

ipsogen[®] JAK2 RGQ PCR Kit

Instructions for Use



Version 2



For In Vitro Diagnostic Use

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



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QIAGEN, GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY



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Intended Use

The *ipsogen*[®] JAK2 RGQ PCR Kit is a quantitative in vitro PCR assay intended for the detection and quantification of the JAK2 V617F/G1849T mutation in genomic DNA extracted from human peripheral whole blood anticoagulated with 2K-EDTA. Results obtained with the *ipsogen* JAK2 RGQ PCR Kit are intended for use as an adjunct to evaluation of suspected Philadelphia (Ph) chromosome negative myeloproliferative neoplasm (MPN) and molecular disease monitoring in MPN patients. Any diagnostic results generated must be interpreted in conjunction with other clinical-pathological findings.

The *ipsogen* JAK2 RGQ PCR Kit is intended to be used only with the QIAGEN Rotor-Gene[®] Q MDx 5plex HRM instrument and other validated workflow components as outlined in the instructions for use. The *ipsogen* JAK2 RGQ PCR Kit is not an automated device; however, the analysis is assisted by a dedicated software.

The *ipsogen* JAK2 RGQ PCR Kit is intended for in vitro diagnostic use.

Intended User

This kit is intended for professional use.

The product is to be used only by professionals specially instructed and trained in molecular biology techniques and familiar with this technology. The device procedure is to be implemented in a molecular biology laboratory environment.

Description and Principle

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), and in approximately 60% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) (5). *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) (6).

The *JAK2 V617F* 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 *JAK2* gene encodes a tyrosine kinase involved in cytokine receptor signaling through the STAT pathway. When constitutively activated, most often by way of the *JAK2 V617F* mutation, the result is transformation of erythroid progenitors, hypersensitivity to erythropoietin, and activation of downstream signaling pathways. Also, it is hypothesized that dysregulated *JAK2* promotes oncogene expression, mitotic recombination, and genetic instability (7).

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 increase in diagnostic accuracy. Detection of the *JAK2 V617F* mutation is part of the reference World Health Organization (WHO) 2016 criteria for the diagnosis of BCR-ABL negative MPN (8) (Table 1), and presence of this mutation is a major criterion for diagnostic confirmation.

Table 1. WHO criteria for the diagnosis of MPN

Criteria for a diagnosis of PV

Major	<ol style="list-style-type: none">1. Hemoglobin (Hgb) >16.5 g/dL (men) or >16.0 g/dL (women) or Hematocrit >49% (men) or >48% (women) or Increased red cell mass >25% above mean normal predicted value.2. Bone marrow (BM) biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)3. Presence of JAK2V617F or JAK2 exon 12 mutation
Minor	Subnormal serum erythropoietin level

Diagnosis of PV requires meeting either all 3 major criteria, or the first 2 major criteria and the minor criterion†.

† Criterion number 2 (BM biopsy) may not be required in cases with sustained absolute erythrocytosis: hemoglobin levels >18.5 g/dL in men (hematocrit, 55.5%) or >16.5 g/dL in women (hematocrit, 49.5%) if major criterion 3 and the minor criterion are present. However, initial myelofibrosis (present in up to 20% of patients) can only be detected by performing a BM biopsy; this finding may predict a more rapid progression to overt myelofibrosis (post-PV MF).

Criteria for a diagnosis of ET

Major	<ol style="list-style-type: none">1. Platelet count $\geq 450 \times 10^9 /L$2. BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers3. Not meeting WHO criteria for <i>BCR-ABL1</i>⁺ CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms4. Presence of <i>JAK2</i>, <i>CALR</i>, or <i>MPL</i> mutation
Minor	Presence of a clonal marker or absence of evidence for reactive thrombocytosis

Diagnosis of ET requires meeting all 4 major criteria, or the first 3 major criteria and the minor criterion.

Criteria for a diagnosis of prePMF

Major	<ol style="list-style-type: none">1. Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade 1, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis2. Not meeting the WHO criteria for <i>BCR-ABL1</i>⁺ CML, PV, ET, myelodysplastic syndromes, or other myeloid neoplasms3. Presence of <i>JAK2</i>, <i>CALR</i>, <i>MPL</i> mutation or in the absence of these mutations, presence of another clonal marker,[†] or absence of minor reactive BM reticulin fibrosis[‡]
Minor	Presence of at least 1 of the following, confirmed in 2 consecutive determinations: <ol style="list-style-type: none">a.) Anemia not attributed to a comorbid conditionb.) Leukocytosis $\geq 11 \times 10^9/L$c.) Palpable splenomegalyd.) Lactate dehydrogenase (LDH) increased to above upper normal limit of institutional reference range

Diagnosis of prePMF requires meeting all 3 major criteria, and at least 1 minor criterion.

† In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, *ASXL1*, *EZH2*, *TET2*, *IDH1/IDH2*, *SRSF2*, *SF3B1*) are of help in determining the clonal nature of the disease.

‡ Minor (grade 1) reticulin fibrosis secondary to infection, autoimmune disorder or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

Criteria for a diagnosis of overt PMF

- Major
1. Presence of megakaryocyte proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3
 2. Not meeting WHO criteria for ET, PV, *BCR-ABL1*⁺ CML, myelodysplastic syndromes, or other myeloid neoplasms
 3. Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker,[†] or absence of reactive myelofibrosis[‡]

- Minor
- Presence of at least 1 of the following, confirmed in 2 consecutive determinations:
- a.) Anemia not attributed to a comorbid condition
 - b.) Leukocytosis $\geq 11 \times 10^9/L$
 - c.) Palpable splenomegaly
 - d.) Lactate dehydrogenase (LDH) increased to above upper normal limit of institutional reference range
 - e.) Leukoerythroblastosis

Diagnosis of overt PMF requires meeting of all 3 major criteria, and at least 1 minor criterion

[†] In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, *ASXL1*, *EZH2*, *TET2*, *IDH1/IDH2*, *SRSF2*, *SF3B1*) are of help in determining the clonal nature of the disease.

[‡] BM fibrosis secondary to infection, autoimmune disorder, or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

Note: CML: Chronic myeloid leukemia; ET : Essential thrombocythemia; PMF: Primary myelofibrosis; PV: Polycythemia vera; WHO: World Health Organization

Moreover, the discovery of *JAK2 V617F* mutation in MPN patients has unveiled a new target for therapies. Molecular disease monitoring measuring the *JAK2 V617F* mutation burden has been shown to be useful to assess response to treatment and to predict relapse on patients submitted to allogeneic stem-cell transplantation (9). The concepts of molecular response are clearly determined by the most recent European LeukemiaNet (ELN) and International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) recommendations (10, 11) and are referenced in the National Comprehensive Cancer Network (NCCN) (12)

and European Society of Medical Oncology (ESMO) guidelines (5). Complete Molecular Response was defined as eradication of a pre-existing molecular abnormality and Partial Molecular Response as a $\geq 50\%$ decrease in *JAK2 V617F* mutant allele burden (partial response applies only to patients with at least 20% *JAK2 V617F* mutant allele burden at baseline) (10,11).

Since 2006, several methods essentially based on PCR techniques or sequencing are available, as laboratory-developed tests to detect the presence and potentially quantify *JAK2 V617F*. 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 and molecular disease monitoring performances.

Given the wide range of potential *JAK2 V617F* mutant allele fractions that may be encountered in MPNs (with levels as low as 1%), laboratories are encouraged to offer *JAK2 V617F* mutation testing at high analytical sensitivity. Suitable techniques should possess a low limit of detection (at least 1% for diagnosis and at least 0.1% for molecular disease monitoring) and a high reproducibility (5,13).

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 for diagnostic and the recommended detection limit of *JAK2 V617F* allele burden of $\leq 0.1\%$ for molecular disease monitoring purposes (5,13). However, it should be noted that some clinical experts consider the presence of any *JAK2 V617F* load as clinically significant at time

of diagnosis and hence the need for a sensitive method, such as qPCR (14). 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.

The QIAGEN 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). 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.

In qPCR, the number of PCR cycles necessary to detect a signal above the threshold is called the Crossing point (Cp) or Cycle threshold (CT) and is directly proportional to the amount of target present at the beginning of the reaction.

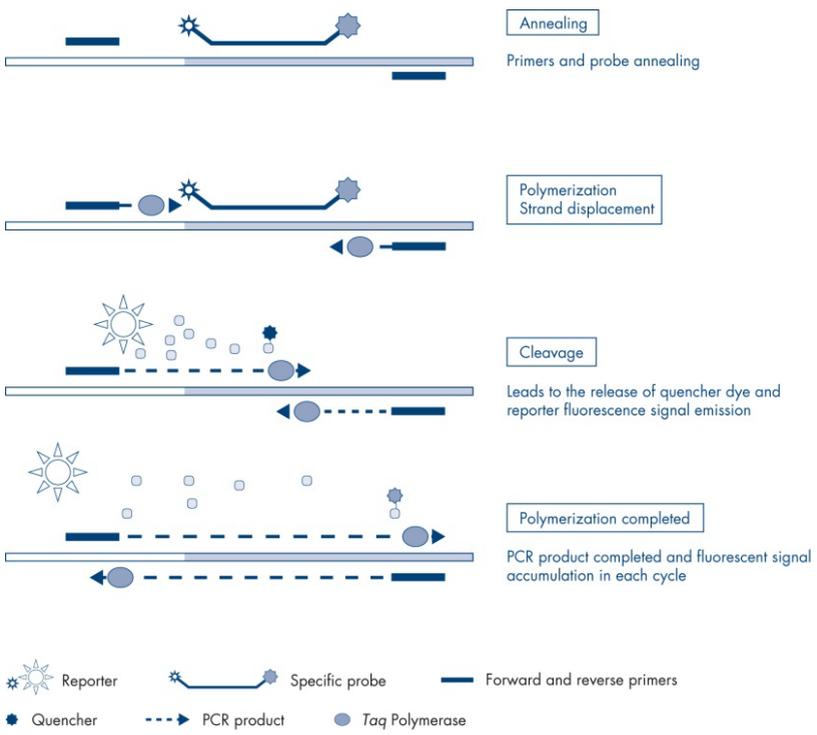


Figure 1. Reaction principle. The quantitative allele-specific PCR technology used in this assay kit allows sensitive, accurate, and highly reproducible detection of SNPs. This technique is based on the use of specific reverse primers, for the wild-type and V617F allele, respectively (15). Only a perfect match between primer and target DNA allows extension and amplification in the PCR reaction (Figure 2).

WT Reaction Mix



MT Reaction Mix

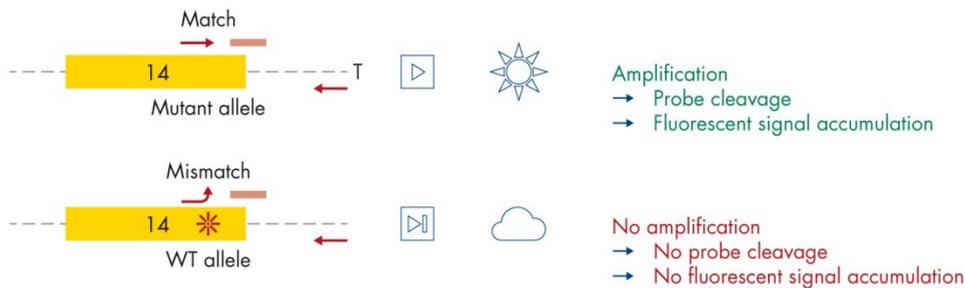


Figure 2. Allele-specific PCR. Use of wild-type or the V617F primers and probe mix allows the specific detection of the wild-type or mutated allele in two separate reactions conducted using the same sample. Results can be expressed as percentage of mutant copies among total JAK2 copies. MT: mutant; WT: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

Materials Provided

Kit contents

ipsogen JAK2 RGQ PCR Kit
Catalog no.

