July 2016

JAK2 RGQ PCR Kit Handbook

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

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

REF 673613

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Intended Use

The JAK2 RGQ PCR Kit is intended for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of products. We recommend all users of QIAGEN® products adhere to the National Institutes of Health (NIH) guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Principle of the Procedure

Several different techniques have been proposed to quantitatively determine the proportion of single nucleotide polymorphisms (SNPs) in DNA samples. Some, such as melting curve analysis and sequencing, are only semi-quantitative. Methods based on real-time quantitative polymerase chain reaction (qPCR) are preferred because of their higher sensitivity. The use of a SNP-specific primer permits the selective amplification of the mutant (MT) or wild-type (WT) allele that is easily detectable using a real-time qPCR instrument. This allows for a sensitivity of <0.1%.

The use of qPCR permits the accurate quantification of PCR products during the exponential phase of the PCR amplification process. Quantitative PCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during and/or after PCR cycling, thereby drastically reducing the risk of contamination of the PCR product. At present, 3 main types of qPCR techniques are available: qPCR analysis using SYBR® Green I Dye, qPCR analysis using hydrolysis probes, and qPCR analysis using hybridization probes.

This assay exploits the qPCR oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. Another dye-linked oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream, 3' dye-free quencher, hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes exploits the 5'→3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer.

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

displaced from the target, and polymerization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR (Figure 1). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

