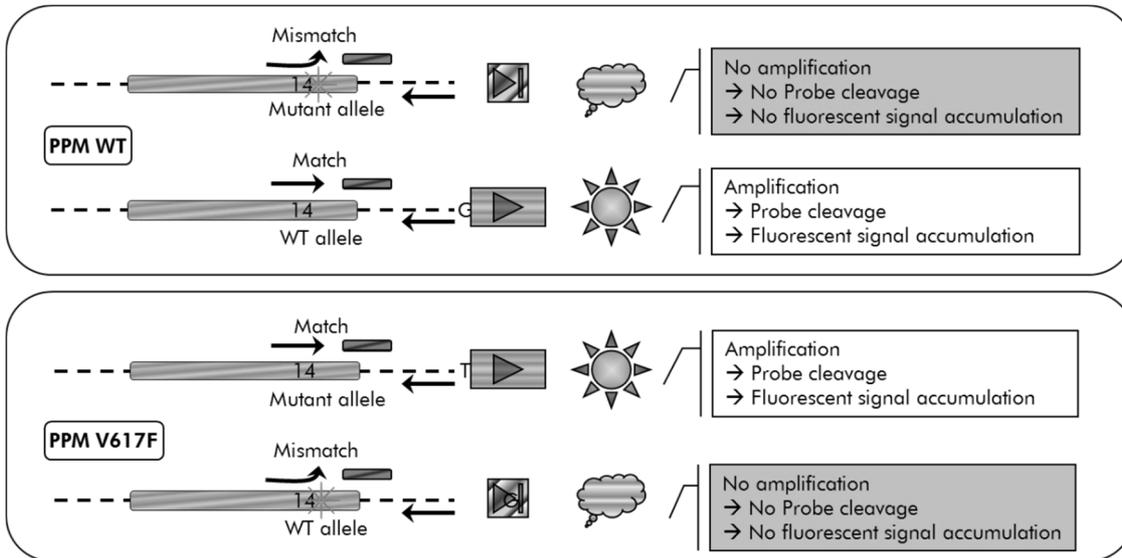


Figure 2. Allele-specific PCR. Use of wild-type or the V617F primers and probe mix allows the specific detection of the wild-type or mutated allele in two separate reactions conducted using the same sample. Results can be expressed as percentage of VF copies among total JAK2 copies.

Materials Provided

Kit contents

ipsogen JAK2 RGQ PCR Kit (24)		
Catalog no.		673623
Number of reactions		24
JAK2 Mutant Control (100% V617F allele)	Red	33 µl
JAK2 WT Control (100% wild-type allele)	Green	33 µl
JAK2 MT Quant Standard 1 (5 x 10 ¹ V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 2 (5 x 10 ² V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 3 (5 x 10 ³ V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 4 (5 x 10 ⁴ V617F copies/5 µl)	Red	20 µl
JAK2 WT Quant Standard 1 (5 x 10 ¹ wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 2 (5 x 10 ² wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 3 (5 x 10 ³ wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 4 (5 x 10 ⁴ wild-type copies/5 µl)	Green	20 µl
JAK2 MT Reaction Mix*	Red	1010 µl
JAK2 WT Reaction Mix†	Green	1010 µl
Taq DNA polymerase (HotStarTaq® 5 units/µl)	Mint	85 µl
TE buffer for sample dilution	White	1.9 ml
Water for no template control (NTC)	White	1.9 ml
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* PCR mix containing all the required components except Taq DNA polymerase and target DNA for the MT allele.

† PCR mix containing all the required components except Taq DNA polymerase and target DNA for the WT allele.

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.

Consumables and reagents for manual DNA extraction

- QIAamp® DSP DNA Blood Mini Kit (cat. no. 61104)
- Ethanol (96–100%)

Note: Do not use denatured alcohol as this contains other substances such as methanol or methylethylketone.

Consumables and reagents for automated DNA extraction

- QIASymphony® DSP DNA Mini Kit (cat. no. 937236)
- Sample Prep Cartridges, 8-well (cat. no. 997002)
- 8-Rod Covers (cat. no. 997004)
- Filter-Tips, 1500 µl (cat. no. 997024)
- Filter-Tips, 200 µl (cat. no. 990332)
- Elution Microtubes CL (cat. no. 19588)
- Tip disposal bags (cat. no. 9013395)
- Micro tubes 2.0 ml Type H (Sarstedt®, cat. no. 72.694, www.sarstedt.com)

Consumables and reagents for PCR

- Nuclease-free aerosol-resistant sterile PCR pipet tips with hydrophobic filters
- 1.5 ml or 2.0 ml nuclease-free PCR tubes
- Strip Tubes and Caps, 0.1 ml, for the Rotor-Gene Q (cat. no. 981103 or 981106)
- Ice

Equipment

- Micropipetter (adjustable)* dedicated for PCR (1–10 µl; 10–100 µl; 100–1000 µl)
- Disposable gloves
- Vortex mixer
- Heating block for lysis of samples at 56°C
- Benchtop centrifuge* with rotor for 0.5 ml/1.5/2.0 ml reaction tubes (capable of attaining 13,000–14,000 rpm)
- Spectrophotometer

Equipment for automated sample preparation

- QIASymphony SP instrument* (cat. no. 9001297), software version 4.0 or higher, provided accessories and Blood_200_V7_DSP protocol
- Tube Insert 3B (Insert, 2.0 ml v2, sample carrier (samplecarr.) (24), Qsym, cat. no. 9242083)

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Equipment for PCR

- Real-time PCR instrument*: Rotor-Gene Q MDx 5plex HRM and provided accessories
- Installed Rotor-Gene AssayManager® v2.1, software 2.1.x (x≥0)
- Installed Rotor-Gene AssayManager Gamma Plug-in v1.0.x (x≥0)
- Imported JAK2 CE Assay Profile (ipsogen_JAK2_blood_CE_V1_0_x (X≥0))

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Warnings and Precautions

For in vitro diagnostic use

When working with chemicals, always wear a suitable 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.

<p>CAUTION</p> 	<p>CAUTION: DO NOT add bleach or acidic solutions directly to the sample or preparation waste.</p>
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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 in vitro diagnostic use. Reagents and instructions supplied in this kit have been validated for optimal performance.

- The test is for use with whole blood samples anti-coagulated with potassium EDTA and stored at 2–8°C for no more than 96 hours until DNA extraction.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Discard sample and assay waste according to your local safety procedures.
- Reagents for *ipsogen* JAK2 RGQ PCR Kit are diluted optimally. Do not dilute reagents further as this may result in a loss of performance.
- Do not use reaction volumes (reaction mix plus sample) of less than 25 µl.
- All reagents supplied in the *ipsogen* JAK2 RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same kit. Do not substitute any reagent from one kit with the same reagent from another *ipsogen* JAK2 RGQ PCR Kit, even from the same batch, as this may affect performance.
- Refer to the Rotor-Gene Q MDx 5plex HRM instrument user manual and RGAM 2.1 user manual for additional warnings, precautions and procedures.

- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Do not use expired or incorrectly stored components.
- Reaction mixes may be altered if exposed to light.
- Use extreme caution to prevent contamination of the mixes with the synthetic materials that are contained in the JAK2 MT and JAK2 WT Quant Standards reagents and with the JAK2 MT and JAK2 WT Control reagents.
- Use extreme caution to prevent DNA or PCR product carryover contamination resulting in a false positive signal.
- Use extreme caution to prevent contamination by DNase, which might cause degradation of the template DNA.
- Use individual, dedicated pipets for setting up reaction mixes and adding templates.
- Do not open the Rotor-Gene Q MDx instrument until the run is finished.
- Do not open Rotor-Gene Q tubes after the run is finished.
- Caution must be observed to ensure correct sample testing with emphasis on wrong sample entry, loading error and pipetting error.
- Make sure the samples are handled in a systematic way to ensure correct identification at all times so maintaining traceability.

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 matrices (DNA, plasmid or PCR products) are introduced. Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).

For safety information relative to the extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) and QIA Symphony DNA DSP Mini Kit (cat. no. 937236), please refer to the corresponding handbooks.