24
674623

Color	Identity	Tube ID	Volume
Red	JAK2 Mutant Control (100% V617F allele)	MT Ctrl	33 μ l
Green	JAK2 WT Control (100% wild-type allele)	WT Ctrl	33 μ l
Red	JAK2 MT Quant Standard 1 (5 x 10 ¹ V617F copies/5 μ l)	MT QS1	20 μ l
Red	JAK2 MT Quant Standard 2 (5 x 10 ² V617F copies/5 μ l)	MT QS2	20 μ l
Red	JAK2 MT Quant Standard 3 (5 x 10 ³ V617F copies/5 μ l)	MT QS3	20 μ l
Red	JAK2 MT Quant Standard 4 (5 x 10 ⁴ V617F copies/5 μ l)	MT QS4	20 μ l
Green	JAK2 WT Quant Standard 1 (5 x 10 ¹ wild-type copies/5 μ l)	WT QS1	20 μ l
Green	JAK2 WT Quant Standard 2 (5 x 10 ² wild-type copies/5 μ l)	WT QS2	20 μ l
Green	JAK2 WT Quant Standard 3 (5 x 10 ³ wild-type copies/5 μ l)	WT QS3	20 μ l

Kit contents (cont'd)

ipsogen JAK2 RGQ PCR Kit
Catalog no.

24
674623

Color	Identity	Tube ID	Volume
Green	JAK2 WT Quant Standard 4 (5 x 10 ⁴ wild-type copies/5 µl)	WT QS4	20 µl
Red	JAK2 MT Reaction Mix	MT Mix	1010 µl
Green	JAK2 WT Reaction Mix	WT Mix	1010 µl
Mint	Taq DNA polymerase (HotStarTaq® 5 units/µl)	Taq	85 µl
White	TE buffer for sample dilution	TE	1.9 ml
White	Water for no template control (NTC)	NTC	1.9 ml
ipsogen JAK2 RGQ PCR Kit Handbook (English)			1

Components of the kit

The principal components of the kit are explained below.

Table 2. Reagents supplied

Reagent	Active Ingredients	Volume
JAK2 Mutant Control	Cell line DNA carrying 100% V617F allele	33 µl
JAK2 WT Control	Cell line DNA carrying 100% wild-type allele	33 µl
JAK2 MT Quant Standards (QS1 to QS4)	Plasmid carrying the V617F allele sequence	20 µl each
JAK2 WT Quant Standards (QS1 to QS4)	Plasmid carrying the WT allele sequence	20 µl each
JAK2 MT Reaction Mix	Oligonucleotides for the detection of the MT allele and the internal control, PCR Buffer, MgCl ₂ , dNTPs	1010 µl
JAK2 WT Reaction Mix	Oligonucleotides for the detection of the WT allele and the internal control, PCR Buffer, MgCl ₂ , dNTPs	1010 µl
Taq DNA polymerase	Hot-start Taq DNA Polymerase in storage buffer	85 µl
TE buffer for sample dilution	Tris-EDTA buffer solution	1.9 ml
Water for no template control (NTC)	Nuclease-free water	1.9 ml

Reagents

The reagents supplied within this kit, listed in Table 2 above, are those needed to dilute test samples to the required input, and to perform the qPCR reactions for the detection and quantification of the *JAK2* mutant and wild-type alleles, in order to determine the mutation percentage. The internal amplification control included in the reaction mixes is used to monitor qPCR inhibition, and to rule out failure of the PCR reaction in case of negative results.

Controls and standards

Two controls are included in the kit: a JAK2 Mutant Control used as a positive control for the JAK2 Mutant (MT) reaction mix, and a JAK2 wild-type (WT) Control used as a positive control for the JAK2 wild-type (WT) reaction mix. Nuclease-free water is provided to perform a no template control for both reaction mixes.

Four JAK2 Mutant (MT) and four JAK2 wild-type (WT) Quantitation Standards (QS) are included in the kit. They are used to calculate the JAK2 MT and WT copy numbers and subsequently the *JAK2 V617F* mutation percentage for the test samples.

Materials Required but Not Provided

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

- Adjustable pipettes* 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 0.5/1.5/2.0 ml reaction tubes (capable of attaining 13,000–14,000 rpm)
- Spectrophotometer*

Equipment for sample preparation

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

Equipment for real-time PCR

- Real-time PCR instrument*: Rotor-Gene Q MDx 5plex HRM Platform (cat. no. 9002032) or Rotor-Gene Q MDx 5plex HRM System (cat. no. 9002033) and provided accessories
- Installed Rotor-Gene AssayManager® software version 2.1.x (x≥0)
- Installed Rotor-Gene AssayManager Gamma Plug-in version 1.0.x (x≥0)
- Imported ipsogen_JAK2_blood_CE_IVDR Assay Profile (AP_ipsogen_JAK2_blood_CE_IVDR_V2_0_x.iap (x≥1))

* Prior to use, ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Warnings and Precautions

Please be aware that you may be required to consult your local regulations for reporting serious incidents that have occurred in relation to the device to the manufacturer and/or its authorized representative and the regulatory authority in which the user and/or the patient is established.

Safety information

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

- Specimens and samples are potentially infectious. Discard sample and assay waste according to your local safety procedures.

<p>CAUTION</p> 	<p>DO NOT add bleach or acidic solutions directly to the sample or preparation waste.</p>
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Emergency information

CHEMTREC

Outside USA & Canada +1 703-527-3887

Precautions

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

This kit is intended for in vitro diagnostic use. Reagents and instructions supplied in this kit have been validated for optimal performance.

- The test is for use with whole blood samples anti-coagulated with potassium EDTA (K₂-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 optimally diluted. 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 Rotor-Gene Q MDx 5plex HRM instrument User Manual, Rotor-Gene AssayManager v2.1 Core Application User Manual, Gamma Plug-In User Manual, and QIAasymphony SP instrument 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 Mutant 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 to maintain 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 QIASymphony DNA DSP Mini Kit (cat. no. 937236), please refer to the corresponding handbooks.

Reagent Storage and Handling

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

Shipping conditions

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

For storage information relative to the 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.

In-use 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 to -15°C for up to 12 months. 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 indicated on the tube and box label.
- **Note:** Tubes from different batches should not be mixed. All *ipsogen* JAK2 RGQ PCR Kit components used for a test must be from the same batch. 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 Storage and Handling

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 (K₂-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 –30 to –15°C for no more than 1 month

Note: Temperature changes between storage at the collection site and shipment must be avoided. Storage conditions at the testing site should be the same as shipment or lower.

All samples should be treated as potentially infectious. Discard sample and assay waste according to your local safety procedures.

Genomic DNA samples

Once genomic DNA is extracted, DNA samples can be stored and shipped at –30 to –15°C for no more than 24 months. Freeze–thaw cycling should be avoided. Do not exceed a maximum of four freeze–thaw cycles.

Protocol: Genomic DNA Extraction and Preparation from Whole Blood

Important points before starting

- 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*.

Handling reagents

- When preparing the Wash Buffers for this protocol, always mix the reconstituted Wash buffer by inverting the bottle several times before starting the procedure.
- Use pipet tips with aerosol barriers when pipetting Elution Buffer from the bottle and replace the cap immediately after to avoid contamination.
- When handling viscous liquids, use extra caution and a suitable pipet to ensure correct volumes are pipetted.
- Avoid touching the QIAamp Mini spin column membrane with the pipet tip.

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

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.
- Prepare 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).
- Prepare 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.
- Prepare 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.
- Prepare 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 of Procedure.

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

Note: To ensure 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, ensure that you add the correct volume of Lysis Buffer (AL) by pipetting carefully and by using a suitable pipet.



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

4. Incubate at 56°C ($\pm 1^\circ\text{C}$) for 10 minutes (± 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 µl ethanol (96–100%) to the lysis tube (LT), close the lid, and mix thoroughly using a pulse-vortex for ≥ 15 seconds.
7. Centrifuge the lysis tube (LT) for ≥ 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 x 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 x g (8000 rpm), centrifuge again at full speed (up to 20,800 x 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 µl 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 x 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 µl 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 x 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 QIASymphony DSP DNA Mini Kit

Automated genomic DNA extraction must be performed with the QIASymphony instrument using the Sample Preparation (SP) module in combination with the QIASymphony DSP DNA Mini Kit (cat. no. 937236) and by following the instruction in the *QIASymphony DSP DNA Kit Handbook*. Protocol features specific to a use with the *ipsogen* JAK2 RGQ PCR Kit are highlighted with the sign  in the procedure below.

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

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

Important points before starting

-  Whole blood volume to be extracted is 300 µl.

Setting up

- Ensure that you are familiar with operating the QIASymphony SP. Refer to the user manuals supplied with your instrument for operating instructions.

Handling reagents

- 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.

Maintenance

- Optional maintenance of the QIASymphony SP is not mandatory, but is highly recommended to reduce risk of contamination.

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.
- Ensure 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, ensure that 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 QIASymphony SP.

Procedure

1. Close all drawers and the hood.
2. Switch on the QIAAsymphony SP, and wait until the “Sample Preparation” screen appears and the initialization procedure is finished.

Note: The power switch is located at the bottom left corner of the QIAAsymphony SP.

3. Log on to the instrument.
4. Ensure that 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.

Important: Do not load a 96-well plate onto “Elution slot 4”.

Only use “Elution slot 1” with the corresponding cooling adapter.

Note: When using a 96-well plate, ensure 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 the 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 nuclease-free 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 the default tube format. To do this, click **Select All**. Then, select **Sarstedt reference 72.694** from the **Tube Insert** sheet.

- **Protocol to be run:** Click **Select All**. Then, in the category, click **DNA Blood > Blood_200_V7_DSP** (or later version) for whole blood sample.



- **Elution volume and output position:** 100 µl for the whole blood protocol.
Note: After entering the batch information, the status changes from **LOADED** to **QUEUED**. As soon as one batch is queued, the **Run** button becomes enabled.

10. Start the run.

10a. To start the run, click **Run**.

10b. 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 QIASymphony SP carrier status changes to **RUNNING**.

Important: 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).

11. Click **Run** to start the purification procedure.

12. 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 QIASymphony 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:

- 12a. 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.

- 12b. 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.
13. Export the QIASymphony SP result file: this report is generated for each elution plate.
- 13a. Insert the USB stick into one of the USB ports at the front of the QIASymphony SP.
- 13b. Click **Tools**.
- 13c. Select **File Transfer**.
- 13d. In the In-/Output Files tab, click **Results Files > Transfer**.
- The name of the exported file should have the following format:
- yyyy-mm-ddhh:mm:ss_Elution rack ID**
14. Check the "Validity of result" column for each sample on the QIASymphony SP result file.
- **Valid and unclear status:** Proceed to Qualification and quantification of DNA.
 - **Invalid status:** Sample is rejected. Reprocess the extraction step.
15. 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.
16. Discard used sample tubes, plates and waste according to your local safety regulations.
17. 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.
18. 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/or 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.

Each PCR reaction on the Rotor-Gene Q MDx 5plex HRM is optimized for 50 ng of purified genomic DNA diluted in a final sample volume of 5 μl . A total of 100 ng per tested sample is needed, to perform the mutant and wild-type reactions.

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.
- The *ipsogen* JAK2 RGQ PCR Kit specifically requires the Gamma Plug-in. This plug-in can be downloaded from the QIAGEN website: resources.qiagen.com/674623. 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 dedicated to the *ipsogen* JAK2 RGQ PCR Kit on the QIAGEN website: resources.qiagen.com/674623. The assay profile must be imported into the Rotor-Gene AssayManager v2.1 software.
- 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.

Setting up

- Download and install the Rotor-Gene AssayManager v2.1. See "Installation of the Rotor-Gene AssayManager v2.1 core software", page 38 for the detailed information.
- Download and install the Gamma plug-in. See "Installation of Gamma Plug-in and importing assay profile", page 39 for the detailed information.

- We recommend testing eight genomic DNA samples in the same experiment to optimize use of the controls, standards and reaction mixes. See “Sample processing on Rotor-Gene Q MDx 5plex HRM instrument with 72-tube rotor”, page 42 for the detailed information.