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

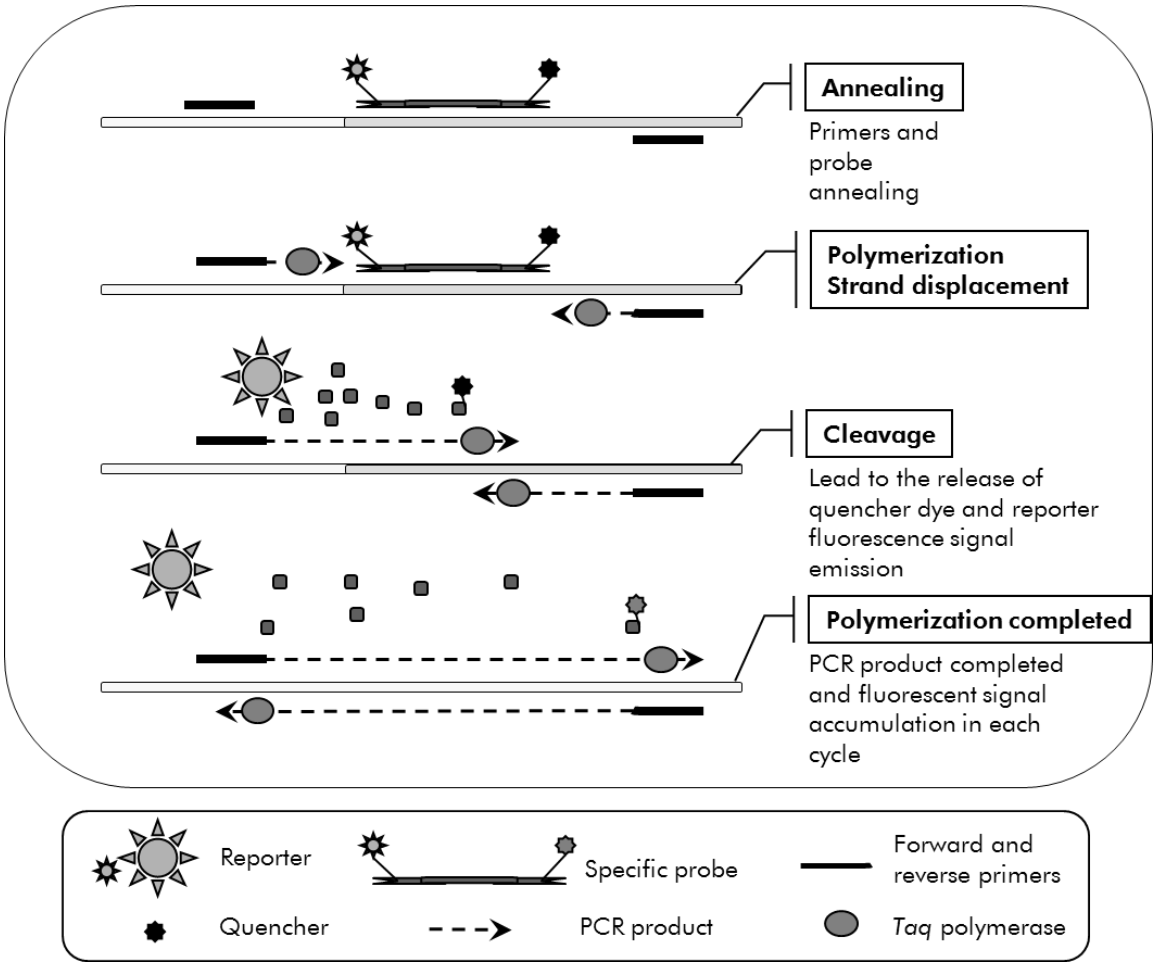

Figure 1. Reaction principle.

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

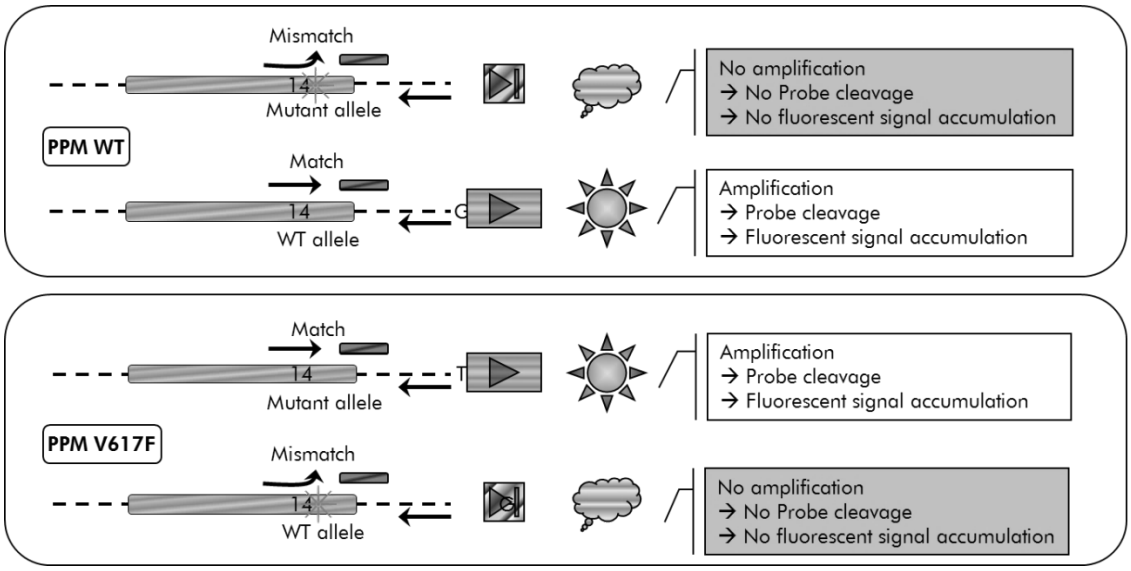

Figure 2. Allele-specific PCR. Use of wild-type or the V617F primers and probe mix allows the specific detection of the wild-type or mutated allele in two separate reactions conducted using the same sample. Results can be expressed as percentage of VF copies among total JAK2 copies.

The JAK2 RGQ PCR Kit provides reagents intended for the detection of the JAK2 V617F/G1849T allele in genomic DNA extracted from whole blood.