Reagent Storage and Handling

Shipping conditions

The *ipsogen* JAK2 PGQ PCR Kit is shipped on dry ice. If any component of the *ipsogen* JAK2 RGQ PCR Kit (apart from the enzyme) is not frozen on arrival, the outer packaging has been opened during transit or the shipment does not contain a packing note, handbook or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Storage conditions

The *ipsogen* JAK2 RGQ PCR Kit must be stored immediately upon receipt at -30°C to -15°C upon receipt in a constant-temperature freezer and protected from light.

For storage information relative to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) and the QIASymphony DNA DSP Mini Kit (cat. no. 937236), please refer to corresponding handbooks.

Stability

When stored under the specified storage conditions, the *ipsogen* JAK2 RGQ PCR Kit is stable until the stated expiration date on box label.

Once opened, reagents can be stored in their original packaging at -30°C to -15 until the stated expiration date shown on the box label. Repeated thawing and freezing should be avoided. Do not exceed a maximum of five freeze-thaw cycles.

For stability information relative to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) and QIASymphony DNA DSP Mini Kit (cat. no. 937236), please refer to corresponding handbooks.

- Gently mix by inverting the tube 10 times and centrifuge all tubes except the enzyme before opening.
- Expiration dates for each reagent are indicated on the individual component labels. Under correct storage conditions, the product will maintain performance for the stability time as long as the same batches of components are used.
- Quality control procedures at QIAGEN employ functional kit release testing for each individual kit lot. Therefore, do not mix reagents from different kits, even from the same lot.

Specimen Handling and Storage

Whole blood samples

The *ipsogen* JAK2 RGQ PCR Kit is for use with genomic DNA samples extracted from whole blood samples anti-coagulated with potassium EDTA either stored as follows:

- At 2–8°C for no more than 96 hours
- At 15–25°C for no more than 96 hours
- Frozen at –15°C to –30°C for no more than 1 month

Note: Whole blood samples must be shipped under the same conditions as storage to avoid temperature changes during storage and shipment.

Genomic DNA samples

Once genomic DNA is extracted, samples can be stored and shipped at –30°C to –15°C for no more than 15 months, either directly after extraction or after being diluted in TE buffer.

Procedure

Genomic DNA extraction and preparation from whole blood

Genomic DNA should be extracted using either the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) or the QIASymphony SP instrument in combination with the QIASymphony DSP DNA Mini Kit (cat. no. 937236).

Ensure reagents to be used have not expired and have been transported and stored under the correct conditions.

Note: The *ipsogen* JAK2 RGQ PCR Kit has only been validated in combination with the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) or the QIASymphony DSP DNA Mini Kit (cat. no. 937236). Do not use any other DNA extraction product.

Manual genomic DNA extraction using the QIAamp DSP DNA Blood Mini Kit

Manual genomic DNA extraction must be performed with the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) according to the corresponding *QIAamp DSP DNA Blood Mini Kit Handbook*.

Things to do before starting

- **Equilibrate blood samples to room temperature (15–25°C), and ensure that they are well homogenized.**
- **Prepare the Lysis Buffer**
If a precipitate has formed in Lysis Buffer (AL), dissolve by incubating at 56°C.
- **Preparing QIAGEN Protease**
Add 1.2 ml Protease Solvent (PS) to the vial of lyophilized QIAGEN Protease (QP) and mix carefully. To avoid foaming, mix by inverting the vial several times. Ensure that the QIAGEN Protease (QP) is completely dissolved.
Note: Do not add QP directly to Lysis Buffer (AL).
- **Preparing Wash Buffer 1**
Using a measuring cylinder, add 25 ml ethanol (96–100%) to the bottle containing 19 ml Wash Buffer 1 (AW1) concentrate. Store the reconstituted Wash Buffer 1 (AW1) at room temperature (15–25°C).
Note: Always mix the reconstituted Wash Buffer 1 (AW1) by inverting the bottle several times before starting the procedure.
- **Preparing Wash Buffer 2**
Using a measuring cylinder, add 30 ml ethanol (96–100%) to the bottle containing 13 ml Wash Buffer 2 (AW2) concentrate. Store the reconstituted Wash Buffer 2 (AW2) at room temperature (15–25°C).
Note: Always mix the reconstituted Wash Buffer 2 (AW2) by inverting the bottle several times before starting the procedure.
- **Preparing the Elution Buffer**
One bottle of Elution Buffer (AE) is provided with the kit. To prevent contamination of Elution Buffer (AE), we strongly recommend using pipet tips with aerosol barriers when pipetting Elution Buffer (AE) from the bottle and replacing the cap of the bottle immediately afterwards. Equilibrate Elution Buffer (AE) to room temperature (15–25°C).
- Set a heating block to 56°C for use in step 4.

Procedure

1. Pipet 20 µl QIAGEN Protease (QP) into a lysis tube (LT).
Note: Check the expiration date of the reconstituted protease before use.
2. Add 200 µl blood sample to the lysis tube (LT).

3. Add 200 μ l Lysis Buffer (AL) to the lysis tube (LT), close the lid and mix using a pulse-vortex for 15 seconds.

Note: To ensure efficient lysis, it is essential that the sample and Lysis Buffer (AL) are mixed thoroughly to yield a homogenous solution.

Note: Since Lysis Buffer (AL) has a high viscosity, be sure to add the correct volume of Lysis Buffer (AL) by pipetting carefully or by using a suitable pipet.



Do not add QIAGEN Protease (QP) directly to Lysis Buffer (AL).

4. Incubate at 56°C (\pm 1°C) for 10 minutes (\pm 1 minute).
5. Centrifuge the lysis tube (LT) for about 5 seconds at full speed to remove drops from the inside of the lid.
6. Add 200 μ l ethanol (96–100%) to the lysis tube (LT), close the lid and mix thoroughly using a pulse-vortex for \geq 15 seconds.
7. Centrifuge the lysis tube (LT) for \geq 5 seconds at full speed to remove any drops of liquid from the inside of the lid.
8. Carefully apply the entire lysate from step 7 to the QIAamp Mini spin column without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.

Note: If processing several samples, open only one lysis tube (LT) at a time.
9. Close the lid of the QIAamp Mini spin column, and centrifuge at approximately 6000 \times g for 1 minute. Place the QIAamp Mini spin column in a clean wash tube (WT), and discard the tube containing the filtrate.

Note: If the lysate has not completely passed through the membrane after centrifugation at 6000 \times g (8000 rpm), centrifuge again at full speed (up to 20,800 \times g) for 1 minute.

Note: If the lysate still does not pass through the membrane during centrifugation, discard the sample and repeat the isolation and purification with new sample material.
10. Carefully open the QIAamp Mini spin column, and add 500 μ l Wash Buffer 1 (AW1) without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.
11. Close the lid of the QIAamp Mini spin column, and centrifuge at approximately 6000 \times g (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean wash tube (WT), and discard the tube containing the filtrate.
12. Carefully open the QIAamp Mini spin column, and add 500 μ l Wash Buffer 2 (AW2) without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.
13. Close the lid of the QIAamp Mini spin column, and centrifuge at full speed (approximately 20,000 \times g or 14,000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean wash tube (WT), and discard the tube containing the filtrate.

14. Centrifuge at full speed (approximately 20,000 x g or 14,000 rpm) for 3 minutes to dry the membrane completely.
15. Place the QIAamp Mini spin column in a clean elution tube (ET) and discard the wash tube (WT) containing the filtrate. Carefully open the lid of the QIAamp Mini spin column, and apply 50–200 µl Elution Buffer (AE) to the center of the membrane. Close the lid and incubate at room temperature (15–25°C) for 1 minute. Centrifuge at approximately 6000 x g (8000 rpm) for 1 minute to elute the DNA.
16. Discard used sample tubes, plates and waste according to your local safety regulations.

Automated genomic DNA extraction using the QIAasymphony DSP DNA Mini Kit

Automated genomic DNA extraction must be performed with the QIAasymphony instrument using the Sample Preparation module in combination with the QIAasymphony DSP DNA Mini Kit (cat. no. 937236) and by following the instruction in the *QIAasymphony DSP DNA Kit Handbook*. JAK2 protocol features are highlighted with the sign  in the procedure below.