Installation of the Rotor-Gene AssayManager v2.1 core software

The Rotor-Gene AssayManager v2.1 software must be installed on the computer connected to the Rotor-Gene Q MDx 5plex HRM instrument and can be downloaded from the QIAGEN website: resources.qiagen.com/674623. For details about the installation of the Rotor-Gene AssayManager v2.1 core software, including computer requirements, please refer to *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

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 defined for the closed mode:

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

Installation of Gamma Plug-in and importing 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*.

To install the Gamma Plug-in

1. Download both the Gamma Plug-in and the latest version of the *ipsogen* JAK2 CE IVDR assay profile from the QIAGEN website.
2. Double-click the **RGAM_V2_1_Gamma_Plug-in.Installation.V1_0_x** .msi file (where $x \geq 0$). Follow 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, click 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.

3. After the successful installation of the plug-in, a user with Administrator rights for the Rotor-Gene AssayManager v2.1 software will need to import the *ipsogen_JAK2_blood_CE_IVDR* assay profile.

To import the *ipsogen_JAK2_blood_CE_IVDR* assay profile

1. Click the icon  for Rotor-Gene AssayManager v2.1 to open the software.
2. Log in as user with Administrator rights in Closed mode (Figure 3).

The log-in window opens (Figure 4).



Rotor-Gene AssayManager

2.1.0.7

User ID

Password

Mode
Closed

OK Cancel

Figure 3. Rotor-Gene AssayManager login window. 1: Closed mode.

Available work lists | Manage or apply work lists

Manually created work lists

Work list name	# samp...	Assay profiles	Rotor type	Volume	Author	Creation date	Actions	Apply
20200914_JAK2_testing	3	JAK2_b	72-Well Rotor	25 µl	admin	20/10/2020 17:09:42	[Icons]	[Apply]

Automatically generated work lists

Enter assay rack ID: Import type: QIAasymplicity [Import] [New manual work list]

2: Setup icon, 3: Approval icon, 4: Archive icon, 5: Service icon, 6: Configuration icon, 7: Rotor-Gene Q icon

Figure 4. Rotor-Gene AssayManager v.2.1.1. 2: Setup environment. Used for creation, management and application of work lists. 3: Approval environment. Used to search for unreleased or partially released experiments and for the approval of dedicated samples. Experiment reports are created on release of a sample. 4: Archive environment. Used to search for fully and partially released experiments and to generate experiment reports using predefined report profiles. 5: Service environment. Contains the Audit Trail and Re-usable Data tabs. 6: Configuration. Used to adjust the settings of Rotor-Gene AssayManager. 7: Rotor-Gene Q icon. Used to stop or finish a run and to release a cyclor after a run is finished (and to check instrument connection).

3. Click the Configuration environment (Figure 4, box 6) (Figure 5, box 8).
4. Click the Assay Profiles tab (Figure 5, box 9).
5. Click **Import** (Figure 5, box 10).
6. In the Select assay profile to import dialog box, select the ipsogen_JAK2_blood_CE_IVDR assay profile. Click **Open** (Figure 5, box 11).

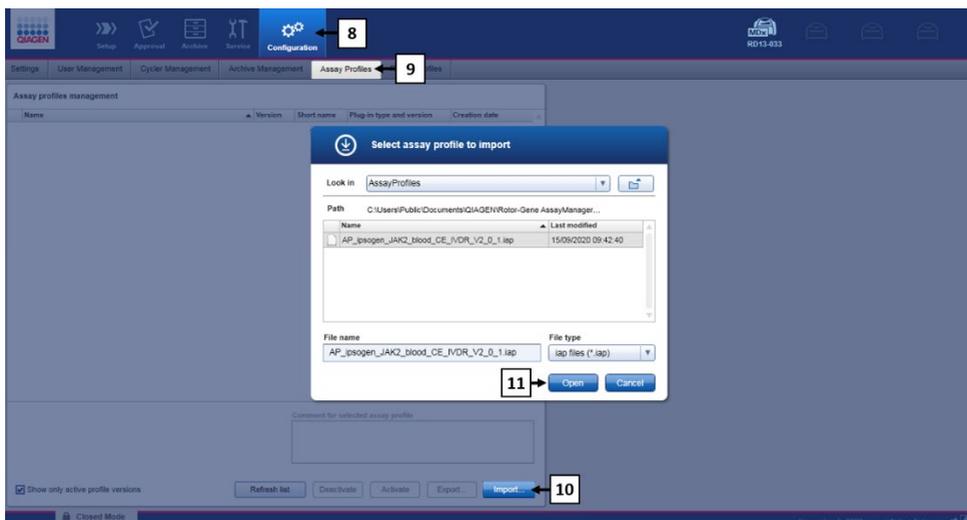


Figure 5. Assay profile import. 8: Configuration environment, 9: Assay profile tab, 10 : Import button, 11 : Open button.

7. After the assay profile is successfully imported, it can be used in the Setup environment (Figure 4, box 2).

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

Sample processing on Rotor-Gene Q MDx 5plex HRM instrument 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 3 provides the number of reactions that can be run using the 72-tube rotor.

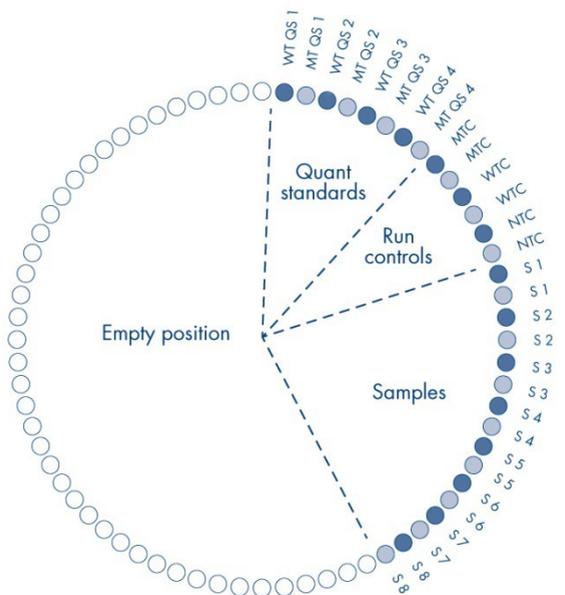
The scheme shown in Figure 6 provides an example of the loading block and rotor setup for an experiment with the *ipsogen* JAK2 RGQ PCR Kit.

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

Table 3. Number of reactions for Rotor-Gene Q MDx 5plex HRM instrument with 72-tube rotor

Samples	Number of reactions
With the JAK2 MT Reaction Mix	
8 genomic DNA samples	8
JAK2 MT Quant Standards (mutant)	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 QS 1	9	MTC	17	S 2	25	S 6	33		41		49		57		65	
2	MT QS 1	10	MTC	18	S 2	26	S 6	34		42		50		58		66	
3	WT QS 2	11	WTC	19	S 3	27	S 7	35		43		51		59		67	
4	MT QS 2	12	WTC	20	S 3	28	S 7	36		44		52		60		68	
5	WT QS 3	13	NTC	21	S 4	29	S 8	37		45		53		61		69	
6	MT QS 3	14	NTC	22	S 4	30	S 8	38		46		54		62		70	
7	WT QS 4	15	S 1	23	S 5	31		39		47		55		63		71	
8	MT QS 4	16	S 1	24	S 5	32		40		48		56		64		72	



● WT reaction mix ● MT reaction mix ○ Empty position

Figure 6. Plate and rotor setup for an experiment with the ipsogen JAK2 RGQ PCR Kit. WTC: JAK2 WT Control; MTC: JAK2 mutant (MT) Control; WT-QS: JAK2 WT Quant 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 6 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: All unused positions should be filled with empty capped strip tubes.

qPCR on Rotor-Gene Q MDx 5plex HRM instrument with 72-tube rotor

Things to do before starting:

- Create a work list for the samples to be processed.

To create a work list

1. Switch on Rotor-Gene Q MDx 5plex HRM instrument.
2. Open the Rotor-Gene AssayManager v2.1 software and log in as a user with the operator role in Closed mode (Figure 3, box 1).
3. Check that the Rotor-Gene Q MDx 5plex HRM instrument is correctly detected by the software before launching the run (Figure 7).



Figure 7. Rotor-Gene Q connection status.

4. Click **“New manual work list”** in the Setup environment (Figure 8, box 1).

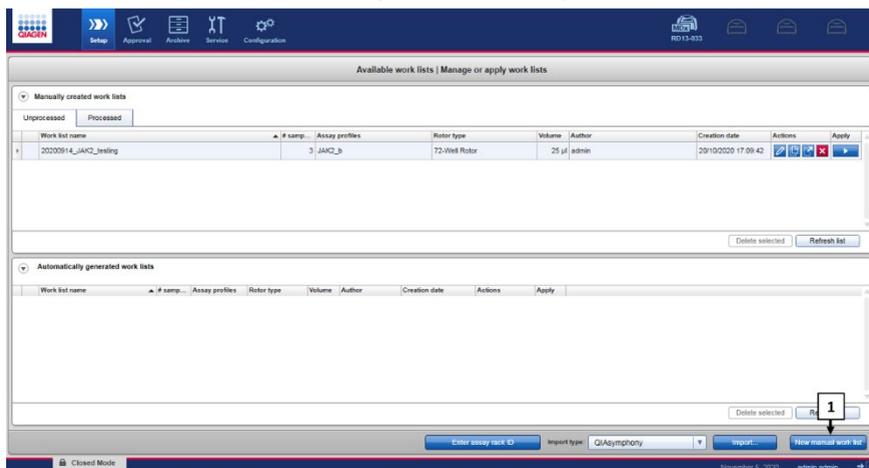


Figure 8. Work list creation. 1: New work list creation button.

5. Select the “ipsogen_JAK2_blood_CE_IVDR” assay profile from the list of available assay profiles in the “Assay” step (Figure 9, box 2).

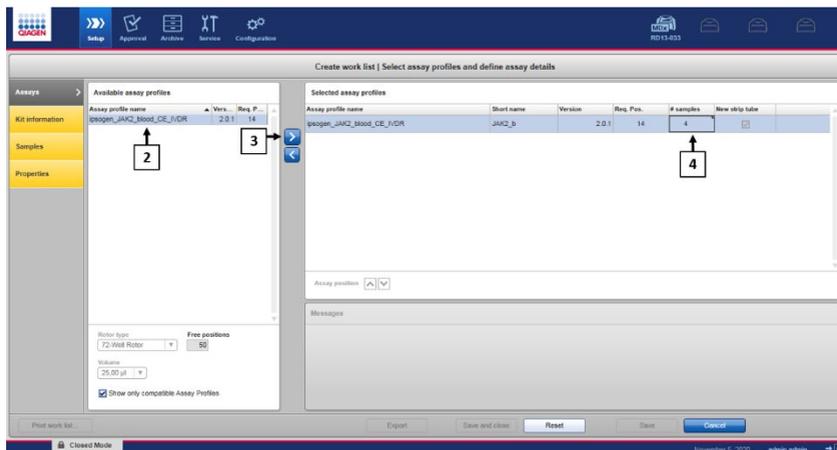


Figure 9. Work list creation – Assay Profile selection. 2: Available assay profiles. 3: Assay profile transfer to worklist. 4: Enter number of samples.

6. Click “>” to transfer the selected assay profile to the “Selected assay profiles” list (Figure 9, box 3). The assay profile should now be displayed in the “Selected assay profiles” list.
7. Enter the number of samples in the corresponding field (Figure 9, box 4).
8. Click the “Kit Information” step and manually enter the following JAK2 kit information, which is printed on the lid of the box:
 - Material number 1120216 (Figure 10, box 6)
 - Valid expiry date (Figure 10, box 7)
 - Lot number (Figure 10, box 8)

Note: Alternatively, the kit bar code (Figure 10, box 5) can be entered or scanned.

