

January 2018

ipsogen[®] JAK2 RGQ PCR Kit

Instructions for Use (Handbook)



Version 1

IVD

For in vitro diagnostic use

Rx only (For prescription use only)

For use with QIAasymphony[®] SP and QIAasymphony DSP DNA Mini Kit

For use with Rotor-Gene[®] Q MDx

REF

673633



QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden,
GERMANY

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Handbook Revision History

Document	Changes	Date
HB-2354-001	Initial release	April 2017
HB-2354-002	<p>Change of intended use from an adjunct to evaluation of suspected Polycythemia Vera (PV), in conjunction with other clinicopathological factors to Myeloproliferative Neoplasm (MPN), in conjunction with other clinicopathological factors.</p> <p>Inclusion of two new tables regarding diagnostic criteria for Essential Thrombocythemia (ET; Table 2) and Primary Myelofibrosis (PMF; Table 3).</p> <p>Software: Update of Rotor-Gene AssayManager v1.0 to Rotor-Gene AssayManager v2.1 and the JAK2 plug-in to the Gamma MDx plug-in. New screenshots associated with software changes throughout.</p> <p>New and updated software flags (Table 7).</p> <p>Update to "Limitations" section, page 51.</p> <p>Changes to Performance Characteristics section: Updates to "Limit of bank" (page 53), new section "Analytical accuracy" (page 60) and deletion of old section "Secondary performance outcome: Accuracy".</p>	January 2018

Intended Use

The *ipsogen* JAK2 RGQ PCR Kit is a qualitative in vitro diagnostic test for the detection of the JAK2 V617F/G1849T allele in genomic DNA extracted from EDTA whole blood. The *ipsogen* JAK2 RGQ PCR Kit is a real time PCR test performed on the QIAGEN® Rotor-Gene Q MDx instrument. The test is intended for use as an adjunct to evaluation of suspected Myeloproliferative Neoplasm (MPN), in conjunction with other clinicopathological factors.

This test does not detect less common JAK2 mutations associated with Myeloproliferative Neoplasm including mutations in exon 12 and is not intended for standalone diagnosis of Myeloproliferative Neoplasm.

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 Myeloproliferative Neoplasms. JAK2 is a critical intracellular signaling molecule for a number of cytokines, including erythropoietin.

The JAK2 V617F mutation is detected in >95% of patients with Polycythemia Vera (PV), 50–60% of patients with Essential Thrombocythemia (ET) and in 50% of patients with Primary Myelofibrosis (PMF). JAK2 V617F has been also detected in some rare cases of chronic myelomonocytic leukemia, myelodysplastic syndrome, systemic mastocytosis, and chronic neutrophilic leukemia, but not in chronic myeloid leukemia (5).

The mutation corresponds to a single-nucleotide change of JAK2 nucleotide 1849 in exon 14, resulting in a unique valine (V) to phenylalanine (F) substitution at position 617 of the protein (JH2 domain). It leads to constitutive activation of JAK2, hematopoietic transformation in vitro and erythropoietin-independent erythroid colony (EEC) growth in all patients with PV and a large proportion of ET and PMF patients (3). JAK2 V617F represents a key driver in the transformation of hematopoietic cells in MPN, but the exact pathological mechanisms leading with the same unique mutation to such different clinical and biological entities remain to be fully elucidated.

Traditionally, the diagnosis of MPNs was based on clinical, biological, bone marrow histology and cytogenetic criteria. The discovery of a disease-specific molecular marker resulted in both simplification of the diagnostic process and increased diagnostic accuracy. Detection of the JAK2 V617F mutation is part of the WHO 2016 guideline for the classification of myeloid neoplasms and acute leukemia (Table 1, next page). The presence of the JAK2V617F mutation or the JAK2 exon 12 mutations is one of the major diagnostic criteria (see Table 1 and reference 6 for details).

Table 1. WHO 2016 criteria for the diagnosis of PV (6)

Criteria for a diagnosis of Polycythemia Vera (PV)	
Major	<ol style="list-style-type: none"> 1. Hemoglobin (Hgb) >16.5 g/dl in men, hemoglobin >16.0 g/dl in women or, hematocrit >49% in men, hematocrit >48% in women or, increased red cell mass (RCM)*. 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	<ol style="list-style-type: none"> 1. Subnormal serum erythropoietin level. <p>Diagnosis of PV requires meeting either all three major criteria, or the first two major criteria and the minor criterion†.</p>

* More than 25% above mean normal predicted value.

† Criterion number two (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 number three 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).

Table 2. WHO 2016 criteria for the diagnosis of ET (6)

Criteria for a diagnosis of Essential Thrombocythemia (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 fibers. 3. Not meeting WHO criteria for BCR-ABL1 + CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms. 4. Presence of JAK2, CALR or MPL mutation.
Minor	<ol style="list-style-type: none"> 1. Presence of a clonal marker or absence of evidence for reactive thrombocytosis. <p>Diagnosis of ET requires meeting all four major criteria or the first three major criteria and the minor criterion.</p>

Table 3. WHO 2016 criteria for the diagnosis of PMF (6)

Criteria for a diagnosis of Primary Myelofibrosis (PMF)	
Major	<div><div>1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3.*</div><div>2. Not meeting WHO criteria for ET, PV, BCR-ABL1+ CML, myelodysplastic syndromes or other myeloid neoplasm.</div><div>3. Presence of JAK2, CALR or MPL mutation or in absence of these mutations, presence of another clonal marker,[†] or absence of reactive myelofibrosis.[‡]</div></div>
Minor	<div>Presence of at least one of the following, confirmed in two consecutive determinations:</div> <div><div>1. Anemia not attributed to a comorbid condition.</div><div>2. Leukocytosis $\geq 11 \times 10^9/L$.</div><div>3. Palpable splenomegaly.</div><div>4. LDH increased to above upper normal limit of institutional reference range.</div><div>5. Leukoerythroblastosis.</div></div> <div>Diagnosis of PMF requires meeting all three major criteria, and at least one minor criterion.</div>

* See reference 6.

[†] In the absence of any of the three major clonal mutations, the search for the most frequent accompanying mutations (e.g., ASXL1, EZH2, TET2, IDH1/2, SRSF2, SF3B1) helps determine 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.

Since 2006, laboratory-developed tests or commercial kits have been available to detect the presence of, and eventually quantify, JAK2V617F. These were based initially on bi-directional sequencing, but are now based on PCR techniques. Methods have different analytical performances, especially with regard to precision and level of sensitivity. This may impact on the need for bone marrow analysis, time required to establish a final diagnosis, and potentially on diagnostic performance.

Principle of the Procedure

The *ipsogen* JAK2 RGQ PCR assay is based on the single nucleotide polymorphisms (SNPs)-specific primer technique which permits the selective amplification of the mutant (MT) or wild-type (WT) allele that is easily detectable using a real-time qPCR instrument (Figure 1). This allows a sensitivity <0.1%, which is in line with the currently accepted JAK2 cutoff of 1% (equal or higher) used for clinical positivity (7).

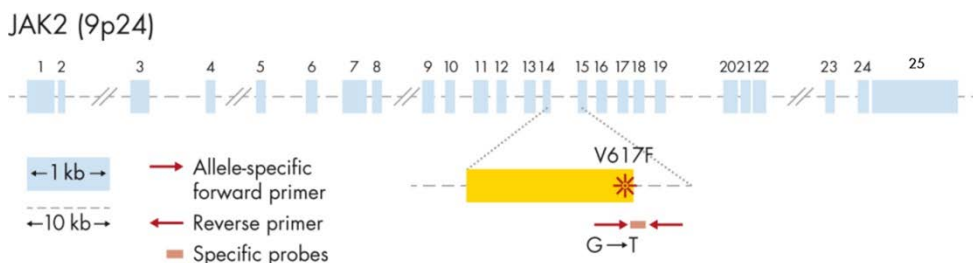


Figure 1. Human JAK2 gene and position of the V617F point mutation on exon 14.

This assay exploits the qPCR oligonucleotide hydrolysis principle using TaqMan[®] probes. 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, leading to an increase in detectable reporter fluorescence and polymerization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR (Figure 2). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

The increase in fluorescence signal is detected only if the target sequence is complementary to the 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 TaqMan technology, the number of PCR cycles necessary to detect a signal above the threshold is called the Crossing point (C_p) or Cycle threshold (C_t) and is directly proportional to the amount of target present at the beginning of the reaction.