With the QIAasymphony SP, the QIAasymphony DSP DNA Mini Kit enables automated DNA purification from human whole blood (using the Blood_200_V7_DSP protocol on the QIAasymphony).

- No pre-treatment is required
- Tubes are directly transferred to QIAasymphony SP
- Purification of DNA is performed with magnetic particles

Important points before starting

-  Whole blood volume to be extracted is 300 µl.
- Ensure that you are familiar with operating the QIAasymphony SP. Refer to the user manuals supplied with your instrument for operating instructions.
- Optional maintenance is not mandatory for instrument function, but is highly recommended to reduce risk of contamination.

- Before using a reagent cartridge for the first time, check that Buffer QSL1 and Buffer QSB1 do not contain a precipitate. If necessary, remove the troughs containing Buffer QSL1 and Buffer QSB1 from the reagent cartridge and incubate for 30 minutes at 37°C with occasional shaking to dissolve precipitate. Make sure to replace the troughs in the correct positions. If the reagent cartridge is already pierced, make sure that the troughs are sealed with Reuse Seal Strips and incubate the complete reagent cartridge for 30 minutes at 37°C with occasional shaking in a water bath.
- Try to avoid vigorous shaking of the reagent cartridge (RC) otherwise foam may be generated, which can lead to liquid-level detection problems.

Things to do before starting

- Before starting the procedure, ensure that the magnetic particles are fully resuspended. Vortex the trough containing the magnetic particles vigorously for at least 3 minutes before first use.
- Make sure that the piercing lid is placed on the reagent cartridge and that the lid of the magnetic-particle trough has been removed or, if using a partially used reagent cartridge, make sure the Reuse Seal Strips have been removed.
- Make sure to open the enzyme tubes.
- If samples are bar coded, orient samples in the tube carrier so that the bar codes face the bar code reader at the left side of the QIA Symphony SP.

Procedure

1. Close all drawers and the hood.
2. Switch on the QIA Symphony SP, and wait until the "Sample Preparation" screen appears and the initialization procedure has finished.

Note: The power switch is located at the bottom, left corner of the QIA Symphony SP.

3. Log on to the instrument.
4. Ensure the "Waste" drawer is prepared properly, and perform an inventory scan of the "Waste" drawer, including the tip chute and liquid waste container. Replace the tip disposal bag if necessary.
5. Load the required elution rack into the "Eluate" drawer.

Do not load a 96-well plate onto "Elution slot 4"

Only use "Elution slot 1" with the corresponding cooling adapter.

When using a 96-well plate, make sure that the plate is in the correct orientation, as incorrect placement may cause sample mix-up in downstream analysis.

6. Load the required reagent cartridge(s) and consumables into the “Reagents and Consumables” drawer.

Note: Ensure that pipetting tips are correctly fixed.

7. Perform an inventory scan of the “Reagents and Consumables” drawer.

8.  Transfer **300 µl** of the whole blood sample to be extracted to a Micro tube (2.0 ml Type H) and place the tube into the 3b 2 ml adapter on the tube sample carrier. Load the sample tubes into the “Sample” drawer.

9. Using the touchscreen, enter the required information for each batch of samples to be processed:

- Sample information: change default tube format (choose “Select All” button and select “Sarstedt reference 72.694” from the “Tube Insert” sheet)
- Protocol to be run: choose “Select All” button and select the category “DNA Blood” → Blood_200_V7_DSP for whole blood sample

-  Elution volume and output position: 100 µl for the whole blood protocol.

Note: After information about the batch has been entered, the status changes from “LOADED” to “QUEUED”. As soon as one batch is queued, the “Run” button appears.

10. Start the run

- To start the run, press the “Run” button.
- Read and confirm the message that appears.

Note: We recommend waiting beside the instrument until it has performed liquid-level detection of the internal control tubes and the QIAAsymphony SP carrier status changes to “RUNNING”.

Note: Do not pause or stop the run during processing (unless an emergency occurs), as this will lead to the samples being flagged as “unclear”.

Note: It is possible to continuously load samples and add them to this run (until reagents are loaded). Press the “Run” button to start the purification procedure.

11. At the end of the protocol run, the status of the batch changes from “RUNNING” to “COMPLETED”. Retrieve the elution rack containing the purified nucleic acids from the “Eluate” drawer.

We recommend removing the eluate plate from the “Eluate” drawer immediately after the run has finished. Depending on temperature and humidity, elution plates left in the QIAAsymphony SP after the run is completed may experience condensation or evaporation.

Note: In general, magnetic particles are not carried over into eluates. If any eluate contains black particles, the magnetic particles can be removed as follows:

Apply the tube containing the DNA to a suitable magnetic separator (e.g., QIAGEN 12-Tube Magnet, cat. no. 36912) until the magnetic particles are separated. If DNA is in microplates, apply the microplate to a suitable magnetic separator (e.g., QIAGEN 96-Well Magnet Type A, cat. no. 36915) until the magnetic particles are separated. If no suitable magnetic separator is available, centrifuge the tube containing the DNA for 1 minute at full speed in a microcentrifuge to pellet any remaining magnetic particles.

12. Export the QIASymphony SP result file: this report is generated for each elution plate.

- Insert the USB stick into one of the USB ports at the front of the QIASymphony SP.
- Click the “Tools” button.
- Select “File Transfer”.
- On the “In-/Output Files” tab, select “Results Files” and click “Transfer”.

The name of the exported file should have the following format: yyyy-mm-ddhh:mm:ss_Elution rack ID

13. Check the “Validity of result” column for each sample on the QIASymphony SP result file.

- Valid and unclear status: proceed to DNA qualification and quantification.
- Invalid status: sample is rejected. Reprocess the extraction step.

14. If a reagent cartridge is only partially used, seal it with the provided Reuse Seal Strips and close tubes containing proteinase K with screw caps immediately after the end of the protocol run to avoid evaporation.

15. Discard used sample tubes, plates and waste according to your local safety regulations.

16. Clean the QIASymphony SP.

Follow the maintenance instructions in the user manuals supplied with your instrument. Make sure to clean the tip guards regularly to minimize the risk of cross-contamination.

17. Close the instrument drawers, and switch off the QIASymphony SP.

Qualification and quantification of DNA

A blank of ATE or AE buffer should be used to calibrate the spectrophotometer. It is necessary to use these buffers because elution buffers used in genomic DNA extraction kits contain the preservative sodium azide, which absorbs at 260 nm.

- The A_{260}/A_{280} ratio must be ≥ 1.7 as smaller ratios usually indicate protein contamination or the presence of organic chemicals and affect the PCR step.
- DNA quantity is determined by measuring optical density at 260 nm.
- Total amount of DNA purified = concentration x volume of sample in μl .
- If A_{260}/A_{280} ratio is below 1.7 and if genomic DNA concentration is below 10 ng/ μl , the sample must not be further processed.

Genomic DNA sample normalization

The DNA must be diluted to 10ng/μl in TE buffer provided in the *ipsogen* JAK2 RGQ PCR Kit.

The Rotor-Gene Q PCR is optimized for 50 ng of purified genomic DNA diluted in a final volume of 5 μl.

Protocol: qPCR on Rotor-Gene Q MDx 5plex HRM instrument

Important points before starting

- The *ipsogen* JAK2 RGQ PCR Kit must be run on the Rotor-Gene Q MDx 5plex HRM instrument using Rotor-Gene AssayManager v2.1. Take time to familiarize yourself with the Rotor-Gene Q MDx instrument before starting the protocol. See the user manuals for the instrument, the Rotor-Gene AssayManager v2.1 and the Gamma Plug-in for details.
- Rotor-Gene AssayManager v2.1 enables automated interpretation of the PCR results. The cycling parameters are locked for the run.