Note: All fields must be filled in. Fields become blue when valid information is entered (i.e., kit not expired, valid material and lot numbers entered).

Figure 10. Work list creation - Entering Kit information. 5: Kit bar code (can be scanned or entered manually; if the bar code is entered, the other fields are filled automatically). 6: Material number. 7: Kit expiry date. 8: Lot number. This information is available on the kit box.

9. Click the “Samples” step.

A list with the sample details is shown. This list represents the expected layout of the rotor.

10. Enter the sample identification numbers (ID) (Figure 11, box 9) in this list as well as any optional sample information (Figure 11, box 10) as a comment for each sample.

Pos.	Style	Sample ID	Status	Sample type	Targets	Assay	Sample comment
2					FAM_Mt_HEX_Mt	JAK2_b	
3		Q02		QS	FAM_VT_HEX_VWT	JAK2_b	
4		Q03		QS	FAM_Mt_HEX_Mt	JAK2_b	
5		Q03		QS	FAM_VT_HEX_VWT	JAK2_b	
6		Q04		QS	FAM_Mt_HEX_Mt	JAK2_b	
7		Q04		QS	FAM_VT_HEX_VWT	JAK2_b	
8		Q04		QS	FAM_Mt_HEX_Mt	JAK2_b	
9		Q04		QS	FAM_VT_HEX_VWT	JAK2_b	
9		MutantControl		PC	FAM_VT_HEX_VWT	JAK2_b	
10					FAM_Mt_HEX_Mt	JAK2_b	
11		WildTypeControl		PC	FAM_VT_HEX_VWT	JAK2_b	
12					FAM_Mt_HEX_Mt	JAK2_b	
13		NTC		NTC	FAM_VT_HEX_VWT	JAK2_b	
14					FAM_Mt_HEX_Mt	JAK2_b	
15		Sample 1		Test	FAM_VT_HEX_VWT	JAK2_b	
16					FAM_Mt_HEX_Mt	JAK2_b	
17		Sample 2		Test	FAM_VT_HEX_VWT	JAK2_b	
18					FAM_Mt_HEX_Mt	JAK2_b	
19		Sample 3		Test	FAM_VT_HEX_VWT	JAK2_b	
20					FAM_Mt_HEX_Mt	JAK2_b	
21					FAM_VT_HEX_VWT	JAK2_b	
22				Test	FAM_Mt_HEX_Mt	JAK2_b	

Figure 11. Work list creation Entering sample information. 9: Sample ID. 10: Sample comments (optional)

11. Click the “Properties” step. Enter a work list name (Figure 12, box 11).
12. Check the “work list is complete (can be applied)” box (Figure 12, box 12).

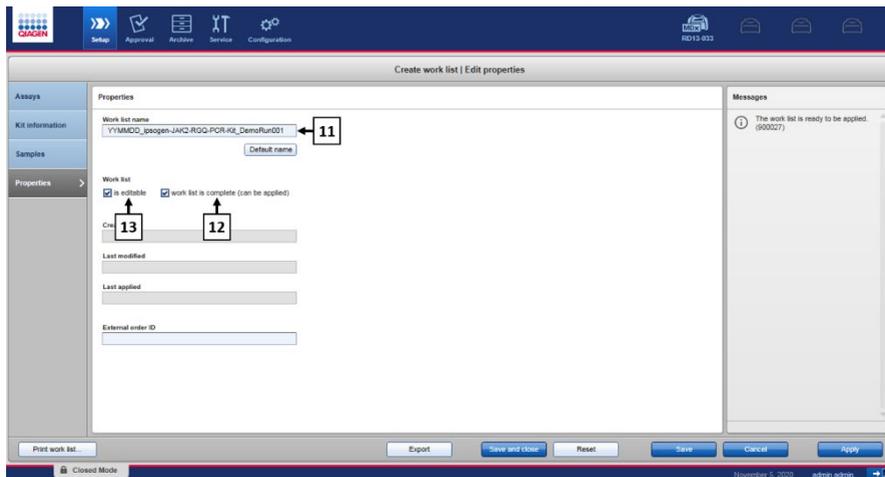


Figure 12. Work list creation – Properties. 11: Work list name. 12: Check “work list is complete” option. 13: Clear the “is editable” box only if the work list shall not be changed.

Note: The “**is editable**” checkbox (Figure 12, box 13) defines whether the work list is still editable or not. If the work list is applicable and shall not be changed afterwards, clear the **is editable** check box.

13. Save the work list.
14. The work list can be printed and this may help with the preparation and setup of the qPCR. To print the work list, click “**Print work list**”. The sample details are included as part of this work list.

Note: The work list can be saved and run later, or can be created upon loading the experiment in the instrument and applied directly.

Procedure

Setting up the qPCR experiment

1. 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.

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

2. Clean the bench area dedicated for PCR mix preparation to ensure no template or nuclease contamination.
3. Gently mix tubes containing standards, controls, and reaction mixes by inverting 10 times, and briefly centrifuge before use.
4. Prepare the following qPCR master mixes according to the number of samples to be processed.

Note: All concentrations are for the final volume of the reaction.

Table 4 and Table 5 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 4. Preparation of qPCR master 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 6)	5	5 each	–
Total volume	25	25 each	–

* An extra reaction volume is included as dead volume.

Table 5. Preparation of qPCR master mixes for JAK2 WT sequence detection

Component	1 reaction (µl)	15 + 1* reactions (µl)	Final concentration
JAK2 WT Reaction Mix	19.8	316.8	1x
Taq DNA polymerase	0.2	3.2	1x
Sample (to be added at step 6)	5	5 each	–
Total volume	25	25 each	–

* An extra reaction volume is included as dead volume.

Important: Vortex and briefly centrifuge qPCR master mix before dispensing 20 µl into each strip tube.

5. Add the water no template control (NTC) into the corresponding tubes and close them.
6. **Important: Vortex and briefly centrifuge** DNA (genomic DNA samples plus QS and controls). Then, add 5 µl of material to be quantified into its corresponding strip tube to reach a total volume of 25 µl. 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. Start by adding the test samples, then the standards and controls.

7. Return all the *ipsogen* JAK2 RGQ PCR Kit components to the freezer to avoid any material degradation.

Starting the run

1. Prepare the Rotor-Gene Q MDx, and start the run as follows.
 - 1a. Place a 72-well rotor on the Rotor-Gene Q MDx rotor holder.
 - 1b. Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Figure 6 (page 43), 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 6.
 - 1c. Attach the locking ring.
 - 1d. Load the Rotor-Gene Q MDx instrument with the rotor and locking ring, and close the instrument lid.
 - 1e. Within Rotor-Gene AssayManager v2.1 software, either select the corresponding work list from the work list manager and click **“Apply”** (Figure 13, box 14) or, if the work list is still open, click **“Apply”**.



Figure 13. Run setup - Work list selection. 14: Apply button.

Note: If the experiment dedicated work list has not been created, log in the Rotor-Gene AssayManager v2.1 and follow the process in “To create a work list”, page 45 before proceeding as follows.

- Enter the experiment name (Figure 14, box 15).
- Select the cyclers to be used in the **“Cycler selection”** list (Figure 14, box 16). "
- Check correct attachment of locking ring and confirm on the screen that the locking ring is attached (Figure 14, box 17).
- Click **“Start run”** (Figure 14, box 18).

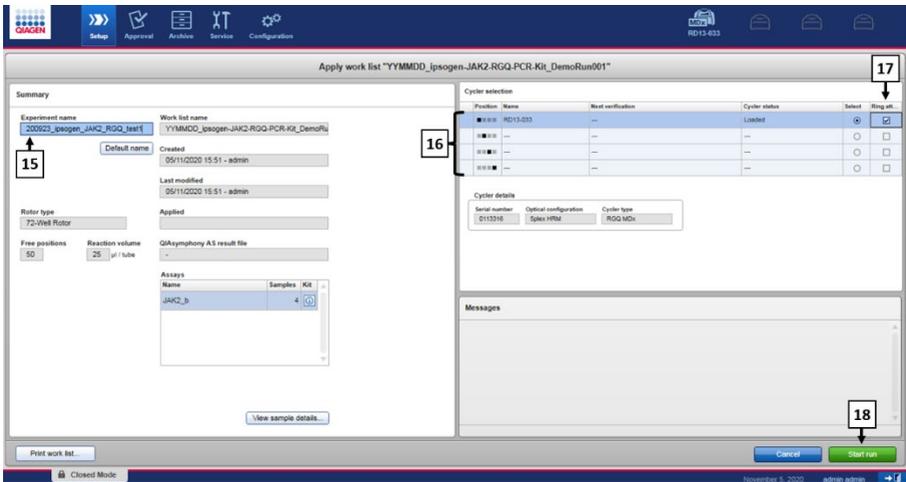


Figure 14. Run setup – Run settings. 15: Experiment name. 16: Cycler selection. 17: Check and confirm locking ring is attached. 18: Start run button.

1f. The JAK2 RGQ PCR run should start.

Ending, releasing and approving the run

1. When the run is finished, click **“Finish run”**.

Note: Until this step is completed, the experiment is not saved in the internal database.

Note: The analysis of the acquired data is performed automatically depending on the plug-in corresponding to the assay profile and the rules and parameter values defined by the assay profile.

2. 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”**.

Note: The general functionality of the Approval environment is described in the Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual.

3. Release results.

- If **“Release and go to approval”** is clicked, the results for the experiment are displayed in the **“Approval”** environment.
- If **“Release”** is clicked, a user with the **“Approver”** role will need to log in and select the **“Approval”** environment.
- Apply the filter options (Figure 15, box 19) to select the experiment to be approved (Figure 15, box 20). Then, click **“Apply”** (Figure 15, box 21).

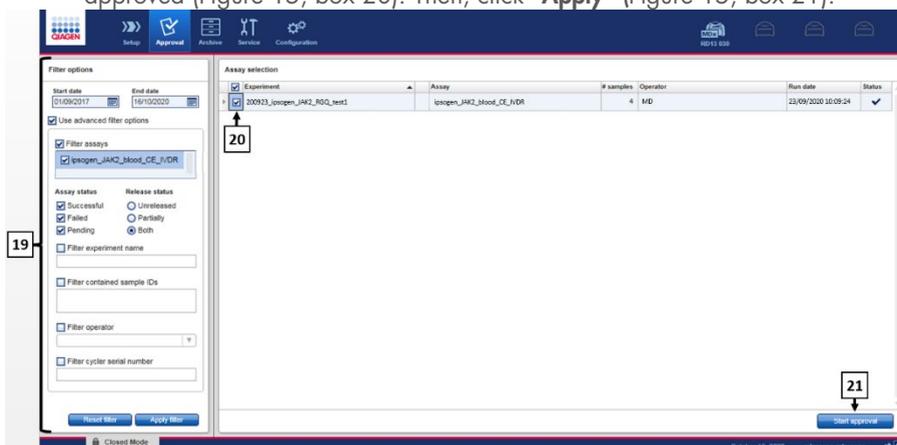


Figure 15. Run approval - Experiment selection. 19: Filter options. 20: Assay selection. 21: Start approval button.

- The following AUDAS (Automatic Data Scan) alert appears (Figure 16). In the **“Plots and Information”** section, manually check the fluorescence curves of the HEX targets for anomalies (e.g., spikes caused by hardware errors).

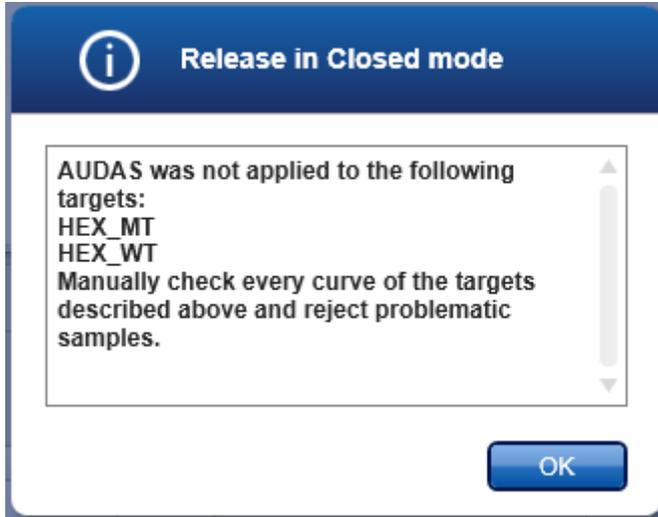


Figure 16. AUDAS alert.