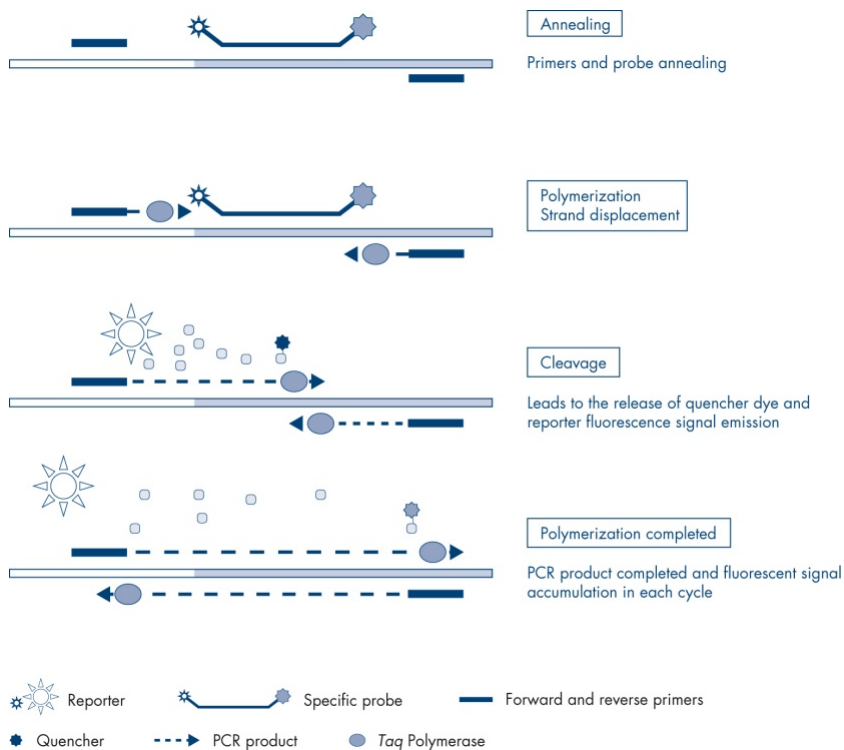


Figure 2. Reaction principle.

The allele-specific PCR technology used in this assay allows a 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 (8). Only a perfect match between the primer and the target DNA allows extension and amplification in the PCR reaction (Figure 3).

WT Reaction Mix



MT Reaction Mix

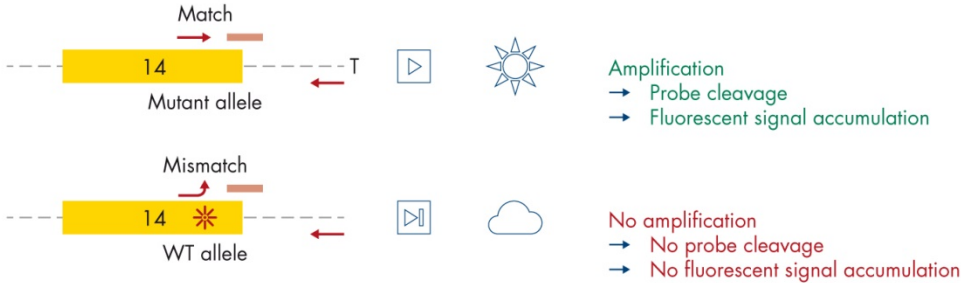


Figure 3. Allele-specific PCR. Use of wild-type or the V617F primers and probe mix allows the specific detection of the wild-type or mutated allele in two separate reactions conducted using the same sample. Results can be expressed as percentage of mutant copies among total JAK2 copies. **MT:** mutant; **WT:** wild type.

Materials Provided

Kit contents

<i>ipsogen JAK2 RGQ PCR Kit (24)</i>		(24)
Catalog no.		673633
Number of reactions		24
Contents	Cap color	Volume
JAK2 Mutant Control (100% V617F allele)	Red	33 µl
JAK2 WT Control (100% wild-type allele)	Green	33 µl
JAK2 MT Quant Standard 1 (5 x 10 ¹ V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 2 (5 x 10 ² V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 3 (5 x 10 ³ V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 4 (5 x 10 ⁴ V617F copies/5 µl)	Red	20 µl
JAK2 WT Quant Standard 1 (5 x 10 ¹ wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 2 (5 x 10 ² wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 3 (5 x 10 ³ wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 4 (5 x 10 ⁴ wild-type copies/5 µl)	Green	20 µl
JAK2 MT Reaction Mix*	Red	1010 µl
JAK2 WT Reaction Mix†	Green	1010 µl
<i>Taq</i> DNA polymerase (5 units/µl)	Mint green	85 µl
TE buffer for sample dilution	White	1.9 ml
Water for no template control (NTC)	White	1.9 ml
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* PCR reaction mix containing all the required components except *Taq* and target DNA for the MT allele.

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

Materials Required but Not Provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Consumables and reagents for automated DNA extraction

- QIAasympohy 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)
- Nuclease-free, low DNA-binding microcentrifuge tubes with flat caps

Consumables and reagents for PCR

- Nuclease-free aerosol-resistant sterile PCR pipette 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 MDx instrument (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
- Water bath at 37°C, if necessary, to remove the formation of an precipitate in Buffers QSL1 and QSB1
- Spectrophotometer*

Equipment for automated sample preparation

- QIA Symphony SP instrument* (cat. no. 9001297) and accessories supplied
- QIA Symphony software version 4.0
- Insert, 2.0 ml v2, samplecarr. (24), Qsym (cat. no. 9242083)
- Optional: QIAGEN 96-Well Magnet Type A, (cat. no. 36915)

Equipment for real-time PCR

- Rotor-Gene Q MDx (US) Platform* (cat. no. 9002035) or Rotor-Gene Q MDx (US) System* (cat. no. 9002036)
- Rotor-Gene AssayManager® software version 2.1.x.x[†]
- Rotor-Gene AssayManager Gamma MDx Plug-in installed, version 1.0.x[†]
- JAK2 Assay Profile (from file AP_ipsogen_JAK2_blood_US_V1.0.x.iap)[†]

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

[†] x ≥ 0, where x corresponds to the latest version available on www.qiagen.com.

Warnings and Precautions

For in vitro diagnostic use

Rx only (For prescription use only)

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.

CAUTION



DO NOT add bleach or acidic solutions directly to the sample preparation waste

General precautions

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

- The test is for use with whole blood samples anti-coagulated with potassium EDTA (K_2 -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 the Rotor-Gene Q MDx and QIAsymphony SP instrument user manuals for additional warnings, precautions and procedures.

Note: The *ipsogen* JAK2 RGQ PCR Kit requires the use of 300 µl whole blood in the DNA extraction step to prevent any extraction failure during initial pipetting.

- 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 may cause degradation of the template DNA.
- Use individual, dedicated pipettes for setting up reaction mixes and adding templates.
- Do not open the Rotor-Gene Q MDx instrument until the run is finished.
- Do not open Rotor-Gene Q tubes after the run is finished.
- Caution must be observed to ensure correct sample testing with emphasis on wrong sample entry, loading error, and pipetting error.
- Make sure the samples are handled in a systematic way to ensure correct identification at all times so maintaining traceability.

We therefore recommend the following:

- Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipette tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Prepare pre-PCR master mix with dedicated material (pipettes, 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 (pipettes, tips, etc.).

For safety information for the QIAasymphony DSP DNA Mini Kit (cat. no. 937236), see the *QIAasymphony DSP DNA Handbook*.

Reagent Storage and Handling

Shipping conditions

The *ipsogen* JAK2 PGQ PCR Kit is shipped on dry ice. If any component of the *ipsogen* JAK2 RGQ PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, handbooks, 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°C to -15°C upon receipt in a constant-temperature freezer and protected from light.

For storage information for the QIAasymphony DSP DNA Mini Kit (cat. no. 937236), see the *QIAasymphony DSP DNA Handbook*.

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 . Repeated thawing and freezing should be avoided. Do not exceed a maximum of three freeze-thaw cycles.

For stability information for the QIAasymphony DSP DNA Mini Kit (cat. no. 937236), see the *QIAasymphony DSP DNA Handbook*.

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

Specimen Handling and Storage

Whole blood samples

The *ipsogen* JAK2 RGQ PCR Kit is for use with genomic DNA samples extracted from whole blood samples anti-coagulated with potassium EDTA (K₂-EDTA) either stored:

- At 2–8°C for no more than 96 hours
- At room temperature (15–25°C) for no more than 96 hours

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

gDNA samples

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

Note: gDNA extracted from whole blood samples collected in K₂-EDTA tubes is stable at 2–8°C for up to 5 days post-DNA extraction.

Procedure

Genomic DNA extraction and preparation from whole blood

Genomic DNA should be extracted using the QIAasymphony SP instrument in combination with the QIAasymphony 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 QIAasymphony DSP DNA Mini Kit (cat. no. 937236). Do not use any other DNA extraction product.


Automated gDNA extraction using the QIAasymphony DSP DNA Mini Kit

Automated gDNA extraction must be performed with the QIAasymphony instrument using the Sample Preparation (SP) module in combination with the QIAasymphony DSP DNA Mini Kit (cat. no. 937236) and by following the procedure below.