Things to do before starting

Rotor-Gene AssayManager v2.1 software must be installed on the computer connected to the Rotor-Gene Q and can be downloaded from the QIAGEN website: www.qiagen.com/Products/Rotor-GeneAssayManager_v2.1.aspx. For details about the installation of the Rotor-Gene AssayManager v2.1 core software, please refer to *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

- The *ipsogen* JAK2 RGQ PCR Kit specifically requires the Gamma Plug-in. This plug-in can be downloaded from the QIAGEN website page: www.qiagen.com/shop/detection-solutions/personalized-healthcare/ipsogen-jak2-rgq-pcr-kit-ce/#resources. This plug-in must be installed on a computer that already has Rotor-Gene AssayManager v2.1 installed.
- The *ipsogen* JAK2 RGQ PCR Kit also requires an assay profile. This assay profile (.iap file) contains all parameters needed for cycling and analyzing the qPCR assay. It can be downloaded from the webpage on the QIAGEN website that is dedicated to the *ipsogen* JAK2 RGQ PCR Kit: www.qiagen.com/shop/detection-solutions/personalized-healthcare/ipsogen-jak2-rgq-pcr-kit-ce/#resources. The assay profile must be imported into the Rotor-Gene AssayManager v2.1 software.

Note: The *ipsogen* JAK2 RGQ PCR Kit can run only when certain configuration settings in the Rotor-Gene AssayManager v2.1 software are programmed.

For system wide process safety, the following required configuration settings must be set for the closed mode:

- "Material number required"
- "Valid expiry date required"
- "Lot number required"

Installation of Gamma Plug-in and importing the assay profile

The installation and importing of the Gamma Plug-in and the assay profile are detailed in the *Rotor-Gene AssayManager v2.1 Core Application User Manual* and the *Gamma Plug-in User Manual*.

- Download both the Gamma Plug-in and the latest version of the JAK2 CE assay profile from the QIAGEN website.
- Start the installation process by double-clicking the RGAM_V2_1_Gamma_Plug-in.Installation.V1_0_0.msi file and following the installation instructions. For a detailed description of this process, refer to the “Installing Plug-ins” section in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

Note: For system-wide process safety, select the “Settings” tab and check the boxes for “Material number required”, “Valid expiry date required” and “Lot number required” for the closed mode (section Work list). If these are not enabled (checked), click to enable.

- After successful installation of the plug-in, a person with administrator rights for the Rotor-Gene AssayManager v2.1 software will need to import the ipsogen_JAK2_blood_CE assay profile as follows:
 1. Log in to the Rotor-Gene AssayManager v2.1 software as user with administrator rights.
 2. Select the configuration environment.
 3. Select the “Assay Profiles” tab.
 4. Click the “Import” button.
 5. Select the ipsogen_JAK2_blood_CE assay profile to be imported in the dialog and click “Open”.
 6. Once the assay profile has been successfully imported, it can be used in the “Setup” environment.

Note: The same version of an assay profile cannot be imported twice.

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

We recommend testing eight genomic DNA samples in the same experiment to optimize use of the controls, standards and reaction mixes.

Table 2 provides the number of reactions that can be run using the 72-tube rotor.

The scheme shown in Figure 3 provides an example of the loading block or rotor setup for an experiment with the *ipsogen* JAK2 RGQ PCR Kit.

Numbers denote positions in the loading block and indicate final rotor position.

Table 2. Number of reactions for Rotor-Gene Q MDx instruments with 72-tube rotor

Samples	Number of reactions
With the JAK2 MT Reaction Mix	
8 genomic DNA samples	8
JAK2 MT Quant Standards	4
JAK2 MT Control (mutant)	1
JAK2 WT Control (wild-type)	1
Water for no template control (NTC)	1
With the JAK2 WT Reaction Mix	
8 genomic DNA samples	8
JAK2 WT Quant Standards (wild-type)	4
JAK2 MT Control (mutant)	1
JAK2 WT Control (wild-type)	1
Water for NTC	1

1	WT QS1	9	MT C	17	S2	25	S6	33		41		49		57		65	
2	MT QS1	10	MT C	18	S2	26	S6	34		42		50		58		66	
3	WT QS2	11	WT C	19	S3	27	S7	35		43		51		59		67	
4	MT QS2	12	WT C	20	S3	28	S7	36		44		52		60		68	
5	WT QS3	13	NT C	21	S4	29	S8	37		45		53		61		69	
6	MT QS3	14	NT C	22	S4	30	S8	38		46		54		62		70	
7	WT QS4	15	S1	23	S5	31		39		47		55		63		71	
8	MT QS4	16	S1	24	S5	32		40		48		56		64		72	

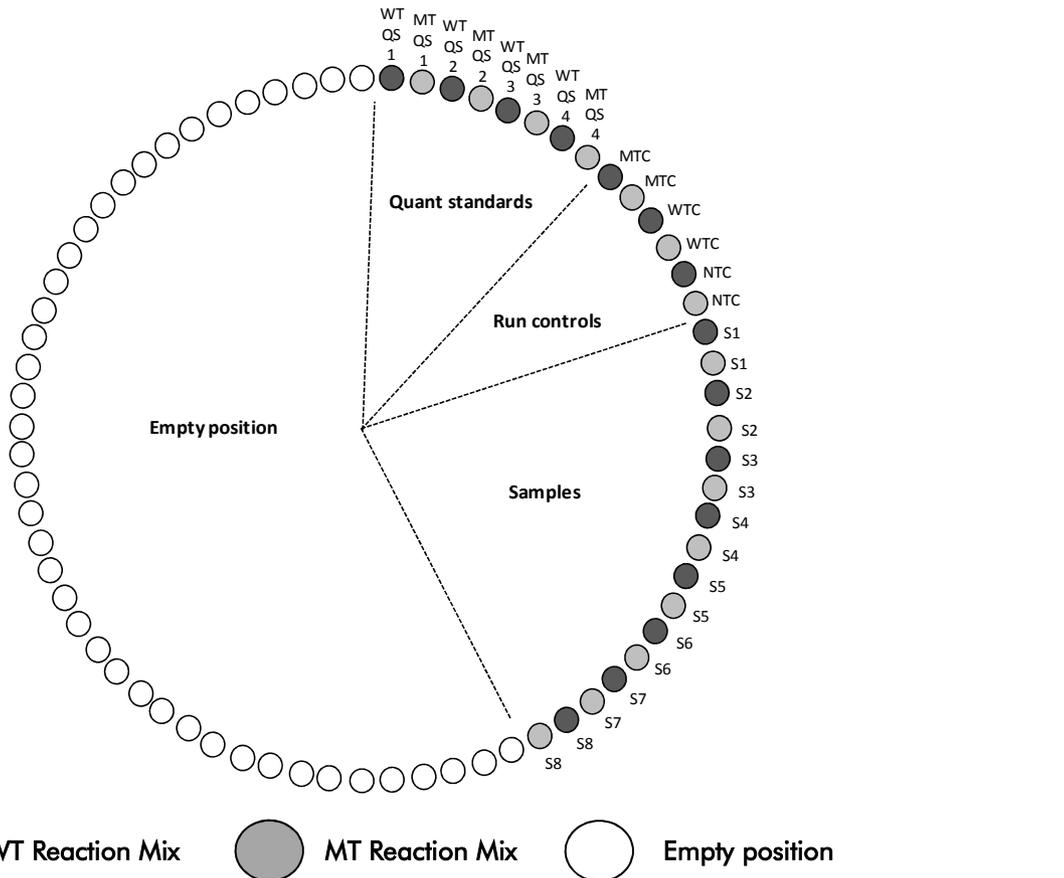


Figure 3. Plate and rotor setup for an experiment with the *ipsogen* JAK2 RGQ PCR Kit. WTC: JAK2 WT Control; MTC: JAK2 MT Control; WT-QS: JAK2 WT standards; MT-QS: JAK2 MT Quant Standards; S: genomic DNA sample; NTC: no template control (water).



Tubes must be inserted into the rotor as indicated in Figure 3 as the automated analysis set in the assay profile is based on this organization. If a different layout is used, aberrant results will be obtained.