Note: The curves of the internal control HEX targets do not exhibit typical sigmoid shapes (as in the example curves in Figure 17) and must be considered as valid curves. Please note that all other internal control validity criteria (e.g., C_T cutoffs) are automatically checked by the software.

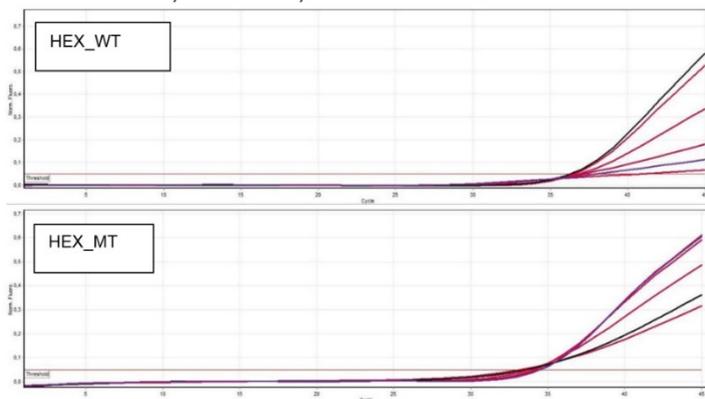


Figure 17. Internal Control HEX curves.

The results of the test samples are automatically analyzed by Rotor-Gene AssayManager v2.1 but must be approved and released by the user logged in with the role of approver. Initially, all test samples of a finished experiment have the status "Undefined". Sample results to be approved have three approval buttons at the end of the dedicated row. These buttons are used to interactively accept or reject the sample results (Figure 18 and Figure 19).

Note: A sample reported as "INVALID" by Rotor-Gene AssayManager v2.1 cannot be reassigned as "VALID" even if the sample result is rejected.

Note: A sample should be rejected if the user does not agree with the result and wishes to retest (e.g. if an anomaly is observed on the curves of the internal control HEX targets).

- Review result (Figure 19, box 22) and click "Release/Report data" (Figure 19, box 23).

Background color	Status of test sample
	Undefined
	Accepted
	Rejected

Figure 18. Sample approval status definition.

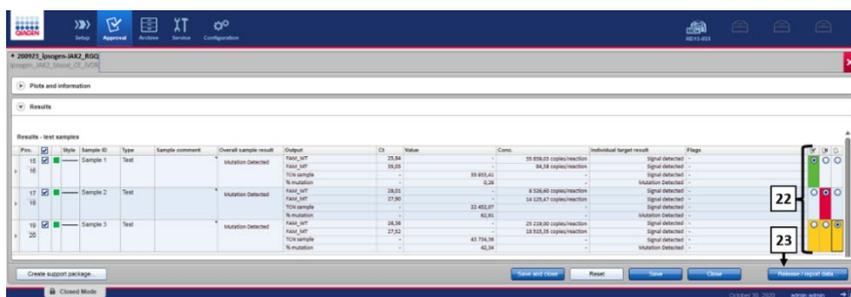


Figure 19. Release and report data. 22: Samples approval buttons (to approve (✓) or reject (✗) results for each sample). 23: Release and report data button.

- Enter password if required, check the **“Create report”** box and click **“OK”** (Figure 20, boxes 24 and 25). The report will be generated in .pdf format and automatically stored in the pre-defined folder.

The default folder path is:

C: > Users > Public > Documents > QIAGEN > Rotor-Gene AssayManager > Export > Reports

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

- At the same time, a LIMS file is automatically created and stored in the predefined folder. The default folder path is: **C: > Users > Public > Documents > QIAGEN > Rotor-Gene AssayManager > Export > LIMS.**

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

- Close the pdf file and return to the Rotor-Gene AssayManager. Click **“OK”** when prompted (Figure 20, box 26).

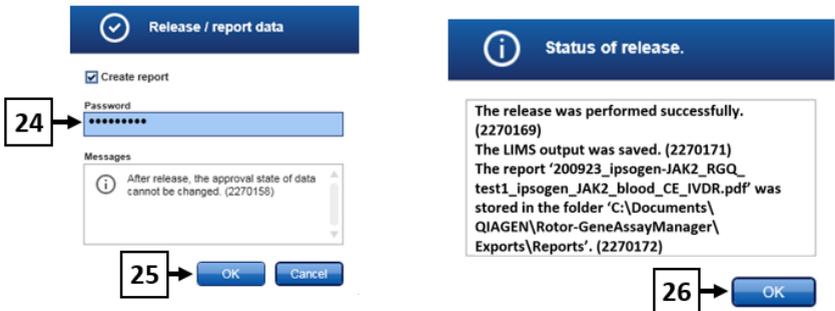


Figure 20. Release and report data. 24: User password. 25-26: OK button to select.

- Upon entering user password, a PDF report is generated and opens. Close the PDF report. A LIMS file is then automatically generated, and a statement of release appears. Click **“OK”**. The Assay is now fully released. Click **“OK”** to go to the “Archive” environment.
- Click the “Archive” tab to export the .rex file corresponding to the raw data. Find your experiment using the filter options (Figure 21, boxes 27 and 28) and click “Show assays” (Figure 21, box 29).

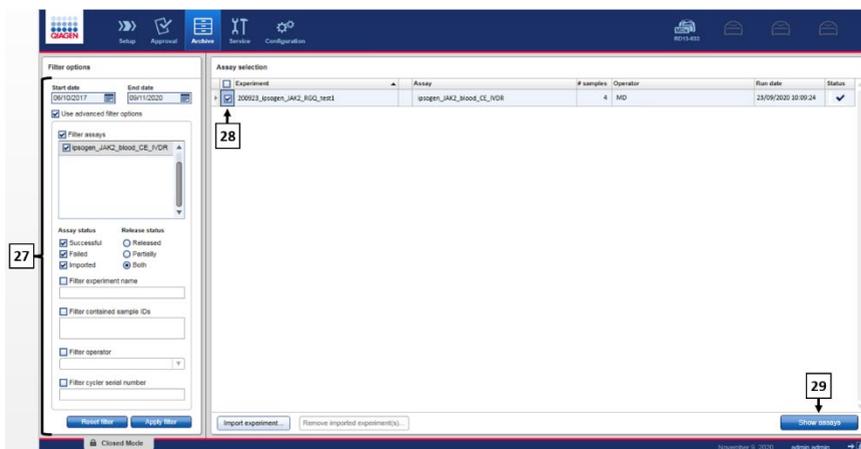


Figure 21. Archive environment. 27: Filter options. 28: Assay selection. 29: Show assays button.

- Experiment results are displayed. In the bottom-right corner of the screen, select “.rex-file” as the type of file to be exported. Click “Export”. Click “OK” to save. The software automatically saves the .rex file in the following predefined folder: **C: > Users > Public > Documents > QIAGEN > Rotor-Gene AssayManager > Export > Experiments.**

Note: This path and folder can be changed in the “Specify the .rex file export destination” tab.

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).

4. 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.

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).
- WT and MT QS: The validation of the quantitation standards is based on the R^2 and the slope values of each standard curve.
- 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.

The internal control (IC) must be amplified in all wells containing controls and quantitation standards, and must be within the predefined range for controls.

Note: The report generated at the end of each run shows the results obtained on run controls. All invalid data will be associated with an invalidating flag (Table 6).

* Enabled only for FAM targets.

If any of these run controls does not conform, the flag "ASSAY_INVALID" will be used. If this flag is highlighted, the run should be considered invalid and the experiment will need to be repeated.

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

- The internal control (IC) must be amplified in all wells containing samples and must fall within the predefined range.
- The total copy number must be high enough in a given sample for the results to be interpreted.
- The JAK2 mutation percentage will then be calculated and the result will be displayed. A CT 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 for each sample.
- Table 6 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 each flag means.
- Table 7 (page 65) provides the warning sample flags and description of terms.

Table 6. 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.

Table continued on next page

Table continued from previous page

Table 6. Invalidating sample flags and description of terms (cont'd)

Flag	Description
MC_NO_VALUE	The mutation percentage of the mutant control has no value.
MC_UNEXPECTED_EARLY_CT (MT)	The detected C_T is lower than expected for the mutant control with the mutant reaction mix.
MC_UNEXPECTED_EARLY_CT (WT)	The detected C_T is lower than expected for the mutant control with the wild-type reaction mix.
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.

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Table 6. Invalidating sample flags and description of terms (cont'd)

Flag	Description
QS_HIGH_SLOPE (MT)	The upper limit of the mutant slope is exceeded.
QS_HIGH_SLOPE (WT)	The upper limit of the wild-type slope is exceeded.
QS_IC_NO_CT (MT)	No detectable C_T for the internal control in the same tube as one or more of the mutant quantification standards.
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.
QS_UNEXPECTED_EARLY_CT (MT)	The detected C_T is lower than expected for one or more of the mutant quantification standards.
QS_UNEXPECTED_EARLY_CT (WT)	The detected C_T is lower than expected for one or more of the wild-type quantification standards.
RUN_FAILED	Assay is set to invalid due to a problem with the cyclor or the cyclor connection.
RUN_STOPPED	Assay is set to invalid because the run has been stopped manually.
SAMPLE_LOW_CN	The total 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.

Table continued on next page

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Table 6. Invalidating sample flags and description of terms (cont'd)

Flag	Description
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_UNEXPECTED_EARLY_CT (MT)	The detected C_T is lower than expected for a test sample with the mutant reaction mix.
SAMPLE_UNEXPECTED_EARLY_CT (WT)	The detected C_T is lower than expected for a test sample with the wild-type reaction mix.
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.

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Table 6. Invalidating sample flags and description of terms (cont'd)

Flag	Description
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.
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_NO_CN	No copy number for the wild-type control.
WTC_NO_VALUE	The mutation percentage of the wild-type control has no value.
WTC_UNEXPECTED_EARLY_CT (MT)	The detected C_T is lower than expected for the wild-type control with the mutant reaction mix.
WTC_UNEXPECTED_EARLY_CT (WT)	The detected C_T is lower than expected for the wild-type control with the wild-type reaction mix.

Table 7. 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.

Limitations

The kit is intended for professional use.

The product is to be used only by professionals specially instructed, trained for molecular biology techniques and familiar with the device technology. The device procedure is to be implemented in a molecular biology laboratory environment.

The *ipsogen* JAK2 RGQ PCR Kit is not an automated device; however, the analysis is assisted by a dedicated software for automatic mutation quantification.

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 19.

Attention should be paid to expiration dates printed on the box label and the tube labels. 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 human peripheral whole blood anticoagulated with 2K-EDTA collected from patients with suspected or diagnosed MPN.

The *ipsogen* JAK2 RGQ PCR Kit is validated only for use with the QIA Symphony 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 QIA Symphony 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-pathological findings. The absence of the JAK2 V617F/G1849T mutation does not exclude the presence of other JAK2 mutations. The test can report false negative results in case of additional mutations located in nucleotides 88504 to 88622 (16).

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

Analytical Performance

Limit of blank

Limit of blank (LOB) was determined following the CLSI/NCCLS EP17-A2 standard, on 30 healthy donor whole blood samples with a wild-type (WT) JAK2 status, using three reagent lots (120 measurements/lot).

The LOB results are summarized in Table 8. This corresponds to the expected value in a normal population using the *ipsogen* JAK2 RGQ PCR Kit.

Table 8. Summary of the LOB results

	Measured LOB	Final limit of blank
Lot 1	0%	
Lot 2	0%	0%
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-A2 standard. In this study, 6 low-levels of mutation were analyzed for 3 independent samples (MPN whole blood DNA spiked into wild-type (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 9.