In combination with the QIAasymphony SP, the QIAasymphony DSP DNA Mini Kit enables automated DNA purification from human whole blood (using the Blood_200_V7_DSP protocol or higher version on the QIAasymphony SP).

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

Important points before starting

-  Whole blood volume to be extracted is 200 µl. However, we recommend starting with 300 µl whole blood to prevent any extraction failure during initial pipetting.
- Ensure that you are familiar with operating the QIAasymphony SP. Refer to the user manuals supplied with the instrument for operating instructions.
- Optional maintenance is not mandatory for instrument function, but is highly recommended to reduce risk of contamination.
- Before using a reagent cartridge for the first time, check that Buffers QSL1 and QSB1 do not contain a precipitate. If necessary, remove the troughs containing Buffers QSL1 and QSB1 from the reagent cartridge and incubate for 30 minutes at 37°C with occasional shaking to dissolve precipitate. Make sure to replace the troughs in the correct positions. If the reagent cartridge is already pierced, make sure that the troughs are sealed with Reuse Seal Strips and incubate the complete reagent cartridge for 30 minutes at 37°C with occasional shaking in a water bath.
- Try to avoid vigorous shaking of the reagent cartridge (RC) otherwise foam may be generated, which can lead to liquid-level detection problems.

Things to do before starting

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

Procedure

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

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

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

5. Load the required elution rack into the "Eluate" drawer.


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

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

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

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

Note: Ensure that pipetting tips are correctly inserted into position.

7. Perform an inventory scan of the "Reagents and Consumables" drawer.
8.  Transfer 300 µl of the whole blood sample to be extracted into a 2 ml nuclease-free microcentrifuge tube with flat cap and place this into the Insert 2.0 ml v2, samplecarr. (24), Qsym. 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 by clicking "Select All" and selecting "Sarstedt reference 72.694" from the "Tube Insert" sheet.

Protocol to be run: Click "Select All", then select "Select "DNA Blood", followed by "Blood_200_V7_DSP" (or higher version) for whole blood sample.

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

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

10. Start the run.

To start the run, press "Run".

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 QIAasympphony SP carrier status changes to "RUNNING".

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

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

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

We recommend removing the eluate plate from the "Eluate" drawer immediately after the run has finished. Depending on temperature and humidity, elution plates left in the QIAasympphony 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 shows black particles, the magnetic particles can be removed as follows.

- Apply the tube containing the DNA to a suitable magnetic separator (e.g., QIAGEN 12-Tube Magnet, cat. no. 36912) until the magnetic particles are separated. If DNA is in microplates, apply the microplate to a suitable magnetic separator (e.g., QIAGEN 96-Well Magnet Type A, cat. no. 36915) until the magnetic particles are separated. If no suitable magnetic separator is available, centrifuge the tube containing the DNA for 1 minute at full speed in a microcentrifuge to pellet any remaining magnetic particles.
12. Export the QIAAsymphony SP result file; this report is generated for each elution plate.
 13. Insert a USB stick into one of the USB ports at the front of the QIAAsymphony SP. Click “Tools” and then select “File Transfer”. On the “In-/Output Files” tab, select “Results Files” and click “Transfer”. Keep the name of file export, in the following format: yyyy-mm-dd hh:mm:ss_Elution rack ID.
 14. Check the “Validity of result” column for each sample on the QIAAsymphony SP result file.
Valid status: Proceed to DNA qualification and quantification.
Invalid and unclear status: Sample is rejected. Reprocess the extraction step.
 15. If a reagent cartridge is only partially used, seal it with the Reuse Seal Strips provided 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 QIAAsymphony SP.
Follow the maintenance instructions in the user manuals supplied with the 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 QIAAsymphony SP.

Qualification and quantification of DNA

A blank of ATE buffer should be used to calibrate the spectrophotometer. This is because elution buffers used in gDNA extraction kits contain the preservative sodium azide, which absorbs at 260 nm.

- The OD_{260}/OD_{280} ratio must be within the range 1.7–2, 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 OD_{260}/OD_{280} ratio is below 1.7 and above 2 or if gDNA concentration is below 10 ng/ μl , the sample must not be processed further. A new blood sample should be used for further analysis.

Genomic DNA sample normalization

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

Each PCR reaction of the Rotor-Gene Q MDx is optimized for 50 ng of purified gDNA diluted in a final volume of 5 μl . A total of 100 ng per tested sample is needed.

Protocol: qPCR on Rotor-Gene Q MDx instrument

Important points before starting

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

Things to do before starting

Rotor-Gene AssayManager software v2.1 must be installed on the computer connected to the Rotor-Gene Q MDx. For details about the installation of the Rotor-Gene AssayManager v2.1 core software, please refer to the *Rotor-Gene AssayManager v2.1 MDx Core Application User Manual*.

- The *ipsogen* JAK2 RGQ PCR Kit requires a specific plug-in – the “Gamma MDx plug-in”. This plug-in can be downloaded from the QIAGEN web page:
<https://www.qiagen.com/us/shop/detection-solutions/personalized-healthcare/ipsogen-jak2-rgq-pcr-kit-pma-us/?#resources>. This plug-in must be installed on a computer that already has at least Rotor-Gene AssayManager v2.1 installed.
- The *ipsogen* JAK2 RGQ PCR Kit also requires an assay profile. This assay profile contains all parameters needed for cycling and analyzing the qPCR assay. The JAK2 assay profile corresponds to the file AP_ipsogen_JAK2_blood_US_V1.0.x.iap. It can be downloaded from the *ipsogen* JAK2 RGQ PCR Kit web page:
<https://www.qiagen.com/us/shop/detection-solutions/personalized-healthcare/ipsogen-jak2-rgq-pcr-kit-pma-us/?#resources>. The assay profile must be imported in Rotor-Gene AssayManager v2.1 software.

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

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

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

Installation of Gamma MDx plug-in and importing the assay profile

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

- Download both the Gamma MDx plug-in and the latest version of the JAK2 assay profile from the QIAGEN website.

Start the installation process by double-clicking on the RGAM_V2_1_Gamma_MDx_Plug-in.Installation.V1_0_x.msi file (where x is ≥ 0) and following the installation instructions. For a detailed description of this process, please refer to the section about “installing plug-ins” in the *Rotor-Gene AssayManager v2.1 MDx Core Application User Manual*.

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

- After successful installation of the plug-in, a person with administrator rights for the Rotor-Gene AssayManager v2.1 software will need to import the latest assay profile version, as follows.

1. Open the Rotor-Gene AssayManager v2.1 software by clicking the icon.



The following window opens.

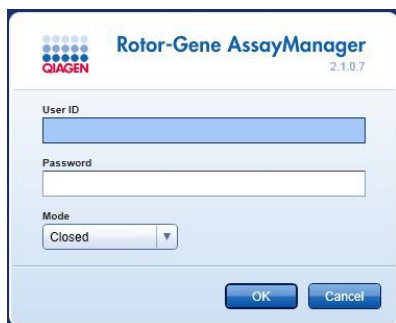


Figure 4. Rotor Gene AssayManager v2.1.

2. Log-in to Rotor-Gene AssayManager v2.1 software with your user ID and password. Do not change the "Closed mode". Click "OK". The following window opens.

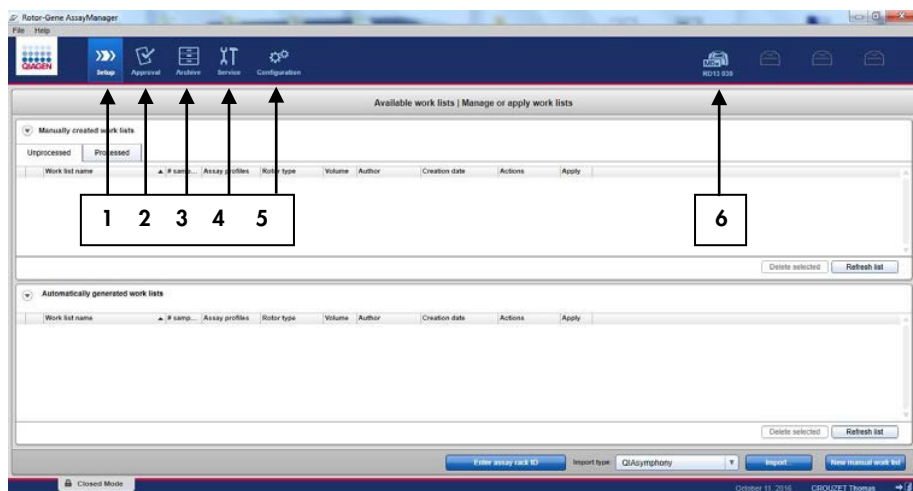


Figure 5. Rotor Gene AssayManager v2.1.1. 1 = Setup tab. This tab allows you to manage or apply work. 2 = Approval tab. This tab enables you to find previous experiments. 3 = Archive tab. This tab allows you to find old experiments already approved. 4 = Service tab. In this tab, an audit trail of each file generated by the software is reported. 5 = Configuration tab. This tab allows configuration of all software parameters. 6 = Rotor-Gene Q (RGQ) icon.