Note: Fill all resting positions with empty tubes.

qPCR on Rotor-Gene Q MDx instruments with 72-tube rotor

1. Create a work list for the samples to be processed as follows.
 - Switch on the Rotor-Gene Q MDx instrument.
 - Open the Rotor-Gene AssayManager v2.1 software and log in as a user with the operator role in the closed mode.
 - Click the “New manual work list” button in the work list manager (“Setup” environment).
 - Select the “JAK2 CE assay profile” from the list of available assay profiles in the “Assay” step.
 - Click the “Move” button to transfer the selected assay profile to the list of “Selected assay profiles”. The assay profile should now be displayed in the “Selected assay profiles” list.
 - Enter the number of samples in the corresponding field.
 - Select the “Kit information” set and enter the following JAK2 kit information, which is printed on the lid of the box
 - Material number: 1079182
 - Valid expiry date
 - Lot numberAlternatively, the kit bar code can be entered or scanned.
 - Select the “Samples” step. A list with the sample details is shown. This list represents the expected layout of the rotor.
 - Enter the sample identification number(s) in this list as well as any optional sample information as a comment for each sample.
 - Select the “Properties” step and enter a work list name.
 - Enable the “is applicable” check box.
 - Save the work list.
 - The work list can be printed and this may help with the preparation and setup of the qPCR. To print the work list, press the “Print work list” button. The sample details are included as part of this work list.

Note: The work list can be created once the experiment is set up in the instrument or before adding the samples into the instrument as the work list file can be saved.

2. Set up the qPCR experiment.

- Thaw all necessary components except the *Taq* DNA polymerase, which must be kept in the freezer when it is not being used. Place the tubes containing the components to be thawed on ice.

Note: Do not exceed 30 minutes for the thawing step to avoid any material degradation.

- Clean the bench area dedicated for PCR mix preparation to ensure no template or nuclease contamination.
- Gently mix tubes containing standards, controls and reaction mixes by inverting 10 times, and briefly centrifuge before use.

3. Prepare the following qPCR mixes according to the number of samples to be processed.

All concentrations are for the final volume of the reaction.

Table 3 and Table 4 describe the pipetting scheme for the preparation of one MT and one WT reagent mixes, calculated to achieve final reaction volumes of 25 μ l. Extra volumes are included to compensate for pipetting error and to accommodate 8 samples as well as controls.

Table 3. Preparation of qPCR mixes for JAK2 MT sequence detection

Component	1 reaction (μ l)	15 +1* reactions (μ l)	Final concentration
JAK2 MT Reaction Mix	19.8	316.8	1x
<i>Taq</i> DNA polymerase	0.2	3.2	1x
Sample (to be added at step 4)	5	5 each	–
Total volume	25	25 each	–

* An extra reaction volume is included as dead volume.

Table 4. Preparation of qPCR mixes for JAK2 WT sequence detection

Component	1 reaction (μ l)	15 +1* reactions (μ l)	Final concentration
JAK2 WT Reaction Mix	19.8	316.8	1x
<i>Taq</i> DNA polymerase	0.2	3.2	1x
Sample (to be added at step 4)	5	5 each	–
Total volume	25	25 each	–

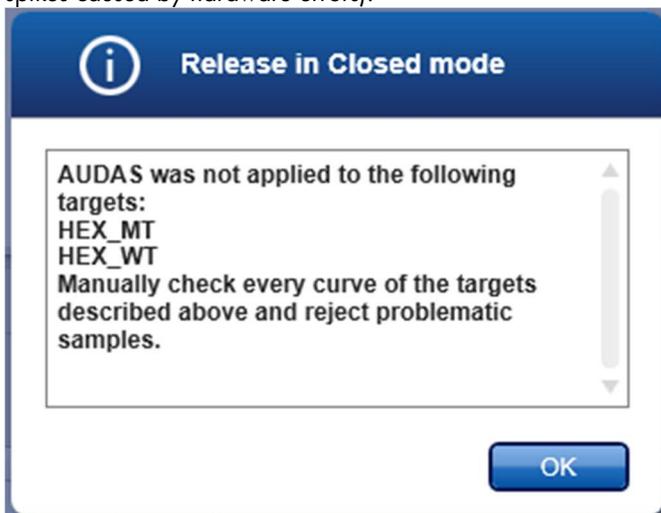
* An extra reaction volume is included as dead volume.

- Vortex and briefly centrifuge before dispensing 20 μ l of the qPCR pre-mix per strip tube.
- Vortex and briefly centrifuge DNA (genomic DNA samples plus QS and controls). Then, add 5 μ l of material to be quantified into its corresponding tube to give a total volume of 25 μ l. Mix gently by pipetting up and down.
- **Note:** Be careful to change tips between each tube to avoid any non-specific template or reaction mix contamination and, therefore, false-positive results.
- Return all the *ipsogen* JAK2 RGQ PCR Kit components to the freezer to avoid any material degradation.

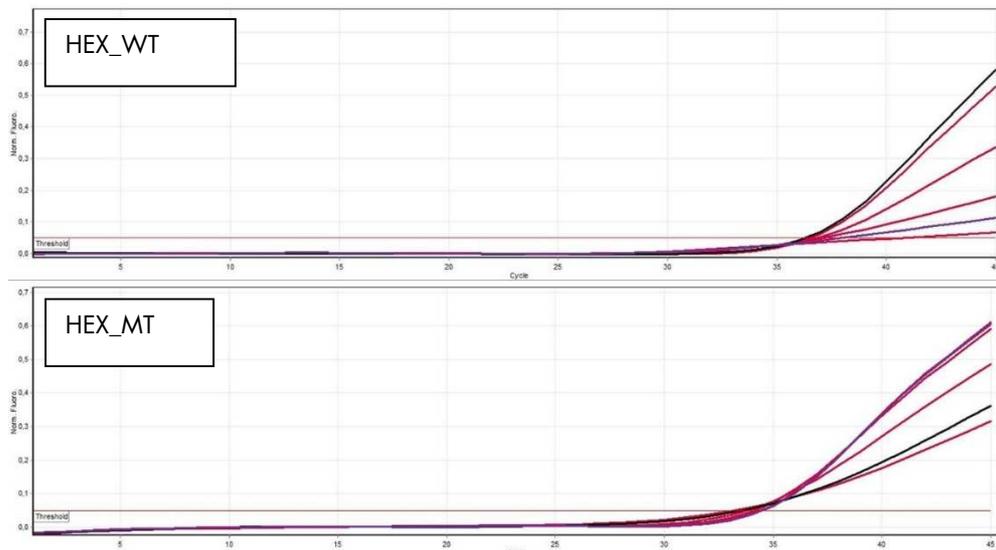
-
4. Prepare the Rotor-Gene Q MDx, and start the run as follows.
- Place a 72-well rotor on the Rotor-Gene Q MDx rotor holder.
 - Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Figure 3 (page 26), with empty capped strip tubes placed into all unused positions.
Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions as shown in Figure 3.
 - Attach the locking ring.
 - Load the Rotor-Gene Q MDx instrument with the rotor and locking ring, and close the instrument lid.
 - Within the Rotor-Gene AssayManager v2.1 software, either select the corresponding work list from the work list manager and click the “Apply” button or, if the work list is still open, click the “Apply” button.
Note: If the experiment dedicated work list has not been created, log in the Rotor-Gene AssayManager v2.1 and follow step 2 before proceeding as follows.
 - Enter the experiment name.
 - Select the cyclers to be used in the “Cycler selection”.
 - Check correct attachment of locking ring and confirm on the screen that the locking ring is attached.
 - Click the “Start run” button.
 - The JAK2 RGQ PCR run should start.
5. Perform the following to end the run.
- When the run has finished, click “Finish run”.
 - Release and approve the run:
 - For users logged in with the Approver role: Click “Release and go to approval”.
 - For users logged in with the Operator role: Click “Release”.

6. Release results.

- If “Release and go to approval” was clicked, the results for the experiment are displayed.
- The following AUDAS (Automatic Data Scan) alert appears. Check the HEX targets manually in the “Plots and Information” section of the raw data curves for anomalies (e.g., spikes caused by hardware errors).



Note that the curves of the internal control HEX targets do not exhibit typically sigmoid shapes (as in the example curves below) and must be considered as valid curves. Please note that all other internal validity criteria (e.g., C_t cutoffs) are automatically checked by the software.



- If “Release” was clicked by a user with user role, someone with an “Approver” role will need to log in and select the “Approval” environment.

- Filter for the assay to be approved by selecting the filter options and clicking the “Apply” button.
- The AUDAS (Automatic Data Scan) alert above appears. Check the HEX targets manually in the “Plots and Information” section of the raw data curves for anomalies (e.g., spikes caused by hardware errors).
- Note that the curves of the internal control HEX targets do not exhibit typically sigmoid shapes (as in the example curves above) and must be considered as valid curves. Please note that all other internal validity criteria (e.g., C_T cutoffs) are automatically checked by the software.
- Review results and click the “Release/Report data” button.
- Click “OK”. The report will be generated in .pdf format and automatically stored in the pre-defined folder.