Table 9. Summary of the LOD results

	Measured LOD	Final limit of detection
Lot 1	0.041%	
Lot 2	0.029%	0.042%
Lot 3	0.042%	

Limit of quantification

Limit of quantitation (LOQ) definition and determination was based on the CLSI/NCCLS EP17-A2 guideline. LoQ was defined as the lowest JAK2 V617F mutation percentage level that can be accurately distinguished from the *ipsogen* JAK2 RGQ PCR Kit's LoD with a confidence interval of 95% (error risk $\alpha = 0.05$). Data from the single-site repeatability study were used to calculate the *ipsogen* JAK2 RGQ PCR Kit's LoQ. The results obtained indicate the LoQ is 0.233% of JAK2 V617F mutation.

In the context of molecular disease monitoring, this implies that if the measured JAK2 V617F mutation percentage is below 0.233% at a given point in time, a JAK2 V617F allele burden reduction cannot be reliably quantified at the next time point.

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, which corresponds to the expected values in the affected population, as long as the quantified DNA sample concentration is close to 10 ng/ μ l (between 5 and 20 ng/ μ l).

Repeatability and reproducibility

The single-site precision study design meets the requirements of the CLSI/NCCLS EP5-A3 standard. Testing was performed on 11 levels of mutation, from 0.07% to 72.67%, using serial dilutions of a clinical sample from a MPN patient. For each level of mutation, 108 measurements were obtained by three operators over 27 days (two replicates per run and two runs per day) using three lots of *ipsogen* JAK2 RGQ PCR Kit and three Rotor-Gene Q MDx 5plex HRM instruments. The precision for the 100% level is expressed by comparison to the precision determined for the 72.67% level, based on trend analyses supported by additional data obtained on a 100% JAK2 V617F sample consisting of DNA from the MUTZ-8 cell line (38 measurements).

The results are summarized in Table 10.

Table 10. Precision results: repeatability (single-site study)

Sample	Mean JAK2 mutation percentage	SD _{R+}	SD _{RUN++}	SD _{TOTAL+++}	CV _{TOTAL}
S0	100	ND	ND	≤ 5.45	≤ 7.50%
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%

SD: Standard deviation

R+: Repeatability

RUN++: Between run precision

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

CV_{TOTAL}: Coefficient of variation for the total precision in percentage

ND: Not determined

The inter-laboratory precision study design meets the requirements of the CLSI/NCCLS EP5-A3 standard. The study involved four sites (France, Germany, and two sites in the USA). Testing was performed on seven levels of mutation, from 1.21% to 67.64%, using dilutions of the MUTZ-8 cell line in healthy donor whole blood (i.e. contrived samples). Each site performed three DNA extraction runs using the QIASymphony SP instrument and a unique batch of the QIASymphony DSP DNA Mini Kit. Each DNA extract was tested in eight qPCR runs (two runs per day and per site over four non-consecutive days) using a unique batch of *ipsogen* JAK2 RGQ PCR Kit, giving rise to 96 expected measurements per sample over all sites.

The L2 sample was invalid in one extraction run leading to a total number of 88 qPCR tests instead of 96. In addition, one qPCR run was invalid, leading to three invalid tests for all samples (except L2, i.e. 2 invalid results). Moreover, the L7 sample was invalid in one qPCR run and L4 was invalid in two qPCR runs leading to two additional invalid tests (Table 11).

The precision for the 100% level is expressed by comparison to the precision determined for the 67.64% level, based on trend analyses supported by additional data obtained on a 100% JAK2 V617F sample consisting of DNA from the MUTZ-8 cell line (38 measurements).

Table 11. Precision results: reproducibility (inter-laboratory study)

Sample	Total tests	Total invalid tests	JAK2%MT mean	Within run, SD, %CV	Between run within day, SD, %CV	Between day, SD, %CV	Between site, SD, %CV	Total, SD, %CV
L0	N/A	N/A	100	N/A	N/A	N/A	N/A	≤4.074, ≤6.02
L1	96	3	67.64	2.616, 3.87	2.060, 3.05	1.999, 2.96	1.530, 2.26	4.074, 6.02
L2	88	2	40.03	3.482, 8.70	1.011, 2.53	2.389, 5.97	0.986, 2.46	4.387, 10.96
L3	96	3	22.26	3.318, 14.90	1.256, 5.64	1.257, 5.64	0.803, 3.61	3.807, 17.10
L4	96	5	8.02	1.770, 22.06	0.516, 6.44	0.000, 0.00	0.000, 0.00	1.841, 22.95
L5	96	3	4.35	0.706, 6.23	0.547, 12.57	0.000, 0.00	0.197, 4.53	0.906, 20.82
L6	96	3	2.03	0.246, 12.15	0.365, 18.00	0.063, 3.11	0.000, 0.00	0.441, 21.76
L7	96	4	1.21	0.104, 8.62	0.057, 4.72	0.211, 17.43	0.000, 0.00	0.189, 15.64

JAK2%MT: JAK2 mutation percentage; **SD:** Standard deviation; **CV:** Coefficient of variation in percentage; **N/A:** Not applicable

An additional inter-laboratory study was conducted across three test sites (one in Europe and two in the USA), on four whole blood samples from MPN patients (i.e. clinical samples). Each site performed three DNA extraction runs. Each DNA extract was tested in 12 qPCR runs (one replicate per run per sample, two runs per day per operator at each site - two operators per site were involved - over three non-consecutive days) on one Rotor-Gene Q MDx instrument using a single lot of *ipsogen* JAK2 RGQ PCR Kit. For each sample, 36 measurements were obtained (Table 12).

Table 12. Additional inter-laboratory study results

Sample	N	JAK2%MT mean	Within run, SD, %CV	Between run within day, SD, %CV	Between day, SD, %CV	Between site, SD, %CV	Total, SD, %CV
Sample 1	36	95.19	0.995, 1.04	0.000, 0.00	0.541, 0.57	0.000, 0.00	1.130, 1.19,
Sample 2	36	22.83	3.988, 17.47	0.000, 0.00	1.707, 7.48	1.552, 6.80	4.501, 19.72
Sample 3	36	14.44	2.257, 15.63	1.398, 9.68	0.000, 0.00	1.422, 9.84	2.890, 20.01
Sample 4	36	4.03	0.186, 4.63	0.835, 20.74	0.000, 0.00	0.608, 15.09	0.922, 22.91

JAK2%MT: JAK2 mutation percentage; **N:** Number of measurements; **SD:** Standard deviation; **CV:** Coefficient of variation in percentage

Interfering Substances (Analytical Specificity)

The study design meets the requirements of the NCCLS standard EP7-A3 “Interference Testing in clinical Chemistry”. A total of 19 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 2K EDTA and 3K EDTA, sodium EDTA, Hgb [human], triglycerides, lisinopril dehydrate, hydroxyurea, acetylsalicylic acid, salicylic acid, thiotepa, anagrelide, interferon alpha 2b).

Substances from the DNA extraction process were also assessed (QSL1, QSB1, QSW1, QSW2 and PK from the QIA Symphony DSP DNA Blood Mini Kit; QIAGEN Protease, ethanol, AW1 and AW2 from the QIAamp DSP DNA Blood Mini Kit).

The obtained results showed no interfering effect for these substances.

Table 13. Interfering substances

Tested substance	Tested concentration
Bilirubin unconjugated	150.3 µg/mL
Hemoglobin [human]	2000 µg/mL
Triglycerides	30000 µg/mL
Busulfan	38.4 µg/mL
Citalopram hydrobromide	0.75 µg/mL
Paroxetine hydrochloride hemihydrate	1.14 µg/mL
Sertraline hydrochloride	0.67 µg/mL
Fluoxetine hydrochloride	3.87 µg/mL
Acetaminophen [paracetamol]	200.7 µg/mL
Lisinopril dehydrate	0.33 µg/mL
Hydroxyurea	28.2 µg/mL
Acetylsalicylic acid	651.6 µg/mL
Salicylic acid	0.6 µg/mL
Thio-tepa	48 µg/mL
Anagrelide	6 µg/mL
Interferon alpha 2b*	1.8 MU/L
Potassium EDTA (2K-EDTA)	2X (3600 µg/mL)
Potassium EDTA (3K-EDTA) †	1X (1800 µg/mL), 3X (5400 µg/mL)

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Table 13. Interfering substances (cont'd)

Tested substance	Tested concentration
Sodium EDTA (2Na-EDTA) †	1X (3000 µg/ml), 3X (9000 µg/ml)
QSL1	2% of total sample volume
QSB1	2% of total sample volume
QSW1	2% of total sample volume
QSW2	2% of total sample volume
proteinase K (PK) ‡	2% of total sample volume
proteinase K (PK) ‡	2x the expected remaining volume after the extraction process 3x the expected remaining volume after the extraction process
QIAGEN Protease	1,29E-05 % of total sample volume
Ethanol (EtOH)	1,29E-03 % of total sample volume
Buffer AW1	1,00 E-01 % of total sample volume
Buffer AW2	1,00 % of total sample volume

* The recommended dosage for PV patients is 3 MU, which is assumed to be distributed in 5L of blood (80Kg individual), resulting in a concentration of 0.6 MU/L. Following recommendations from the NCCLS standard EP7-A2, three times this concentration was tested, i.e. 1.8 MU/L.

† 1x concentration according to provider

‡ PK causes an interfering effect when tested at 2% of the total sample volume (unlikely to occur); further testing confirmed PK is removed during the extraction process: no interference is expected under normal use conditions.

Testing of WHO International Reference Panel for Genomic JAK2 V617F (NIBSC, panel code 16/120)

The WHO 1st International Reference Panel for Genomic JAK2 V617F developed by the National Institute for Biological Standards and Control (NIBSC, panel code 16/120) was tested using three lots of the *ipsogen* JAK2 RGQ PCR Kit (three replicates per level of the reference panel and per reagent lot). The experiments were performed over three days by one operator, using one Rotor-Gene Q 5plex HRM instrument. The concordance between the *ipsogen* JAK2 RGQ PCR Kit results and the consensus values published in the Reference Panel's Instructions for Use was assessed using an ordinary linear regression (slope: 1.003, 95%CI [0.997 ; 1.010] – intercept: 0.677, 95%CI [0.212 ; 1.289]) and a Passing-Bablok regression (slope: 1.01, 95%CI [1.00 ; 1.021] – intercept: 0.00, 95%CI [-0.02 ; 0.010]) (Figure 22). Concordance is confirmed, demonstrating the suitability of the kit in providing JAK2 V617F data which are in agreement with other commonly used diagnostic techniques.

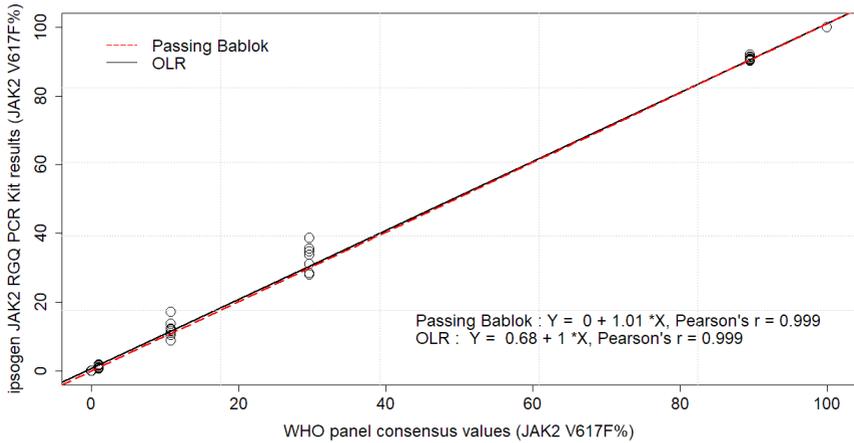


Figure 22. Concordance between the ipsogen JAK2 RGQ PCR Kit results and the WHO International Reference Panel for Genomic JAK2 V617F (NIBSC, panel code 16/120) consensus values. Concordance was assessed using an ordinary Linear Regression (OLR) and a Passing Bablock Regression. The panel comprises seven JAK2 V617F levels: 100%, 89.5%, 29.6%, 10.8%, 1.00%, 0.03% and 0%. The WHO consensus values were determined using a range of commonly used techniques as part of an international collaborative study; the reference values attributed to each JAK2 V617F% level are median values (more information on <https://www.nibsc.org>).