Not connected



Connected

3. Select the configuration environment.
4. Select the tab "Assay Profiles".
5. Click "Import".
6. Select the assay profile to be imported in the dialog and click "Open".
7. Once the assay profile has been successfully imported, it can be used in the "Setup" environment.

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

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

We recommend testing eight gDNA samples in the same experiment to optimize use of the controls, standards, and reaction mixes (Table 4).

Table 4. Number of reactions for Rotor-Gene Q MDx instruments with 72 tube rotor

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

The scheme shown in Figure 6 provides an example of the loading block or rotor setup for an experiment with the *ipsogen* JAK2 RGQ PCR Kit. Numbers denote positions in the loading block and indicate final rotor position.

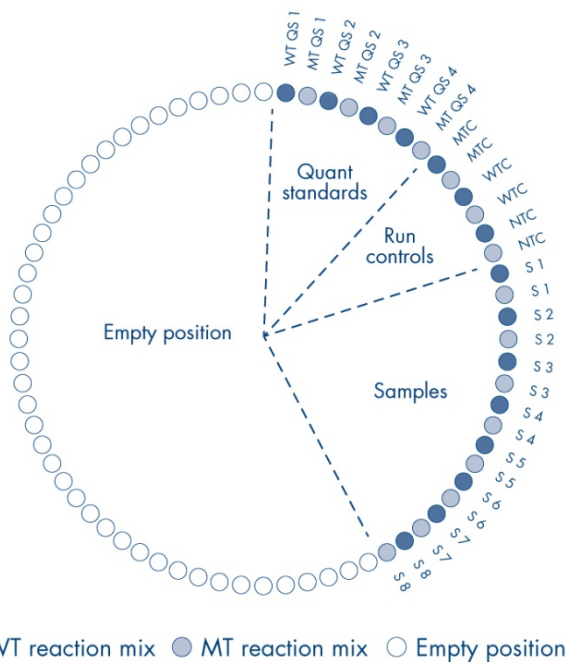
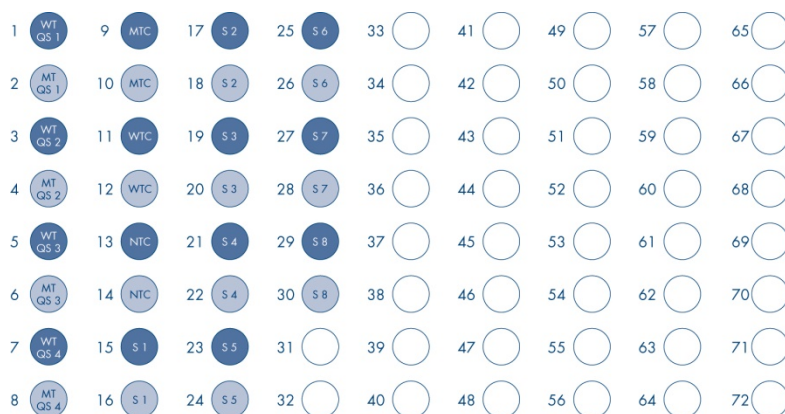


Figure 6. Plate and rotor setup for an experiment with the *ipsogen* JAK2 RGQ PCR Kit. WTC: JAK2 WT Control; MTC: JAK2 MT Control; WT-QS: JAK2 wild-type standards; MT-QS: JAK2 MT Quant Standards; S: gDNA sample; NTC: No-template control (water).

⚠ 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: Fill all unused positions with empty tubes.

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

1. Create a work list for the samples to be processed as follows.

- Switch on the Rotor-Gene Q MDx instrument.
- Open the Rotor-Gene AssayManager v2.1 software and log-in as user with the operator role in the closed mode.
- Click “New manual work list” in the work list manager (“Setup” environment; Figure 7).

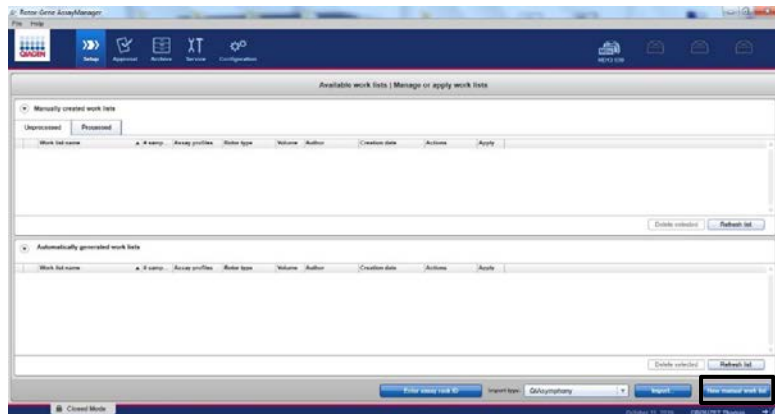


Figure 7. Creating a new work list.

- Select the “JAK2 assay profile” from the list of available assay profiles (Figure 8, box 1).

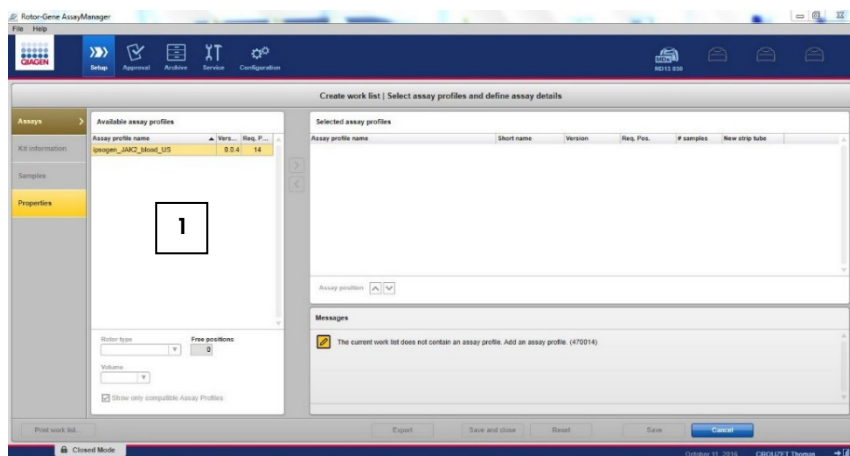


Figure 8. Selecting an assay profile. 1 = Available assay profiles.

- Click "Move" to transfer the selected assay profile to the list of "Selected assay profiles" (Figure 9, box 2). The assay profile should now be displayed in the "Selected assay profiles" list.

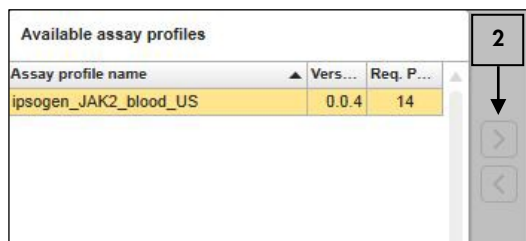


Figure 9. Validation of assay profile selected.

- Enter the number of samples in the corresponding field (Figure 10, box 3).



Figure 10. Creation of the work list: definition of assay details. 3 = Number of samples.

- Enter the following JAK2 kit information, which is printed on the lid of the box
Material number: 1079210 (Figure 11, box 5)
Valid expiry date (Figure 11, box 6)
Lot number (Figure 11, box 7)

Note: The “Scan bar code” option (when available) can be used by clicking “Scan bar code”. The information is then filled automatically.

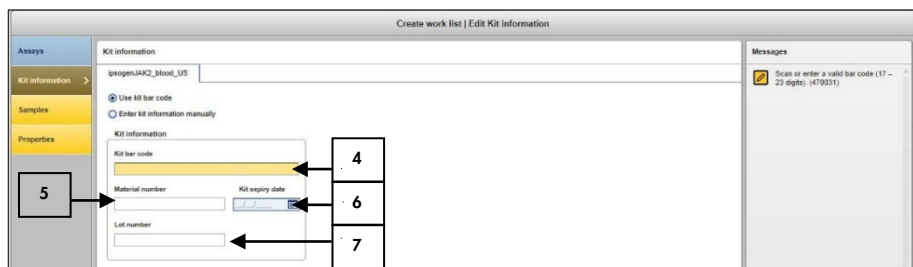


Figure 11. Creation of the work list: Edit kit information. 4 = Kit bar code. This tab indicates the bar code of the kit, if the bar code is entered; the other fields are filled automatically). 5 = Material number. 6 = Kit expiry date. 7 = Lot number. This information is available on the lid of the kit box.

Note: All fields must be filled in and will become blue when valid information is entered. The user can click “OK” when all information is validated (i.e., kit not expired, valid material and lot numbers entered).

- Select the “Samples” step. A list with the sample details is shown. This list represents the expected layout of the rotor.