By default, this folder path is:

QIAGEN > Rotor-Gene AssayManager > Export > Reports

Note: This path and folder can be changed in the “Configuration” environment.

Note: For troubleshooting, a support package from the run is required. Support packages can be generated from the approval or archive environment (*Rotor-Gene AssayManager v2.1 Core Application User Manual*, “Troubleshooting” section, “Creating a support package”). In addition, the audit trail from the time of incident ± 1 day might be helpful. The audit trail can be retrieved in the Service environment (*Rotor-Gene AssayManager v2.1 Core Application User Manual*, Section 1.5.5.5).

7. Unload the Rotor-Gene Q MDx instrument and discard the strip tubes according to your local safety regulations.

Interpretation of Results

The analysis is entirely automated.

Rotor-Gene AssayManager v2.1 first* analyzes amplification curves and may invalidate non-conforming curves, depending on their shape and noise amplitude. If this is the case, a flag will be associated with the invalidated curve.

The results of the test samples are automatically analyzed and set by Rotor-Gene AssayManager v2.1 but must be approved and released by the user logged in with the role of approver. Sample results to be approved have three additional approval buttons at the end of the dedicated row.

* Enabled only for FAM targets.

These buttons are used to interactively accept or reject the sample results. For further information, please refer to the *Gamma Plug-in User Manual*.

Rotor-Gene AssayManager v2.1 will then analyze the run controls:

- NTC: The NTC is checked for the absence of specific amplification (JAK2 WT and JAK2 MT) and the presence of amplification of the internal control.
- WT and MT QS: The validation is based on the R^2 and the slope values of each.
- WTC: The JAK2 total copy number (TCN) must be high enough for this control to be interpreted. If this is the case, the JAK2 mutation percentage will be calculated. This run control is validated if its status is WT according to the test.
- MTC: The JAK2 total copy number must be high enough for this control to be interpreted. If this is the case, the JAK2 mutation percentage will be calculated. This run control is validated if its status is highly positive for the JAK2 mutation.

Note: The report generated at the end of the run shows the results obtained on run controls, with invalidating flags in front of invalid data.

If all the controls in the run conform, then Rotor-Gene AssayManager v2.1 will analyze the unknown samples.

- In the sample, the total copy number must be high enough for the results to be interpreted. The JAK2 mutation percentage will then be calculated and the result will be given. If no specific amplification is observed in a tube (either WT or MT), the amplification of the internal control will be checked to ensure this is not an artifact. C_T value must be observed in each tube (WT and MT) for a sample to be validated by Rotor-Gene AssayManager v2.1 and for the corresponding result to be valid.

Note: If both the run controls and the sample results are valid, the report will show the copy number and mutation percentage in front of each sample.

- Table 5 shows the invalidating sample flags that may be assigned to an individual tube during the analysis by Rotor-Gene AssayManager v2.1, along with an explanation of what this flag means.

Table 6 (page 35) provides the warning sample flags and description of terms.

Table 5. Invalidating sample flags and description of terms

Flag	Description
ANALYSIS_FAILED	Assay is set to invalid because the analysis has failed. Contact QIAGEN Technical Services.
ASSAY_INVALID	The assay is invalid because at least one external control is invalid.
CONSECUTIVE_FAULT	Target that was used for calculation of this target is invalid
CURVE_SHAPE_ANOMALY	The raw data amplification curve shows a shape that deviates from the established behavior for this assay. There is a high likelihood of incorrect results or a result misinterpretation.
FLAT_BUMP	The raw data amplification curve shows a shape like a flat bump deviating from the established behavior for this assay. There is a high likelihood of incorrect results or result misinterpretation (e.g., wrong C_T value determination).
INVALID_CALCULATION	Calculation for this target failed.
MC_IC_HIGH_CT (WT)	The detected C_T value is higher than expected for an internal control in the same tube as the mutant control with the wild-type reaction mix.
MC_IC_LOW_CT (WT)	The detected C_T value is lower than expected for an internal control in the same tube as the mutant control with the wild-type reaction mix.
MC_IC_NO_CT (MT)	No detectable C_T for the internal control in the same tube as the mutant control with the mutant reaction mix.
MC_IC_NO_CT (WT)	No detectable C_T for the internal control in the same tube as the mutant control with the wild-type reaction mix.
MC_LOW_CN	Copy number for the mutant control is too low.
MC_LOW_PERCENTAGE	The mutation percentage of the mutant control is too low.
MC_NO_CN	No copy number for the mutant control.
MC_NO_CT (MT)	No detectable C_T for the mutant control with the mutant reaction mix.
MC_NO_VALUE	The mutation percentage of the mutant control has no value.
MULTIPLE_THRESHOLD_CROSSING	The amplification curve crosses the threshold more than once. An unambiguous C_T cannot be determined.
NO_BASELINE	No initial baseline has been found. The subsequent analysis cannot be performed.
NTC_IC_HIGH_CT (MT)	The detected C_T value is higher than expected for an internal control in the same tube as the no template control with the mutant reaction mix.
NTC_IC_HIGH_CT (WT)	The detected C_T value is higher than expected for an internal control in the same tube as the no template control with the wild-type reaction mix.
NTC_IC_NO_CT (MT)	No detectable C_T for the internal control in the same tube as the no template control with the mutant reaction mix.
NTC_IC_NO_CT (WT)	No detectable C_T for the internal control in the same tube as the no template control with the wild-type reaction mix.
NTC_IC_LOW_CT (MT)	The detected C_T value is lower than expected for an internal control in the same tube as the no template control with the mutant reaction mix.
NTC_IC_LOW_CT (WT)	The detected C_T value is lower than expected for an internal control in the same tube as the no template control with the wild-type reaction mix.
NTC_UNEXPECTED_VALUE	C_T have been detected in the no template control.
OTHER_TARGET_INVALID	Another target for the same sample is invalid.
OUT_OF_COMPUTATION_RANGE	The calculated concentration for this sample exceeds the technical limit.
QS_HIGH_SLOPE (MT)	The upper limit of the mutant slope is exceeded.

Flag	Description
QS_HIGH_SLOPE (WT)	The upper limit of the wild-type slope is exceeded.
QS_IC_NO_CT (WT)	No detectable C_T for the internal control in the same tube as one or more of the wild-type quantification standards.
QS_LOW_SLOPE (MT)	The lower limit of the mutant slope is not met.
QS_LOW_SLOPE (WT)	The lower limit of the wild-type slope is not met.
QS_LOW_RSQUARED (MT)	The lower limit of the mutant R^2 is not met.
QS_LOW_RSQUARED (WT)	The lower limit of the wild-type R^2 is not met.
QS_NO_CT (MT)	No detectable C_T for one or more of the mutant quantification standards.
QS_NO_CT (WT)	No detectable C_T for one or more of the wild-type quantification standards.
RUN_FAILED	Assay is set to invalid due to a problem with the cycler or the cycler connection.
RUN_STOPPED	Assay is set to invalid because the run has been stopped manually.
SAMPLE_LOW_CN	The copy number for a test sample is too low.
SAMPLE_MT_IC_HIGH_CT	The detected C_T value is higher than expected for an internal control in the same tube as a test sample with the mutant reaction mix.
SAMPLE_MT_IC_LOW_CT	The detected C_T value is lower than expected for an internal control in the same tube as a test sample with the mutant reaction mix.
SAMPLE_MT_IC_NO_CT	No detectable C_T for the internal control in the same the tube as a test sample with the mutant reaction mix.
SAMPLE_NO_CN	No copy number for a test sample.
SAMPLE_NO_VALUE	The mutation percentage of a test sample has no value.
SAMPLE_WT_IC_HIGH_CT	The detected C_T value is higher than expected for an internal control in the same tube as a test sample with the wild-type reaction mix.
SAMPLE_WT_IC_LOW_CT	The detected C_T value is lower than expected for an internal control in the same tube as a test sample with the wild-type reaction mix.
SAMPLE_WT_IC_NO_CT	No detectable C_T for the internal control in the same the tube as a test sample with the wild-type reaction mix.
SATURATION	The raw data fluorescence is saturating strongly before the inflection point of the amplification curve.
SPIKE_CLOSE_TO_CT	A spike is detected in the amplification curve close to the C_T .
STEEP_BASELINE	A steeply rising baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_BASELINE_DIP	A strong drop in the baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_NOISE	Strong noise is detected outside the growth phase of the amplification curve.
STRONG_NOISE_IN_GROWTH_PHASE	Strong noise is detected in the growth (exponential) phase of the amplification curve.
WAVY_BASE_FLUORESCENCE	Wavy baseline for the raw data fluorescence detected in the amplification curve.
WTC_HIGH_PERCENTAGE	The mutation percentage of the wild-type control is too high.
WTC_IC_HIGH_CT (MT)	The detected C_T value is higher than expected for an internal control in the same tube as the wild-type control with the mutant reaction mix.
WTC_IC_LOW_CT (MT)	The detected C_T value is lower than expected for an internal control in the same tube as the wild-type control with the mutant reaction mix.