Trueness and accuracy

Measurement trueness is inversely related to the systematic measurement error (SE or bias). Bias was calculated based on instructions from the NCCLS guideline EP09c, for each JAK2 V617F% level of the reference panel, for each reagent lot as well as over all reagent lots (Table 14), using data from the above-described study. The highest bias values were obtained with the *ipsogen* JAK2 RGQ PCR Kit lot 2.

The accuracy is the closeness of agreement between a test result and the accepted reference value (in this case, the value assigned to each JAK2 V617F% level of the WHO panel). Accuracy takes into account both trueness and precision, and is inversely proportional to the total error, calculated as shown in Table 14.

Table 14. Bias and measurement error

WHO panel <i>Ampoule code</i> Reference value	<i>ipsogen JAK2</i> RGQ PCR Kit lot	Bias (SE) per lot [95% CI]	Bias (SE) overall [95% CI]	Total error (Accuracy)
15/172 0%	1	0.000 [N/A]	0.001 [-0.001 ; 0.004]	0.010
	2	0.003 [-0.011 ; 0.018]		
	3	0.000 [N/A]		
15/170 0.03%	1	-0.010 [-0.053 ; 0.033]	0.003 [-0.021 ; 0.028]	0.024
	2	0.020 [-0.094 ; 0.134]		
	3	0.000 [-0.075 ; 0.075]		
15/168 1.00%	1	-0.310 [-0.621 ; 0.001]	0.066 [-0.276 ; 0.407]	0.363
	2	0.617 [0.016 ; 1.217]		
	3	-0.110 [-0.261 ; 0.041]		
15/166 10.8%	1	-0.183 [-4.523 ; 4.156]	1.207 [-0.630 ; 3.043]	2.521
	2	3.600 [-2.670 ; 9.870]		
	3	0.203 [-1.387 ; 1.793]		

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Table 14. Bias and measurement error (cont'd)

WHO panel <i>Ampoule code</i>	<i>ipsogen JAK2</i> RGQ PCR Kit lot	Bias (SE) per lot [95% CI]	Bias (SE) overall [95% CI]	Total error (Accuracy)
Reference value				
15/244 29.6%	1	0.970 [-8.238 ; 10.178]	2.874 [0.016 ; 5.733]	5.589
	2	6.347 [0.141 ; 12.552]		
	3	1.307 [-5.767 ; 8.381]		
15/246 89.5%	1	1.000 [-0.295 ; 2.295]	1.381 [0.889 ; 1.873]	≤ 5.622
	2	1.783 [-0.316 ; 3.883]		
	3	1.360 [0.270 ; 2.450]		
15/164 100%	1	-0.017 [-0.031 ; -0.002]	-0.017 [-0.021 ; -0.013]	≤ 5.450
	2	-0.020 [N/A]		
	3	-0.013 [-0.028 ; 0.001]		

SE: Systematic error or bias, i.e. the difference between the average of individual measurements obtained with the ipsogen JAK2 RGQ PCR Kit ($\bar{V}_{\text{JAK2 Kit}}$) and the WHO reference panel consensus value (V_{Ref}).

$$SE (\%) = \frac{\bar{V}_{\text{JAK2 Kit}} - V_{\text{Ref}}}{V_{\text{Ref}}} \times 100$$

The total error (TE) is calculated as $TE = \sqrt{s^2 + SE^2}$, where s is the standard deviation (random error).

95% CI: 95% confidence interval

N/A: not applicable

Analytical accuracy

The purpose of this study was to validate the analytical accuracy of the *ipsogen* JAK2 RGQ PCR Kit under conditions of normal use with clinical samples from subjects suspected of having myeloproliferative neoplasms. This study was performed on gDNA samples extracted from a total of 473 specimens: 276 with suspected PV, 98 with ET and 99 with PMF. The JAK2 V617F status of the patient samples obtained with the *ipsogen* JAK2 RGQ PCR Kit was compared with the JAK2 V617F status obtained with the reference method for JAK2 status determination, i.e., an independently validated bi-directional sequencing (BDS). As the *ipsogen* JAK2 RGQ PCR Kit's LoD is 0.042% of JAK2 V617F, the JAK2 V617F status of a patient sample tested with the *ipsogen* JAK2 RGQ PCR Kit is positive above or at this limit and negative below this limit. Of the 473 specimens, 22 specimens were JAK2-positive with the *ipsogen* JAK2 RGQ PCR Kit while negative with BDS.

The overall agreement is 95.35% (451/473 subjects; 95% CI: 93.04%, 97.06%). The positive agreement was 100% (165/165 subjects; 95% CI: 97.79%, 100%) and the negative agreement was 92.86 % (286/308 subjects; 95% CI: 89.39%; 95.47%). The results are shown in Table 15.

Table 15. Concordance between the *ipsogen* JAK2 RGQ PCR Kit and Sanger bi-directional sequencing in MPN population (combined ET, PMF, and PV populations)

		Sanger bi-directional sequencing		
		JAK2 V617F positive	JAK2 V617F negative	Total
<i>ipsogen</i> JAK2 RGQ PCR Kit	JAK2 V617F positive	165	22	187
	JAK2 V617F negative	0	286	286
	Total	165	308	473

Assessment of analytical accuracy study results in MPN cohorts

The concordance between results obtained for the JAK2 V617F mutation with the *ipsogen* JAK2 RGQ PCR Kit and with Sanger sequencing (BDS) in subjects with ET, PMF, and PV are provided separately:

- For ET, the overall agreement is 89.8% (88/98 subjects; 95% CI: 82.03–95.0%), the positive agreement is 100% (43/43 subjects; 95% CI: 91.78–100%) and the negative agreement is 81.82% (45/55 subjects; 95% CI: 69.1–90.92%).
- For PMF, the overall agreement is 93.94% (93/99 subjects; 95% CI: 87.27–97.74%), the positive agreement is 100% (51/51 subjects; 95% CI: 93.02–100%) and the negative agreement is 87.5% (42/48 subjects; 95% CI: 74.75–95.27%).
- For PV, the overall agreement is 97.83% (270/276 subjects; 95% CI: 95.33–99.2%), the positive agreement is 100% (71/71 subjects; 95% CI: 94.94–100%) and the negative agreement is 97.07% (199/205 subjects; 95% CI: 93.74–98.92%).

The specimens yielding discordant results appeared to have mutation levels below the BDS detection capability (around 10%). Because Sanger sequencing is not as sensitive as the *ipsogen* JAK2 RGQ PCR Kit which can report values as low as 0.042% of JAK2 V617F (i.e. the LoD value), a separate study was conducted using a validated next-generation sequencing (NGS) method to detect JAK2 V617F allele in the 15/22 discordant samples (nine ET, five PMF and one PV), as well as a randomly selected set of 22 JAK2 V617F-positive and -negative concordant specimens. The JAK2 V617F status of patient samples was determined by the NGS method based on its limit of analytical sensitivity (i.e. between 1% and 2% of JAK2 V617F). Therefore, the JAK2 V617F status of a patient sample was positive if the JAK2 V617F mutation was detected by the NGS method and, reciprocally the JAK2 V617F status was negative if the JAK2 V617F mutation was not detected.

All 15 discordant specimens tested positive by NGS, agreeing with the *ipsogen* JAK2 RGQ PCR Kit. All concordant samples tested the same with NGS and in agreement with the *ipsogen*

JAK2 RGQ PCR Kit and BDS. The 7 other samples were considered as discordant as NGS data are not available for these samples.

Conclusion of the analytical accuracy study

After reclassification of the discordant cases using the NGS results, the *ipsogen* JAK2 RGQ PCR Kit demonstrated 98.3% in accuracy for the detection of JAK2 V617F allele in specimens from MPN subjects with JAK2 V617F levels $\geq 0.042\%$ (i.e the LoD value).

Clinical Performance

The clinical performance of the *ipsogen* JAK2 RGQ PCR Kit in the diagnosis of PV was evaluated during a multicenter, international, prospective, interventional study.

The purpose of the study was to demonstrate the accuracy of the *ipsogen* JAK2 RGQ PCR Kit for the detection of the V617F mutation in subjects with suspected PV. The reference for JAK2 status determination was an independently validated bi-directional sequencing (BDS) method.

The detection of the JAK2 V617F mutation has been first introduced in the reference WHO 2008 criteria for the diagnosis of BCR-ABL-negative MPN, and presence of this mutation is a major criterion for diagnostic confirmation (17.)

Presence of JAK2 V617F is one of the two major diagnostic criteria (PV being confirmed if two major and one minor or the first major and two minor criteria are present according to WHO 2008*; (for details see reference 17)

The objective was to evaluate specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratio for diagnoses established by the 2008 WHO

diagnostic criteria* using JAK2 V617F status determination using either the *ipsogen* JAK2 RGQ PCR Kit with a 0.042% cutoff for positivity (i.e kit's LoD) or BDS.

The study was conducted at nine study sites in the US (seven enrolled subjects), 12 study sites in France (all 12 enrolled subjects), and nine study sites in Italy (five enrolled subjects). Subjects were screened and selected based on inclusion criteria that suggested a diagnosis of PV. All enrolled subjects received blood tests with both the *ipsogen* JAK2 RGQ PCR Kit and the reference test, bi-directional sequencing (BDS) determination of the JAK2 V617F and JAK2 exon 12 status. Subjects with clinical features compatible with the diagnosis of PV (including increased hemoglobin and decreased erythropoietin [EPO] levels), but with negative JAK2 V617F and exon 12 determination by BDS, and subjects with positive JAK2 V617F and exon 12 determination by BDS and normal or high EPO levels, were to undergo a bone marrow biopsy with histologic and cytogenetic analysis, as required by the 2008 WHO diagnostic algorithm for myeloproliferative diseases. The final diagnosis (PV or non-PV) was established based on the results of the non-investigational study procedures (i.e., the 2008 WHO criteria with JAK2 mutation determination using the reference BDS assay).

A total of 216 subjects defined as the evaluable population included all participants that successfully met both the clinical screening criteria and analytical criteria using the reference BDS assay. An additional 67 subjects were not evaluable for the reasons described in Table 16 (some subjects were not evaluable for more than one reason).

* As the clinical performance study was initiated before the 2016 update of the WHO diagnostic criteria, the 2008 WHO diagnostic criteria were used to conduct the clinical performance study.

Table 16. Reasons for exclusions in the enrolled population

Reason	Number of subjects
Failure of inclusion or exclusion criteria	9
Missing EPO results	22
Missing BM biopsy, if required for PV diagnosis	26
Positive JAK2 V617F by BDS and serum EPO within normal limits	15
Positive JAK2 Exon 12 by BDS and serum EPO within normal limits	1
Negative JAK2 V617F by BDS and serum EPO abnormally low	10
Patients did not complete study and/or missing final diagnosis of PV	15

For this study, a total of 221 JAK2 V617F status assessments (including five repeat tests) were performed using the *ipsogen* JAK2 RGQ PCR Kit on the Rotor-Gene Q MDx instrument (Table 17, Table 18).