Materials Provided

Kit contents

JAK2 RGQ PCR Kit		**(24)**
Catalog no.		**673613**
Number of reactions		**24**
JAK2 Mutant Control (100% V617F allele)	Red	33 µl
JAK2 WT Control (100% wild-type allele)	Green	33 µl
JAK2 MT Quant Standard 1 (5 x 10^1 V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 2 (5 x 10^2 V617F copies/5 µl)	Red	20 µl
JAK2 MT Quant Standard 3 (5 x 10^3 V617F copies/5 µl))	Red	20 µl
JAK2 MT Quant Standard 4 (5 x 10^4 V617F copies/5 µl)	Red	20 µl
JAK2 WT Quant Standard 1 (5 x 10^1 wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 2 (5 x 10^2 wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 3 (5 x 10^3 wild-type copies/5 µl)	Green	20 µl
JAK2 WT Quant Standard 4 (5 x 10^4 wild-type copies/5 µl)	Green	20 µl
JAK2 MT Reaction Mix*	Red	1010 µl
JAK2 WT Reaction Mix†	Green	1010 µl
Taq DNA polymerase (HotStarTaq® 5 units/ µl)	Mint	85 µl
TE buffer for sample dilution	White	1.9 ml
Water for no template control (NTC)	White	1.9 ml

* 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.

All reaction mixes contain an internal control assay labeled with HEX™. This controls for the presence of inhibitors that may lead to false results.

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.

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

Additional equipment and materials for manual DNA extraction

- QIAamp® DSP DNA Blood Mini Kit (cat. no. 61104) or QIAamp DNA Blood Mini Kit (cat. no. 51104)
- Ethanol (96–100%)
  Note: Do not use denatured alcohol as this contains other substances such as methanol or methylethylketone.

Additional equipment and materials for automated DNA extraction on the QIAsymphony SP

- QIAsymphony SP instrument* (cat. no. 9001297), software version 4.0 or higher, and provided accessories
- Tube Insert 3B (cat. no. 9242083)
- QIAsymphony® DSP DNA Mini Kit (cat. no. 937236)
- Sample Prep Cartridges, 8-well (cat. no. 997002)
- 8-Rod Covers (cat. no. 997004)

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

- Filter-Tips, 1500 µl (cat. no. 997024)
- Filter-Tips, 200 µl (cat. no. 990332)
- Elution Microtubes CL (cat. no. 19588)
- Tip disposal bags (cat. no. 9013395)
- Micro tubes 2.0 ml Type H (Sarstedt®, cat. no. 72.694)

Additional equipment and materials for PCR on Rotor-Gene Q

- Real-time PCR instrument*: Rotor-Gene Q 5plex HRM and provided accessories
- Adaptor Locking Ring 72-Well Rotor (cat. no. 9018901)
- Rotor-Gene Q Software version 2.1.0 or higher
- Nuclease-free, aerosol-resistant, sterile PCR pipet tips with hydrophobic filters
- 1.5 ml or 2.0 ml nuclease-free PCR tubes
- Strip Tubes and Caps, 0.1 ml, for the Rotor-Gene Q (cat. no. 981103 or 981106)
- Ice

Warnings and Precautions

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

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

General precautions

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

This kit is intended for research use only. Not for use in diagnostic procedures. Reagents and instructions supplied in this kit have been tested for optimal performance.

- 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 the JAK2 RGQ PCR Kit are diluted optimally. Do not dilute reagents further as this may result in a loss of performance.
- Do not use reaction volumes (reaction mix plus sample) of less than 25 µl.
- All reagents supplied in the 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 JAK2 RGQ PCR Kit, even from the same batch, as this may affect performance.
- Refer to the Rotor-Gene Q 5plex HRM instrument user manual for additional warnings, precautions, and procedures.
- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Do not use expired or incorrectly stored components.
- Reaction mixes may be altered if exposed to light.
- Use extreme caution to prevent contamination of the mixes with the synthetic materials that are contained in the JAK2 MT and JAK2 WT Quant Standard reagents, and with the JAK2 Mutant and JAK2 WT Control reagents.
- Use extreme caution to prevent DNA or PCR product carryover contamination resulting in a false positive signal.
- Use extreme caution to prevent contamination by DNase, which might cause degradation of the template DNA.
- Use individual, dedicated pipets for setting up reaction mixes and adding templates.
- Do not open Rotor-Gene Q 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.