Flag	Description
WTC_IC_NO_CT (MT)	No detectable C _T for the internal control in the same tube as the wild-type control with the mutant reaction mix.
WTC_IC_NO_CT (WT)	No detectable C _T for the internal control in the same tube as the wild-type control with the wild-type reaction mix.
WTC_LOW_CN	The copy number for the wild-type control is too low.
WTC_NO_CT (WT)	No detectable C _T for the wild-type control with the wild-type reaction mix.
WTC_NO_CN	No copy number for the wild-type control.
WTC_NO_VALUE	The mutation percentage of the wild-type control has no value.

Table 6. Warning sample flags and description of terms

Flag	Description
LOW_FLUORESCENCE_CHANGE	The percentage fluorescence change for this sample relative to the sample tube with the largest fluorescence change is lower than a defined limit.
LOW_REACTION_EFFICIENCY	The reaction efficiency for this sample has not reached a defined limit.
SPIKE	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the C _T is determined.

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 44).

For troubleshooting information relative to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104) and QIASymphony DNA DSP Mini Kit (cat. no. 937236), please refer to the corresponding handbooks.

	Comments and suggestions
Automated extraction	
Sample flagged as “unclear”	This can be due to a pause during the extraction run. If the extraction run was completed, proceed to the OD ratio and concentration measurement step. If not, repeat the extraction run.
Sample flagged as “unprocessed”	This refers to an initial sample volume error. Verify the blood volume by pipetting. If the volume is too low, increase so the sample is 300 µl and restart the run.
Sample flagged as “invalid”	An error occurred during the extraction run. Repeat the extraction step for this sample.
Cooling block temperature error	An error message at the end of the run about the cooling temperature means that samples were kept at room temperature (15–25°C) from the end of the extraction run. If the samples were kept at room temperature for <12 hours, genomic DNA quality should not be altered and genomic DNA can be quantified. If >12 hours, genomic DNA samples may have been degraded. If this is the case, repeat the extraction.
Elution plate removal error	At the end of the run, an error message may appear if the elution plate was removed without checking the relevant operation on screen. This can be rectified by clicking on the relevant box.
General handling for JAK2 mutation status assessment using <i>ipsogen</i> JAK2 RGQ PCR Kit	
Total copy number does not conform and corresponding sample is invalid: the amplification is too low	
a) Check the A_{260}/A_{280} ratio	If this is <1.7, perform a new DNA extraction.
b) Check the DNA concentration	The <i>ipsogen</i> JAK2 RGQ PCR Kit is optimized for a 10 ng/µl working concentration. If the DNA concentration is not at this concentration, dilute or re-extract DNA from whole blood.
c) If both parameters conform, pipetting volumes may be incorrect	Check and recalibrate the pipets before repeating the qPCR step.
Run control fails on a QS standard	
a) Vial inversion	Check the pipetting scheme and the setup of the reaction.
b) Inversion during distribution	Store kit contents at –30 to –15°C, and keep the reaction mixes protected from light.
c) Cross contamination	Avoid repeated freezing and thawing.
d) Standard partial degradation	
e) PCR reagents partially degraded	
f) Nonspecific amplification	

Comments and suggestions

No or low signal for one standard

- a) Distribution issue
Check the pipetting scheme and the setup of the reaction.
Repeat the PCR run.
- b) Use of the same reaction mix for WT and MT QS

No Template Control (NTC) of H₂O is positive

- a) Cross contamination
Replace all critical reagents.
- b) Reagent contamination
Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carryover contamination.
- c) Strip tube inversion
Keep reaction mixes protection from light.
- d) Probe degradation
Check for false positive on the fluorescence curve.

No signal, even in standard controls

- Pipetting error or omitted reagents
Check pipetting scheme and the setup of the reaction.
Repeat the PCR run.

Absent or low signals in samples, but the controls run ok

- Inhibitory effects of the sample material, caused by insufficient purification
Always check the DNA quality by measuring the A_{260}/A_{280} ratio and concentration before starting.
Repeat DNA preparation.

Wild-Type Control (WTC) is positive, but Mutant Control (MTC) is not positive enough

- Carryover 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 carryover contamination.
Make sure tips are change between pipetting different reagents.

Wild-Type Control (WTC) or Mutant Control (MTC) signal using reciprocal reaction mixes

- a) Cross contamination
Replace all critical reagents.
- b) Reagent contamination
Repeat the experiment with new aliquots of all reagents.
- c) Tube inversion
Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carryover contamination.
Check pipetting scheme and the setup of the reaction.

Inverted detection of the positive control

- a) Cross contamination
Check pipetting scheme and the setup of the reaction.
- b) Distribution inversion of the reaction mix in the tube or premix.

No signal for a sample or control, even for the internal control

- a) Reaction mix not added
Check pipetting scheme and the setup of the reaction. If the internal control is not amplified, the reaction mix was not added or is degraded.
- b) Reaction mix degraded
Repeat the qPCR step with new reaction mix.

Note: If the problem cannot be attributed to any of the above causes or if the suggested corrective action fails to resolve the problem, contact QIAGEN Technical Services for advice.

Quality Control

Quality control of the complete kit has been performed on a Rotor-Gene Q MDx 5plex HRM instrument. This kit is manufactured according to ISO 13485:2012 standard. Certificate of analysis are available on request at www.qiagen.com/support/.

Limitations

The kit is intended for professional use.

The product is to be used only by personnel specially instructed, trained for molecular biology techniques and familiar with this technology.

This kit should be used following the instructions given in this manual, in combination with a validated instrument mentioned in "Materials Required but Not Provided", page 9.

Attention should be paid to expiration dates printed on the box label. Do not use expired components.

All reagents supplied in the *ipsogen* JAK2 RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same kit. Failing to follow this guideline might affect performance.

The *ipsogen* JAK2 RGQ PCR Kit is validated only for whole blood anticoagulated in potassium EDTA collected from patients with suspected MPN.

The *ipsogen* JAK2 RGQ PCR Kit is validated only for use with the QIAasymphony DNA DSP Mini Kit (cat. no. 937236) or the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104).

The *ipsogen* JAK2 RGQ PCR Kit is validated only for use with the Rotor-Gene Q MDx 5plex HRM (for PCR) and the QIAasymphony SP (for sample preparation).

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

Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings. The absence of the JAK2 V617F/G1849T mutation does not exclude the presence of other JAK2 mutations.

It is the user's responsibility to validate system performance for any procedures used in their laboratory that are not covered by the QIAGEN performance studies.

Performance Characteristics

Limit of blank

Limit of blank (LOB) was determined following the CLSI/NCCLS EP17-2A standard on healthy whole blood samples, with a WT JAK2 status, 30 samples, 120 measurements/lot, 3 lots).

The LOB results are summarized in Table 7.

Table 7. Summary of the LOB results

	Measured LOB	Final limit of blank
Validation lot 1	0%	
Validation lot 2	0%	0%
Validation lot 3	0%	

Limit of detection

Limit of detection (LOD or analytical sensitivity) was determined based on the "Probit approach" described in the CLSI/NCCLS EP17-2A standard. In this study, 6 low-levels of mutation were analyzed for 3 independent samples (MPN whole blood DNA spiked into WT whole blood DNA), with 3 lots, 60 measurements per sample and per mutation. The results obtained indicated the analytical sensitivity was 0.042% of JAK2 V617F mutation.