- Enter the sample identification number(s) into this list, as well as any optional sample information as a comment for each sample (Figure 12).

The screenshot shows a software window titled "Create work list | Edit samples". On the left is a sidebar with tabs: "Assays", "Kit information", "Samples", and "Properties". The "Kit information" tab is currently selected. The main area displays a table with the following columns: Pos., Style, Sample ID, Status, Sample type, Targets, Assay, and Sample comment. The table contains 20 rows of data, including quality control samples (QS1-QS4, MutantControl, WildTypeControl, NTC) and three test samples (Sample1, Sample2, Sample3). Each row has a corresponding "Assay" value, mostly "JAK2_m". At the bottom of the window are buttons for "Print work list...", "Export", "Save and close", "Reset", "Save", and "Cancel".

Pos.	Style	Sample ID	Status	Sample type	Targets	Assay	Sample comment
1	■	QS1		QS	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
2							
3	■	QS2		QS	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
4							
5	■	QS3		QS	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
6							
7	■	QS4		QS	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
8							
9	■	MutantControl		PC	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
10							
11	■	WildTypeControl		EC-	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
12							
13	■	NTC		NTC	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
14							
15	■	Sample1		Test	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
16							
17	■	Sample2		Test	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
18							
19	■	Sample3		Test	FAM_WT_HEX_WT FAM_MT_HEX_MT	JAK2_m	
20							

Figure 12. Entering sample information.

- Select the "Properties" step and enter a work list name (Figure 13).


The screenshot shows the "Properties" window. The sidebar on the left has tabs: "Assays", "Kit information", "Samples", and "Properties". The "Properties" tab is selected. The main area contains the following fields and options:

- Work list name:** A text input field with a "Default name" button next to it.
- Work list:** A section with two checkboxes: "☒ is editable" and "

The screenshot shows a 'Properties' window. At the top is the title 'Properties'. Below it is a text field labeled 'Work list name' containing the text 'yymdd_operator_studname_runID'. Underneath the text field are two small boxes labeled '2' and '1'. Box '2' has a downward arrow pointing to a checkbox labeled 'is editable', which is currently checked. Box '1' has a downward arrow pointing to a checkbox labeled 'work list is complete (can be applied)', which is currently unchecked. To the right of these checkboxes is a button labeled 'Default name'.

Figure 14. Properties. 1 = Check “work list is complete” option. 2 = Uncheck “is editable”.

Note: The “is editable” check box (Figure 14) defines whether the work list is still editable or not. So if the work list is applicable and shall not be changed afterwards the user has to uncheck the “is editable” check box. The work list can be applied directly or can be saved and run later.

- Enable the check box “is applicable” and click “Apply”. 
- Save the work list.

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

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

2. Set up the qPCR experiment.

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

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

Clean the bench area dedicated for PCR mix preparation to ensure no template or nuclease contamination.

Do not vortex the tubes containing standards, controls and reaction mixes. Mix gently by inverting 10 times, then briefly centrifuge before use.

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

All concentrations apply to the final reaction volume.

Table 5 and Table 6 describe the pipetting scheme for the preparation of one mutant (MT) and one wild-type (WT) reagent mix, calculated to achieve final reaction volumes of 25 µl. Extra volumes are included to compensate for pipetting error and allow for eight samples plus controls.

Table 5. Preparation of qPCR mixes for JAK2 mutant sequence detection

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

* An extra reaction volume is included.

Table 6. Preparation of qPCR mixes for JAK2 wild-type sequence detection

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

* An extra reaction volume is included.

Important: Vortex and briefly centrifuge before dispensing 20 µl of the qPCR pre-mix per strip tube.

Important: Vortex and briefly centrifuge DNA (gDNA samples plus QS and controls). Then, add 5 µl of material to be quantified into its corresponding tube to give 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 nonspecific template or reaction mix contamination, and therefore false-positive results.

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

4. Prepare Rotor-Gene Q MDx and start run as follows.

- Place a 72-well rotor on the rotor holder.
 - Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Figure 6, 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 (page 33).
 - Attach the locking ring.
 - Load the Rotor-Gene Q MDx instrument with the rotor and locking ring, and close the instrument lid.
 - Within the Rotor-Gene AssayManager v2.1 software, either select the corresponding work list from the work list manager and click “Apply”, or if the work list is still open, click “Apply”.

Note: If the work list for the experiment has not been created, log in to Rotor-Gene AssayManager v2.1 and follow step 2 before proceeding.

- Enter the experiment name.
- Select the cyclers to be used in the “Cycler selection”.
- Check correct attachment of locking ring and confirm on the screen that the locking ring is attached.

- Click “Start run”.
- The *ipsogen* JAK2 RGQ PCR run should start.

5. When the run has finished, click “Finish run”.

6. 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”.

7. Release results.

If “Release and go to approval” was clicked, the results for the experiment are displayed.

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

- Filter the assay to be approved by selecting the filter options and clicking “Apply”
- Review results and click “Release/Report data”
- Click “OK”. The report will be generated in Adobe Portable Document format (.pdf) and automatically stored in the predefined folder. By default, this folder path is: QIAGEN > Rotor-Gene AssayManager > Export > Reports

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

At the same time, the LIMS file is automatically created and stored in the predefined folder.

By default, this folder path is: QIAGEN > Rotor-Gene AssayManager > Export > LIMS.

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

Go to the “Archive” tab to export the .rex file, corresponding to the raw data. Find your experiment using the filter options and click “show assays”. Then, click on “Export .rex file” and save it by clicking “OK”. The software automatically saves the .rex file in the following predefined folder: QIAGEN > Rotor-Gene AssayManager > Export > Experiments

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

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

8. 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 (see Table 7, page 45).

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

- NTC is checked for the absence of specific amplification (JAK2 WT and JAK2 MT), and the presence of amplification of the internal control.
- WT and MT QS: The validation is based on the R^2 and the slope values of each.
- WTC: The JAK2 total copy number 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 valid, 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 valid, if its status is highly positive for the JAK2 mutation.

If any of these run controls does not conform, the flag “ASSAY_INVALID” will be used. If this flag is used, the run should be considered invalid and the experiment will need to be performed again.

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

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

- The total copy number of each sample must be high enough for the results to be interpreted. The JAK2 mutation percentage will then be calculated and the result will be displayed. If no specific amplification is observed in a tube (either WT or MT), the amplification of the internal control will be checked to rule out PCR artefacts. At least one C_T value must be observed in each tube (WT and MT) for a sample in order that the Rotor-Gene AssayManager v2.1 validates the sample result.

Note: If both the run controls and the sample results are valid, and provided that the mutation percentage is above the cutoff, the report will show the mutation percentage for each sample.

If the total copy number is too low, the flag "SAMPLE_LOW_CN" will be used. The sample should be considered as invalid and the sample should be tested again.

Table 7 (next page) shows the invalidating sample flags that may be assigned to an individual tube during the analysis by Rotor-Gene AssayManager v2.1, along with an explanation of what this flag means.

Table 7. 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	Sample 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 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).
IC_INVALID	The internal control is invalid. Target and internal control share the same tube.
IC_NO_SIGNAL	No internal control signal detected. Target and internal control share the same tube.
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.
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.
MC_IC_NO_CT (MT and WT)	No signal is detected for an internal control in the same tube as the mutant control.
MC_LOW_CN	Copy number too low in mutant control.
MC_LOW_PERCENTAGE	Mutant control percentage is too low.
MC_NO_CN	No copy number for the mutant control.
MC_NO_CT (MT)	No detectable C_T for the mutant control with the mutant reaction mix.
MC_NO_VALUE	No detectable value for the mutant control.
MULTIPLE_THRESHOLD_CROSSING	The amplification curve crosses the threshold more than once. An unambiguous C_T cannot be determined.
NTC_IC_HIGH_CT (MT and WT)	High C_T for the internal control in the same tube as the no template control.
NTC_IC_LOW_CT (MT and WT)	Low C_T for the internal control in the same tube as the no template control.
NTC_IC_NO_CT (MT and WT)	No detectable C_T for the internal control in the same tube as the no template control.
NTC_UNEXPECTED_VALUE (MT and WT)	The no template control has a value calculated (contamination).