Table 17. Summary of *ipsogen* JAK2 RGQ PCR test results (evaluable population)

<i>ipsogen</i> JAK2 RGQ PCR Kit	Total (N=216)
JAK2 V617F positive (\geq LoD i.e. 0.042%)	54
JAK2 V617F negative ($<$ LoD i.e. 0.042%)	162

Table 18. Summary of *ipsogen* JAK2 RGQ PCR test results – JAK2 V617F positive population (among evaluable population)

<i>ipsogen</i> JAK2 RGQ PCR Kit	Total (N=54)
JAK2 %MT mean value obtained	51.54
JAK2 %MT minimum value obtained	0.14
JAK2 %MT maximum value obtained	93.43

N: Number of samples; **JAK2 %MT:** JAK2 mutation percentage

Assessment of validation study results: performance outcome

Comparison of final PV and non-PV diagnoses demonstrated that the two methods of diagnosis were concordant: 94.6% of subjects (53/56 subjects) diagnosed with PV by the investigator were also diagnosed with PV using the *ipsogen* JAK2 RGQ PCR Kit and the WHO diagnostic criteria. Similarly, 95.6% of subjects (153/160 subjects) diagnosed as non-PV by the investigator were also diagnosed as non-PV using the *ipsogen* JAK2 RGQ PCR Kit and the WHO diagnostic criteria (Table 19, Table 20).

The mutational statuses of JAK2 V617F and exon 12 by BDS, and JAK2 V617F by the *ipsogen* JAK2 RGQ PCR Kit are summarized in Table 19. A comparison of PV and non-PV diagnoses established using each test method is provided in Table 19.

Table 19. Mutation status (JAK2 V617F by bi-directional sequencing, JAK2 exon 12 by bi-directional sequencing and *ipsogen* JAK2 RGQ PCR Kit) by PV status (evaluable population)

Variable	PV (N=56)	Non-PV (N=160)	Total (N=216)
JAK2 V617F mutation status by BDS			
Positive	48 (85.7%)	1 (0.6%)	49 (22.7%)
Negative	8 (14.3%)	159 (99.4%)	167 (77.3%)
JAK2 exon 12 mutational status by BDS			
Positive	3 (5.4%)	0	3 (1.4%)
Negative	53 (94.6%)	160 (100.0%)	213 (98.6%)
<i>ipsogen</i> JAK2 RGQ PCR Kit status			
Positive	48 (85.7%)	6 (3.8%)	54 (25%)
Negative	8 (14.3%)	154 (96.3%)	162 (75%)

N: Number of patients diagnosed by the investigator (evaluated population).

For each mutation status, the number of patients is expressed as an absolute count, and as a percentage of the evaluated population (between brackets).

Table 20. Final PV diagnosis based on the Investigator’s opinion informed by bi-directional testing and the 2008 World Health Organization criteria using the *ipsogen* JAK2 RGQ PCR Kit

Final diagnosis by investigator based on WHO criteria combined with JAK2 assessment done by BDS

Final diagnosis based on WHO criteria combined with JAK2 assessment done by <i>ipsogen</i> JAK2 RGQ PCR Kit	PV (N=56)	Non-PV (N=160)	Total (N=216)
PV	53 (94.6%)	1 (0.6%)	54 (25%)
Non-PV	1 (1.8%)	153 (95.6%)	154 (71.3%)
Inconclusive	2 (3.6%)	6 (3.8%)	8 (3.7%)

N: Number of patients diagnosed by the investigator (evaluated population)

Numbers are expressed as an absolute count, and as a percentage of the evaluated population (between brackets).

Inconclusive cases

Three subjects were wild type for JAK2 V617F (both with BDS and the *ipsogen* JAK2 RGQ PCR Kit), in addition to having low serum EPO concentrations and inconclusive bone marrow histology (two were diagnosed as PV by the investigator and one as non-PV). Five subjects were JAK2 V617F wild type for BDS and positive using the *ipsogen* JAK2 RGQ PCR Kit and no bone marrow biopsy examination was performed (all five were diagnosed as non-PV by the investigator). Despite the absence or the inconclusive bone marrow histology, these eight cases were included in the calculation of the specificity and sensitivity (Table 21) as discordant.

Discordant cases

For two subjects, the investigator’s diagnosis was different from the diagnosis obtained using the *ipsogen* JAK2 RGQ PCR Kit within the WHO diagnostic criteria. One subject had serum EPO levels within the normal range (at 16.5 IU/l) and no JAK2 V617F or exon 12 mutation. However, the subject was diagnosed as having PV according to the investigator’s opinion. One subject had serum EPO levels below the normal range and a JAK2 V617F mutation by BDS, but was given a diagnosis of non-PV according to the investigator’s opinion. According

to the protocol, the investigator’s diagnosis should have strictly followed the 2008 WHO diagnostic criteria. However, in these two discordant cases, investigators used clinical discretion in interpreting the algorithm.

Overall, as summarized in Table 21, sensitivity of PV diagnosis using the *ipsogen* JAK2 RGQ PCR Kit was 94.64% (53/56 subjects; 95% CI:85.13%, 98.88%), indicating that this assay is expected to detect PV in the vast majority of subjects with the disease. Similarly, the specificity of PV diagnosis using this assay was 95.62% (153/160 subjects; 95% CI: 91.19%, 98.22%), indicating that it is also expected to rule out PV in the vast majority of subjects without PV.

In addition, the positive predictive value (PPV) and negative predictive value (NPV) were also calculated, the PPV of the kit was 88.33% (53/60 subjects; 95% CI:77.27%, 93.57%) and the NPV was 98.08% (153/156 subjects; 95% CI: 94.8%, 99.4%).

Table 21. Sensitivity, specificity, PPV and NPV analysis (evaluable population)

Variable	Estimate (%)	Lower 95% confidence limit (%)	Upper 95% confidence limit (%)
Sensitivity	94.64	85.13	98.88
Specificity	95.62	91.19	98.22
PPV	88.33	77.27	93.57
NPV	98.08	94.8	99.4

The likelihood ratio of a negative test using the *ipsogen* JAK2 RGQ PCR Kit, for the PV diagnosis, within the WHO diagnostic criteria was 21.6 (95% CI; 10.44, 44.71), indicating that the JAK2 V617F positive result is more likely to occur in subjects with PV than in those without PV.

The likelihood ratio of a positive test using the *ipsogen* JAK2 RGQ PCR Kit, for the PV diagnosis, within the WHO diagnostic criteria was 0.06 (95% CI; 0.02, 0.18), indicating

that the JAK2 V617F negative result is less likely to occur in subjects with PV than in those without PV.

Conclusion of the clinical study

The following conclusions can be drawn from the analyses:

- Sensitivity was 94.64% (95% CI; 85.13%, 98.88%) indicating that the *ipsogen* JAK2 RGQ PCR Kit within the WHO diagnostic criteria is expected to detect PV in the vast majority of subjects with the disease.
- Specificity of PV diagnosis using the *ipsogen* JAK2 RGQ PCR Kit within the WHO diagnostic criteria was 95.62% (95% CI; 91.19%, 98.22%), indicating that it is also expected to rule out PV in the vast majority of subjects without PV.
- Using the *ipsogen* JAK2 RGQ PCR Kit within the WHO diagnostic criteria PPV was 88.33% (95% CI; 77.27%, 93.57%)* and NPV was 98.08% (95% CI; 94.8%, 99.4%).
- The likelihood ratio of a negative test using the *ipsogen* JAK2 RGQ PCR Kit, for the PV diagnosis, within the WHO diagnostic criteria was 21.61 (95% CI; 10.44, 44.71), indicating that the JAK2 V617F positive result is more likely to occur in subjects with PV than in those without PV.
- The likelihood ratio of a positive test using the *ipsogen* JAK2 RGQ PCR Kit, for the PV diagnosis, within the WHO diagnostic criteria was 0.06 (95% CI; 0.02, 0.18), indicating that the JAK2 V617F negative result is much less likely to occur in subjects with PV than in those without PV.

* PPV is dependent on prevalence. Because prevalence was low in the study population and sensitivity and specificity are independent of prevalence, sensitivity and specificity are more relevant.

Summary of Safety and Performance

The summary of safety and performance section can be downloaded from the *ipsogen* JAK2 RGQ PCR Kit product webpage: resources.qiagen.com/674623. This can also be found on the EUDAMED website.

Disposal

- Discard sample and assay waste according to your local safety procedures.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially hazardous and must be treated as biohazardous materials.
- Used sample tubes, plates, and waste during DNA extraction must be discarded according to your local safety regulations.
- Strip tubes used during the qPCR protocol must be discarded according to your local safety regulations.

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Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support (for contact information, visit www.qiagen.com).

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; for troubleshooting information relating to Rotor-Gene AssayManager v2.1, please refer to the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

Comments and suggestions

Automated extraction

- | | |
|------------------------------------|---|
| a) 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. |
| b) 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. |
| c) Sample flagged as “invalid” | An error occurred during the extraction run. Repeat the extraction step for this sample. |

Comments and suggestions

- d) 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.
- e) 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 *ipsogen* 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 *ipsogen* 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
- b) Inversion during distribution
- c) Cross contamination
- d) Standard partial degradation
- e) PCR reagents partially degraded
- f) Nonspecific amplification

Check the pipetting scheme and the setup of the reaction. Replace all critical reagents and repeat the experiment using new aliquots. Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carryover contamination.

Store kit contents at -30 to -15°C , and keep the reaction mixes protected from light.

Avoid repeated freezing and thawing.

No or low signal for one standard

- a) Homogeneity issue
- b) Use of the same reaction mix for WT and MT QS

Check the pipetting scheme and the setup of the reaction.

Repeat the PCR run.

No Template Control (NTC) of water shows positive amplification

- a) Cross contamination
- b) Reagent contamination
- c) Strip tube inversion (tubes containing *JAK2 V617F* positive template inserted in NTC position)
- d) Probe degradation

Replace all critical reagents.

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

Check the pipetting scheme and the setup of the reaction.

Keep reaction mixes protection from light.

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 for the IC and/or total copy number (TCN) below validity range, but the run controls are valid

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) amplified with the MT reaction mix (instead of the WT reaction mix) and Mutant Control (MTC) amplified with the WT reaction mix (instead of the MT reaction mix)

- a) Cross contamination
- b) Reagent contamination
- c) Tube inversion (tubes containing WTC inserted in MTC position and vice-versa)

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.

Check pipetting scheme and the setup of the reaction.

Inverted detection of the positive control

- a) Cross contamination
- b) Distribution inversion of the reaction mix in the tube or premix.

Replace all critical reagents and repeat the experiment using new aliquots. 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.

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

- a) Reaction mix or one of its components (e.g. Taq polymerase) not added
- b) Reaction mix degraded

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.

Repeat the qPCR step with new reaction mix.

Symbols

The following symbols appear in the instructions for use or on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	This product fulfills the requirements of the European Regulation 2017/746 for in vitro diagnostic medical devices.
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number (i.e., component labeling)
	Global Trade Item Number
	Unique Device Identifier
	Contains
	Component
	Number

Symbol

Symbol definition

Rn

R is for revision of the Instructions for Use and n is the revision number



Temperature limitation



Manufacturer



Consult instructions for use downloadable from resources.qiagen.com/674623



Protect from light



Caution, consult accompanying documents

Ordering Information

Product	Contents	Cat. no.
<i>ipsogen</i> JAK2 RGQ PCR Kit	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	674623
Related products		
Rotor-Gene Q MDx – for IVD-validated real-time PCR analysis in clinical applications		
Rotor-Gene Q MDx 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	9002032
Rotor-Gene Q MDx 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	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 0.1ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1ml (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
QIAasympphony DSP DNA Mini Kit	For 192 preps of 200 µl each: Includes 2 reagent cartridges and enzyme racks and accessories.	937236
QIAasympphony SP and accessories		
QIAasympphony SP System	QIAasympphony sample prep module: includes installation and training, 1-year warranty on parts and labor	9001751
QIAasympphony SP	QIAasympphony 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 QIAasympphony SP	997002
8-Rod Covers (144)	8-Rod Covers for use with the QIAasympphony SP	997004
Filter-Tips, 200 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube® and the QIAasympphony SP/AS instruments	990332
Filter-Tips, 1500 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIAasympphony 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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Document Revision History

Revision	Description
R1, July 2022	Initial release

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