The LOD results are summarized in Table 8.

Table 8. Summary of the LOD results

	Measured LOD	Final limit of blank
Validation lot 1	0.041%	
Validation lot 2	0.029%	0.042%
Validation lot 3	0.042%	

Linearity

The linearity of the quantification of the JAK2 mutation in MPN patients was assessed according to the CLSI/NCCLS EP06AE standard, with one lot of *ipsogen* JAK2 RGQ PCR Kit and with testing

on 11 levels of mutation for five different DNA inputs. The quantification of the JAK2 mutation burden in MPN samples is linear; i.e., the *ipsogen* JAK2 RGQ PCR Kit is able to quantify samples from the LOD value to 100% mutation as long as the quantified sample concentration is close to 10 ng/μl (between 5 and 20 ng/μl).

Repeatability and reproducibility

The precision study was performed according to the CLSI/NCCLS EP5-A2 standard. Testing was performed on 11 levels of mutation, each level tested in duplicate on 54 runs performed over 27 days, giving rise to 108 measurements per level of mutation. The results are summarized in Table 9.

Table 9. Precision results

Sample	Mean JAK2 mutation percentage	SD _{R+}	SD _{RUN++}	SD _{TOTAL+++}	CV _{TOTAL}
S1	72.67	1.99	2.99	5.45	7.50%
S2	53.96	2.48	3.16	6.52	12.09%
S3	23.13	1.59	1.95	4.51	19.52%
S4	11.97	1.10	1.17	2.79	23.27%
S5	6.01	0.71	0.63	1.57	26.17%
S6	2.39	0.31	0.36	0.70	29.23%
S7	1.23	0.17	0.16	0.34	27.38%
S8	0.63	0.13	0.12	0.24	37.88%
S9	0.13	0.05	0.03	0.07	52.31%
S10	0.07	0.03	0.02	0.04	65.01%
S11	0.007	0.01	0.002	0.01	146.84%

R+: Repeatability.

RUN++: Between run reproducibility.

TOTAL+++: Total precision (including inter-instrument, inter-operator and inter-lot).

CV_{TOTAL}: Coefficient of variation for the total precision (%JAK2 MT).

Interfering substances

The study design was based on recommendations described in the NCCLS standard EP7-A2 “Interference Testing in clinical Chemistry”. A total of 17 substances potentially present in blood samples were chosen for their potential effect on PCR (busulfan, citalopram hydrobromide, paroxetine hydrochloride hemihydrate, sertraline hydrochloride, fluoxetine hydrochloride, acetaminophen [paracetamol], bilirubin unconjugated, potassium EDTA, Hgb [human], triglycerides, lisinopril dehydrate, hydroxyurea, acetylsalicylic acid, salicylic acid, thiotepa, anagrelide, interferon alpha 2b). The obtained results showed no interfering effect for these substances.

Clinical validation and methods comparison

A study including 65 MPN clinical blood samples was performed in two French clinical centers to compare the *ipsogen* JAK2 RGQ PCR Kit with the *ipsogen* JAK2 MutaQuant® Kit from QIAGEN, which was used as the reference method.

A total of 65 MPN blood samples were frozen, thawed and genomic DNA was extracted. All samples passed DNA quality controls for both genomic DNA extraction methods.

Deming regression compared the measured percentages of JAK2 mutations from both methods. There was a strong correlation between the reference method and the *ipsogen* JAK2 RGQ PCR Kit for samples with JAK2 mutations with 0% to 95% mutation levels ($R^2=0.969$), as shown in Figure 4.

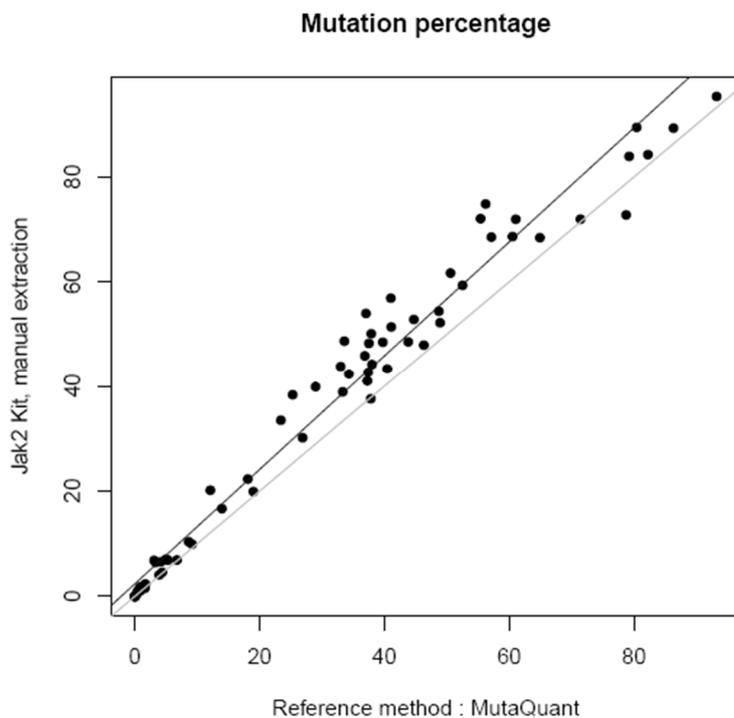


Figure 4. Plot of JAK2 V617F mutation percentages obtained with *ipsogen* JAK2 RGQ PCR Kit and a reference method on the same samples.

The JAK2 mutation percentages obtained with *ipsogen* JAK2 RGQ PCR Kit were globally higher than the percentages obtained with the reference method, highlighting the better sensitivity of this new kit (~ 1 log) (9).

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Symbols

The following symbols may appear on the packaging and labeling:



Contains reagents sufficient for <N> reactions



Use by



In vitro diagnostic medical device



Catalog number



Lot number



Material number



Global Trade Item Number



Temperature limitation



Manufacturer



Protect from light



Consult instructions for use



Warning

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> JAK2 RGQ PCR Kit (24)	For 24 reactions: Wild-type JAK2 Gene Control, JAK2 V617F Control Gene, JAK2 WT Quant Standards, JAK2 MT Quant Standards, JAK2 WT Reaction Mix, JAK2 MT Reaction Mix, Taq DNA polymerase, TE buffer for dilution, water for NTC	673623
Rotor-Gene Q MDx – for IVD-validated real-time PCR analysis in clinical applications		
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9002032
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002033
Rotor-Gene Assay Manager 2.1 Software	Software for routine testing in combination with the Rotor-Gene Q	9024203
Rotor-Gene Assay Manager 2.1 License	Single license software for installation on one computer	9025620
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
QIAamp DSP DNA Blood Mini Kit	For 50 preps: QIAamp Mini Spin Columns, Buffers, Reagents, Tubes, VacConnectors	61104
QIAasymphony DSP DNA Mini Kit	For 192 preps of 200 µl each: Includes 2 reagent cartridges and enzyme racks and accessories.	937236
QIAasymphony SP and accessories		
QIAasymphony SP System	QIAasymphony sample prep module: includes installation and training, 1-year warranty on parts and labor	9001751
QIAasymphony SP	QIAasymphony sample prep module: includes 1-year warranty on parts and labor	9001297
Sample Prep Cartridges, 8-well (336)	8-well sample prep cartridges for use with the QIAasymphony SP	997002
8-Rod Covers (144)	8-Rod Covers for use with the QIAasymphony SP	997004
Filter-Tips, 200 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube® and the QIAasymphony SP/AS instruments	990332
Filter-Tips, 1500 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIAasymphony SP/AS instruments	997024
Elution Microtubes CL (24 x 96)	Nonsterile polypropylene tubes (0.85 ml maximum capacity, less than 0.7 ml storage capacity, 0.4 ml elution capacity); 2304 in racks of 96; includes cap strips	19588
RNase A (17,500 U)	2.5 ml (100 mg/ml; 7000 units/ml, solution)	19101
Buffer ATL (200 ml)	200 ml Tissue Lysis Buffer for 1000 preparations	19076

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