Flag	Description
OTHER_TARGET_INVALID	Another target for the same sample is invalid.
QS_HIGH_SLOPE (MT and WT)	Upper limit of the slope is exceeded.
QS_IC_NO_CT (MT and WT)	No signal is detected for an internal control in the same tube as the quantification standard.
RUN_FAILED	Invalid Assay is set to invalid due to a problem with the cyclor or the cyclor connection.
RUN_STOPPED	Invalid Assay is set to invalid because the run has been stopped manually.
QS_LOW_RSQUARED (MT and WT)	The lower limit of the R^2 is not met.
QS_LOW_SLOPE (MT and WT)	The lower limit of the slope is not met.
QS_NO_CT (MT and WT)	No detectable C_T for one or more of the quantification standards.
SAMPLE_LOW_CN	Test sample copy number too low.
SAMPLE_MT_IC_HIGH_CT	C_T for the internal control in the same tube as a test sample with the mutant reaction mix is too high.
SAMPLE_MT_IC_LOW_CT	C_T for the internal control in the same tube as a test sample with the mutant reaction mix is too low.
SAMPLE_MT_IC_NO_CT	No detectable C_T for the internal control in the same tube as a test sample with the mutant reaction mix.
SAMPLE_NO_CN	No copy number for test sample.
SAMPLE_NO_VALUE	No detectable value for the sample.
SAMPLE_WT_IC_HIGH_CT	C_T for the internal control in the same tube as a test sample with the wild-type reaction mix is too high.
SAMPLE_WT_IC_LOW_CT	C_T for the internal control in the same tube as a test sample with the wild-type reaction mix is too low.
SAMPLE_WT_IC_NO_CT	No detectable C_T for the internal control in the same 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.
SATURATION_IN_PLATEAU	The raw data fluorescence is saturating strongly after the inflection point of the amplification curve.
SPIKE	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the C_T is determined.
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.

Flag	Description
STRONG_BASELINE_DIP	A strong drop in the baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_NOISE	Strong noise outside the growth phase of the amplification curve detected.
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	Percentage for 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.
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.
WTC_IC_NO_CT (MT and WT)	No detectable C_T for the internal control in the same tube as the wild-type control with the mutant reaction mix.
WTC_LOW_CN	Copy number for the wild-type control is too low.
WTC_NO_CN	No copy number for the wild-type control.
WTC_NO_CT	No detectable C_T for the wild-type control with the wild-type reaction mix.
WTC_NO_VALUE	No detectable value for the wild-type control.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

For troubleshooting information relating to QIAsymphony DNA DSP Mini Kit (cat. no. 937236), please refer to the *QIAsymphony DNA DSP Handbook*, and for troubleshooting information relating to Rotor-Gene AssayManager v2.1, please refer to the *Rotor-Gene AssayManager v2.1 MDx 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 the sample volume to 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.
d) Cooling block temperature error	An error message at the end of the run about the cooling temperature means that samples remained at room temperature from the end of the extraction run. If the samples remained at room temperature for <12 hours, gDNA quality should not be altered and gDNA can be quantified. If >12 hours have elapsed, gDNA 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 the relevant box.

Comments and suggestions

General handling for JAK2 mutation status assessment using the *ipsogen* JAK2 RGQ PCR Kit

Total copy number does not conform and corresponding sample is invalid: the amplification is too low

- | | | |
|----|--|---|
| a) | Check the OD ₂₆₀ /OD ₂₈₀ ratio | If this is not within the range 1.7–2.0, perform a new DNA extraction. |
| b) | Check the DNA concentration | The <i>ipsogen</i> JAK2 RGQ PCR Kit is optimized for a 10 ng/μl working concentration.
If the DNA concentration is not at this concentration, dilute or re-extract DNA from whole blood. |
| c) | If both parameters conform, pipetting volumes may be incorrect | Check and re-calibrate the pipettes before repeating the qPCR step. |

Run control fails on a quantitative standard

- | | | |
|----|---------------------------------|--|
| a) | Vial inversion | Check the pipetting scheme and the reaction setup. |
| b) | Inversion during distribution | Check the pipetting scheme and the reaction setup. |
| c) | Cross-contamination | 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. |
| d) | Standard partial degradation | Store kit contents at –30 to –15°C and keep the reaction mixes protected from light.
Avoid repeated freezing and thawing. |
| e) | PCR reagents partially degraded | Store kit contents at –30 to –15°C and keep the reaction mixes protected from light.
Avoid repeated freezing and thawing. |
| f) | Nonspecific amplification | Check the pipetting scheme and the reaction setup. |

No or low signal for one standard

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

Comments and suggestions

NTC of water is positive

- | | |
|--------------------------|---|
| a) Cross-contamination | Replace all critical reagents.
Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carryover contamination. |
| b) Reagent contamination | Replace all critical reagents.
Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carryover contamination. |
| c) Strip tube inversion | Check the pipetting scheme and the reaction setup. |
| d) Probe degradation | Keep reaction mixes protected 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 reaction setup.
Repeat the PCR run. |
|-------------------------------------|---|

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

- | | |
|--|---|
| Inhibitory effects of the sample material, caused by insufficient purification | Always check the DNA quality (by measuring the OD_{260}/OD_{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.
Change pipette tips to pipet different reagents. |
|-------------------------|---|

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

- | | |
|--------------------------|--|
| a) Cross-contamination | 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. |
| b) Reagent contamination | 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. |
| c) Tube inversion | Check pipetting scheme and the reaction setup. |

Comments and suggestions

- | | |
|---|--|
| d) Distribution inversion of the reaction mix in the tube or premix | Check the pipetting scheme and the reaction setup. |
|---|--|

No signal for a sample or control, including internal control

- | | |
|---------------------------|--|
| a) Reaction mix not added | Check the pipetting scheme and the reaction setup. |
| b) Reaction mix degraded | Repeat the qPCR step with new reaction mix. |

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

Limitations

Rx only (For prescription use only)

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

Accuracy for detection of the JAK2 V617F mutation was evaluated in specimens from Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary Myelofibrosis (PMF) subjects and not in other MPN types. This mutation occurs less frequently in Acute Myeloid Leukemia (AML) and other hematologic diseases, such as Myelodysplastic Syndrome (MDS); Myelodysplastic Syndrome/Myeloproliferative Neoplasm, Unclassifiable (MDS/MPN-U); Refractory Anemia with Ring Sideroblasts with Thrombocytosis (RARS-T), and Idiopathic Hypereosinophilic Syndrome, atypical CML, and Chronic Myelomonocytic Leukemia.

JAK2 V617F mutation has been detected in apparently healthy subjects. Testing for JAK2 mutation should not be used to screen individuals for a myeloproliferative disorder.

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

This kit should be used following the instructions given in this manual, in combination with a validated instrument mentioned in “Materials Required but Not Provided”, page 15.

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

All reagents supplied in the *ipsogen* JAK2 RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same kit. This may affect performance.

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

The *ipsogen* JAK2 RGQ PCR Kit is validated only for use with the QIAsymphony DNA DSP Mini Kit (cat. no. 937236).

Only the Rotor-Gene Q MDx (for PCR) and the QIAsymphony SP (for sample preparation) have been validated.

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

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

Performance Characteristics

Limit of blank (LOB)

Limit of blank (LOB) was determined based on the data point corresponding to the upper 95% percentile of the results obtained with blank samples and was determined as a %MT, as described in CLSI/NCCLS EP17-2A. Blank samples correspond to 30 healthy, whole blood samples with wild-type JAK2 status. The LOB results are based on 120 measurements/lot using three different lots. No JAK2 mutation was detected; therefore, the limit of blank is below the level of reporting.

Limit of Detection (LOD)

Limit of detection (LOD or analytical sensitivity) was determined based on the 'Probit approach' described in CLSI/NCCLS EP17-2A. In this study, six low mutation levels were analyzed for three independent samples (PV whole blood DNA spiked into wild-type whole blood DNA), with three lots, 60 measurements per sample and per mutation. The results obtained indicated the analytical sensitivity was 0.042% of JAK2 V617F mutation. Results less than 1% are reported as negative.

Linearity

The linearity of the quantification of the JAK2 mutation in PV patients was assessed according to CLSI/NCCLS EP06-AE, with one lot of *ipsogen* JAK2 RGQ PCR Kit for 11 levels of mutation from five different DNA inputs. The linear range of quantification of the JAK2 mutation burden in PV samples is linear, i.e., LOD to 70% mutation, provided that the sample concentration is close to the recommended input of 10 ng/μl (between 5 and 20 ng/μl). The assay is not linear with DNA inputs outside this range. The % mutation is determined using the *ipsogen* JAK2 RGQ PCR Kit standards (Table 8).

Table 8. Summary of the linearity results

DNA concentration (ng/μl)	Slope (95% confidence intervals)	Intercept (95% confidence intervals)
5	1.024 (1.008; 1.041)	−0.461 (−0.906; −0.017)
10	1.007 (0.991; 1.023)	−0.010 (−0.427; 0.407)
20	0.988 (0.971; 1.006)	0.156 (−0.307; 0.619)

Repeatability and reproducibility

The precision study was performed according to CLSI/NCCLS EP5-A2. Testing was performed on 11 levels of mutation. For each level of mutation, 108 measurements were obtained by three operators including two replicates per run and two runs per day over 27 non-consecutive days using three lots of *ipsogen* JAK2 RGQ PCR Kit and three Rotor-Gene Q MDx instruments (Table 9, next page).

Table 9. Precision results including all variables

Sample	%MT	JAK2 %MT			Between operator, SD, %CV	Between instrument, SD, %CV	Between kit lot, SD, %CV	Between day, SD, %CV	Within day, SD, %CV	Within run, SD, %CV	Total SD, %CV
		Min	Max	Mean							
S1	108	61.70	88.61	72.68	0.117, 0.16	0.000, 0.00	4.615, 6.35	1.423, 1.96	2.987, 4.11	1.998, 2.75	5.398, 7.43
S2	108	22.58	67.54	53.69	0.434, 0.81	0.000, 0.00	5.761, 10.73	0.000, 0.00	3.245, 6.04	4.116, 7.67	7.059, 13.15
S3	108	14.15	33.19	23.13	0.000, 0.00	0.000, 0.00	4.480, 19.37	0.398, 1.72	1.953, 8.44	1.587, 6.86	4.467, 19.31
S4	108	6.82	17.27	11.97	0.472, 3.95	0.000, 0.00	2.572, 21.49	0.673, 5.62	1.168, 9.75	1.099, 9.18	2.757, 23.03
S5	108	3.38	9.63	6.01	0.157, 2.61	0.000, 0.00	1.470, 24.48	0.245, 4.08	0.632, 10.52	0.704, 11.72	1.556, 25.90
S6	108	1.14	4.23	2.39	0.089, 3.74	0.000, 0.00	0.540, 22.57	0.236, 9.85	0.364, 15.22	0.306, 12.79	0.694, 28.97
S7	108	0.60	2.08	1.23	0.000, 0.00	0.000, 0.00	0.270, 21.96	0.101, 8.21	0.156, 12.70	0.169, 13.74	0.335, 27.18
S8	108	0.22	1.44	0.63	0.043, 6.88	0.000, 0.00	0.164, 26.08	0.067, 10.66	0.123, 19.61	0.131, 20.84	0.236, 37.62
S9	108	0.03	0.42	0.13	0.000, 0.00	0.000, 0.00	0.026, 20.20	0.015, 11.86	0.032, 24.87	0.052, 40.67	0.066, 51.75
S10	106	0.00	0.21	0.07	0.012, 17.84	0.008, 11.63	0.020, 30.11	0.000, 0.00	0.021, 30.95	0.033, 48.82	0.044, 65.13
S11	108	0.00	0.05	0.01	0.000, 0.00	0.003, 38.69	0.001, 6.94	0.003, 44.42	0.000, 0.00	0.011, 146.91	0.012, 156.72

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

Across all variables, the total coefficient of variation (CV) was $\leq 37.62\%$, starting from a 0.63% JAK2 V617 mutation burden.

Inter-laboratory study

The inter-laboratory study of the *ipsogen* JAK2 RGQ PCR Kit was realized by testing a healthy, whole blood sample mixed with a JAK2-positive cell line, representing eight different JAK2 mutation percentages. Blood samples were extracted on a QIAAsymphony SP instrument using a unique batch of the QIAAsymphony DSP DNA Mini Kit. Each extracted genomic DNA was tested in qPCR runs on Rotor-Gene Q MDx instruments, using a unique batch of *ipsogen* JAK2 RGQ PCR Kits. The study was conducted across four sites (France, Germany, and two in the USA), each site performed three extraction runs, tested each extracted DNA in eight qPCR runs, leading to an expected number of test per samples of 96.

The L2 sample was invalid in one extraction run leading to a total number of tests of 88 instead of 96. Due to one invalid qPCR run, at least three invalid tests are presented for each sample (except for L2 sample, i.e., 2). Moreover, L7 and L8 samples were invalid in one qPCR run one more invalid test and L4 was invalid in two qPCR runs leading to two additional invalid tests (Table 10, next page).

Table 10. Inter-laboratory results

Sample	No. positive results	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
L1	93	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	86	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	93	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	90	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	93	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	93	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	25*	96	4	1.21	0.104, 8.62	0.057, 4.72	0.211, 17.43	0.000, 0.00	0.189, 15.64
L8	N/A	96	4	N/A	N/A	N/A	N/A	N/A	N/A

* Sample around the 1% qualitative cut off, data are in line with expected positive/negative distribution for such specimens.

JAK2%MT: JAK2 mutation percentage; **SD:** Standard deviation; **CV:** coefficient of variation in percentage; **N/A:** Not applicable, data not available as all observed JAK2 V617F statuses are negative, no JAK2 mutation percentage available for such analysis.

The differences observed during the inter-laboratory study are not statistically significant and do not impact the clinical decision for the *ipsogen* JAK2 RGQ PCR Kit.

An additional inter-laboratory study of the *ipsogen* JAK2 RGQ PCR Kit was realized by testing whole blood samples representing four JAK2 V617F-positive statuses and two JAK2 V617F-negative statuses. The study was conducted across three sites (Europe and two in the USA). At each site, extracted gDNA were tested in singlicate in 12 qPCR runs on one Rotor-Gene Q MDx instruments using a single batch of *ipsogen* JAK2 RGQ PCR Kit.

For each sample, 36 measurements were obtained including two runs per day per operator (two operators per site were involved) over three non-consecutive days (Table 11).

Table 11. 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	N/A	N/A	N/A	N/A	N/A	N/A
Sample 3	36	N/A	N/A	N/A	N/A	N/A	N/A
Sample 4	36	22.83	3.988, 17.47	0.000, 0.00	1.707, 7.48	1.552, 6.80	4.501, 19.72
Sample 5	36	14.44	2.257, 15.63	1.398, 9.68	0.000, 0.00	1.422, 9.84	2.890, 20.01
Sample 6	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 Mean: JAK2 mutation percentage; **N:** Number of sample, **SD:** Standard deviation; **CV:** coefficient of variation in percentage; **N/A:** Not applicable, data not available as all JAK2 V617F statuses are negative, no JAK2 mutation percentage available for such analysis.

Each study sample was assessed with the expected JAK2 V617 status in all three sites. The overall agreement, sensitivity and specificity were assessed to 100%.

Interfering substances

The study design was conducted according to NCCLS standard EP7-A2 'Interference Testing in clinical Chemistry' recommendations. A total of 17 substances potentially present in blood samples were chosen for their potential effect on PCR (Table 12, next page).

Table 12. Interfering substances

Interfering substance	Concentration tested
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	200.7 µg/ml
Bilirubin unconjugated	150.3 µg/ml
K ₂ EDTA	3600 µg/ml
Hemoglobin (human)	2000 µg/ml
Triglycerides	30,000 µg/ml
Lisinopril dihydrate	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

* The recommended dosage for PV patients was 3 MU. This resulted in a test concentration of 1.8 MU. Therefore, the 50-fold concentrated stock solution was 90 MU.

The study was conducted on a PV whole blood sample (85.37% JAK2 mutation percentage) and a healthy donor whole blood sample JAK2 V617F-negative. Each sample was spiked with each of the interfering substances at the concentrations described in Table 12. For each condition and sample, the extracted gDNA were tested in four replicates. A total of nine qPCR runs were performed on a Rotor-Gene Q MDx instrument.

The results obtained showed no interfering effect for these substances (no significant differences in mean percentage mutation between native and spiked samples).

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). Of the 473 specimens, 15 specimens were JAK2-positive with the *ipsogen* assay while negative with BDS.

The overall agreement is 96.8% (458/473 subjects; 95% CI: 94.8%, 98.2%). The positive agreement was 100% (165/165 subjects; 95% CI: 97.8%, 100%) and the negative agreement was 95.1 % (293/308 subjects; 95% CI: 92.1%; 97.2 %). The results are shown in Table 13.

Table 13. 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	15	180
	JAK2 V617F negative	0	293	293
	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 90.8% (89/98 subjects; 95% CI: 83.3–95.7%), the positive agreement is 100% (43/43 subjects; 95% CI: 91.8–100%) and the negative agreement is 83.6% (46/55 subjects; 95% CI: 71.2–92.2%).
- For PMF, the overall agreement is 94.9% (94/99 subjects; 95% CI: 88.6–98.3%), the positive agreement is 100% (51/51 subjects; 95% CI: 93.0–100%) and the negative agreement is 89.6% (43/48 subjects; 95% CI: 77.3–96.5%).
- For PV, the overall agreement is 99.6% (275/276 subjects; 95% CI: 98.0–100%), the positive agreement is 100% (71/71 subjects; 95% CI: 94.9–100%) and the negative agreement is 99.5% (204/205 subjects; 95% CI: 97.3–100%).

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 and the *ipsogen* JAK2 RGQ PCR Kit reported $\geq 1\%$, a separate study was conducted using a validated next-generation sequencing (NGS) method to detect JAK2 V617F allele in the 15 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.

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.

Conclusion of the analytical accuracy study

The *ipsogen* JAK2 RGQ PCR assay was 100% accurate for the detection of JAK2 V617F allele in specimens from subjects with JAK2 V617F levels $\geq 1\%$.

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 assay 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 (Table 14), and presence of this mutation is a major criterion for diagnostic confirmation (9).

Table 14. WHO 2008 criteria for the diagnosis of MPN (adapted from reference 9)

Criteria for a diagnosis of Polycythemia Vera (PV)	
Major	<div>1. Hemoglobin (Hgb) >18.5 g/dl⁻¹ (men) or >16.5 g/dl⁻¹ (women) or Hgb or hematocrit (Hct) >99th percentile of reference range for age, sex or altitude of residence or Hgb >17 g/dl⁻¹ (men) or >15 g/dl⁻¹ (women) if associated with sustained increase of ≥2 g/dl⁻¹ from baseline that cannot be attributed to correction of iron deficiency or Elevated red cell mass >25% above mean normal predicted value.</div> <div>2. Presence of JAK2V617F or similar mutation.</div>
Minor	<div>1. Bone marrow trilineage myeloproliferation.</div> <div>2. Subnormal serum erythropoietin level.</div> <div>3. Endogenous erythroid colony (EEC) growth.</div>

JAK2V617F 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 9).

The primary objective was to evaluate specificity and sensitivity for diagnoses established by the 2008 WHO diagnostic algorithm using JAK2V617F status determination using either the *ipsogen* JAK2 RGQ PCR Kit with a 1% cutoff for positivity or BDS. The secondary objectives were to compare JAK2V617F status determination between the *ipsogen* JAK2 RGQ PCR Kit (1% positivity cutoff) and BDS in a PV population (for positive percentage agreement; PPA) and a non-PV population (for negative percentage agreement; NPA).

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 JAK2V617F 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 JAK2V617F and exon 12 determination by BDS, and subjects with positive JAK2V617F 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 algorithm 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 15 (some subjects were not evaluable for more than one reason).

Table 15. Reasons for exclusions in the all 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 JAK2V617F by BDS and serum EPO within normal limits	15
Positive JAK2 Exon 12 by BDS and serum EPO within normal limits	1
Negative JAK2V617F by BDS and serum EPO abnormal low	10
Patient did not complete study and/or missing final diagnosis of PV	15

For this study, a total of 221 JAK2 V617F status assessment (including five repeat tests) were performed using the *ipsogen* JAK2 RGQ PCR Kit on the Rotor-Gene Q MDx instrument (Table 16, Table 17).

Table 16. Summary of *ipsogen* JAK2 RGQ PCR test results (evaluable population)

<i>ipsogen</i> JAK2 RGQ PCR Kit results	Total (N=216)
JAK2 V617F positive ($\geq 1\%$)	50
JAK2 V617F negative ($< 1\%$)	166

Table 17. Summary of *ipsogen* JAK2 RGQ PCR test results – JAK2 V617F positive population (evaluable population)

<i>ipsogen</i> JAK2 RGQ PCR Kit results	Total
JAK2 %MT mean value observed	55.64
JAK2 %MT minimum value observed	5.87
JAK2 %MT maximum value observed	93.43

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

Assessment of validation study results: Primary performance outcome

Comparison of final PV and non-PV diagnoses demonstrated that the two methods of diagnosis were mostly concordant. Almost all subjects (94.6%; 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 algorithm. Similarly, almost all subjects (98.1%; 157/160 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 algorithm (Table 18, Table 19).

The mutational statuses of JAK2V617F and exon 12 by BDS, and JAK2V617F by the *ipsogen* JAK2 RGQ PCR Kit are summarized in Table 18. A comparison of PV and non-PV diagnoses established using each test method is provided in Table 18.

Table 18. Mutation status (V617F by bi-directional sequencing, 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)
JAK2V617F mutational status by BDS			
Positive	48 (85.7%)	1 (0.6%)	49 (22.7%)
Negative	8 (14.3%)	159 (99.4%)	167 (77.3%)
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%)	2 (1.3%)	50 (23.1%)
Negative	8 (14.3%)	158 (98.8%)	166 (76.9%)

Table 19. Final PV diagnosis based on the Investigator’s opinion informed by bi-directional testing and the 2008 World Health Organization algorithm using the *ipsogen* JAK2 RGQ PCR Kit

Final diagnosis based on WHO algorithm combined with JAK2 assessment with the <i>ipsogen</i> JAK2 RGQ PCR Kit	Final diagnosis by investigator based on WHO algorithm combined with JAK2 assessment with BDS		
	PV (N = 56)	Non-PV (N = 160)	Total (N = 216)
PV	53 (94.6%)	1 (0.6%)	54 (25.0%)
Non-PV	1 (1.8%)	157 (98.1%)	158 (73.1%)
Inconclusive	2 (3.6%)	2 (1.3%)	4 (1.9%)

Inconclusive cases

Three subjects were wild type for JAK2 (both with BDS and the *ipsogen* JAK2 RGQ PCR Kit), in addition to having low serum EPO concentrations and inconclusive bone marrow histology. One subject was JAK2 wild-type for BDS and positive using the *ipsogen* JAK2 RGQ PCR. This subject’s serum EPO concentration was at the lower limit of normal and no bone marrow biopsy examination was performed. Despite the inconclusive bone marrow histology, these four cases were included in the calculation of the specificity and sensitivity (Table 20) as discordant.

Discordant case

The WHO diagnostic algorithm based on the *ipsogen* JAK2 RGQ PCR Kit gave a diagnosis which disagreed with the investigator’s diagnosis in two subjects. One subject had serum EPO levels within the normal range (at 16.5 IU/l) and no JAK2 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 JAK2V617F 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 algorithm. However, in these two discordant cases, investigators used clinical discretion in interpreting the algorithm.

Overall, as summarized in Table 20, sensitivity of PV diagnosis using the *ipsogen* JAK2 RGQ PCR Kit was 94.6% (53/56 subjects; 95% CI:85.1%, 98.9%), 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 98.1% (157/160 subjects; 95% CI: 94.6%, 99.6%), indicating that it is also expected to rule out PV in the vast majority of subjects without PV.

Table 20. Sensitivity and specificity analysis (evaluable population)

Variable	Estimate	Lower 95% Confidence Limit	Upper 95% Confidence Limit
Sensitivity	0.94643	0.851326	0.988813
Specificity	0.98125	0.946184	0.996116

Conclusion of the clinical study

The following conclusions can be drawn from the analyses:














- Sensitivity was 0.94643 (95% CI; 0.851326, 0.988813), indicating that the *ipsogen* JAK2 RGQ PCR Kit within the WHO diagnostic algorithm 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 algorithm was 0.98125 (95% CI; 0.946184, 0.996116), indicating that it is also expected to rule out PV in the vast majority of subjects without PV.



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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number
	Components
	Contains
	Number
R_n	R is for revision of the Instructions for Use (Handbook) and n is the revision number
	Temperature limitation
	Manufacturer
	Protect from light
	Consult instructions for use

Symbol	Symbol definition
	Caution
	Prescription use only

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Ordering Information

Product	Contents	Cat. no.
<i>ipsogen</i> JAK2 RGQ PCR Kit (24)	For 24 reactions: Wild-type JAK2 Gene Control, JAK2 V617F Control Gene, JAK2 WT Quant Standards, JAK2 MT Quant Standards, JAK2 WT Reaction Mix, JAK2 MT Reaction Mix, <i>Taq</i> DNA polymerase, TE buffer for dilution, water for NTC	673633
Rotor-Gene Q MDx and accessories		
Rotor-Gene Q MDx (US) System	Real-time PCR cyclers with 6 channels (the red and HRM channels are not intended for use with FDA cleared or approved nucleic acid tests), laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002036
Rotor-Gene Q MDx (US) Platform	Real-time PCR cyclers with 6 channels, laptop computer, software, accessories, 1-year warranty on parts and labor	9002035
Rotor-Gene AssayManager v2.1	Software for routine testing in combination with Rotor-Gene Q and Rotor-Gene Q MDx instruments	9024203
Rotor-Gene AssayManager v2.1 License (1)	Single license for the installation of Rotor-Gene AssayManager v2.1 software on one computer	9025620

Product	Contents	Cat. no.
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipette in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
DNA preparation		
QIAsymphony DSP DNA Mini Kit	For 192 preps of 200 µl each: Includes 2 reagent cartridges and enzyme racks and accessories.	937236
QIAsymphony SP and accessories		
QIAsymphony SP System	QIAsymphony sample prep module: includes installation and training, 1-year warranty on parts and labor	9001751
QIAsymphony SP	QIAsymphony 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 QIAsymphony SP	997002
8-Rod Covers (144)	8-Rod Covers for use with the QIAsymphony SP	997004
Filter-Tips, 200 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube® and the QIAsymphony SP/AS instruments	990332

Product	Contents	Cat. no.
Filter-Tips, 1500 µl, Qsym SP (1024)	Disposable Filter-Tips, racked; (8 x 128). For use with the QIA Symphony 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

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