We therefore recommend the following:

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Prepare pre-PCR master mix with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrices (DNA, plasmid or PCR products) are introduced. Add template in a separate zone, preferably in a separate room, with specific material (pipets, tips, etc.).

For safety information relating to the extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104), QIAamp DNA Blood Mini Kit (cat. no. 51104), and QIAsymphony DSP DNA Mini Kit (cat. no. 937236), please refer to the respective handbooks.

Reagent Storage and Handling

Shipping conditions

The JAK2 RGQ PCR Kit is shipped on dry ice. If any component of the JAK2 RGQ PCR Kit (apart from the enzyme) is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain the reagents, please contact QIAGEN Technical Services or visit **www.qiagen.com**.

Storage conditions

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

For storage information relating to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104), QIAamp DNA Blood Mini Kit (cat. no. 51104), or QIAsymphony DSP DNA Mini Kit (cat. no. 937236), please refer to the respective handbooks.

Stability

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

Once opened, reagents can be stored in their original packaging at –30°C to –15°C until the stated expiration date shown on the box label. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 3 freeze–thaw cycles.

For stability information relating to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104), QIAamp DNA Blood Mini Kit (cat. no. 51104), and QIAsymphony DSP DNA Mini Kit (cat. no. 937236), please refer to respective handbooks.

- Gently mix (by inverting the tube 10 times) and centrifuge all tubes except the enzyme before opening.
- Under correct storage conditions, the product will maintain performance until the expiration date stated on the kit packaging 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 JAK2 RGQ PCR Kit is for use with genomic DNA samples extracted from whole blood samples anti-coagulated with potassium EDTA either stored:

- At 2°C to 8°C for no more than 96 hours
- At 15°C to 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

Genomic DNA may be stored at 2–8°C for 1 week post-extraction or at –25°C to –15°C if long term storage is required.

Procedure

Genomic DNA extraction and preparation from whole blood

Genomic DNA should be extracted using either the QIAamp DSP DNA Blood Mini Kit (cat. no. 61104), QIAamp DNA Blood Mini Kit (cat. no. 51104), or QIAsymphony DSP DNA Mini Kit (cat. no. 937236).

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

Manual gDNA extraction using the QIAamp DSP DNA Blood Mini Kit or QIAamp DNA Blood Mini Kit

Manual gDNA extraction should be performed with the QIAamp DSP DNA Blood Mini Kit (cat. no. 51104) or QIAamp DNA Blood Mini Kit (cat. no. 61104) according to the corresponding *QIAamp DSP DNA Blood Mini Kit Handbook* or *QIAamp DNA Blood Mini Kit Handbook*.

Things to do before starting

- **Equilibrate blood samples to room temperature (15–25°C), and make sure that they are well homogenized.**
- **Prepare the Lysis Buffer**

  If a precipitate has formed in Lysis Buffer (AL), dissolve by incubating at 56°C.
- **Preparing QIAGEN Protease**

  Add 1.2 ml Protease Solvent (PS) to the vial of lyophilized QIAGEN Protease (QP) and mix carefully. To avoid foaming, mix by inverting the vial several times. Make sure that the QIAGEN Protease (QP) is completely dissolved.

  Note: Once dissolved, the QIAGEN Protease (QP) is stable for up to 2 months when stored at 2–8°C. To prolong the life of QIAGEN Protease (QP), storage at –20°C is recommended, but repeated freezing and thawing should be avoided. For this reason, storage of aliquots of QIAGEN Protease (QP) is recommended.
- **Preparing Wash Buffer 1**

  Using a measuring cylinder, add 25 ml ethanol (96–100%) to the bottle containing 19 ml Wash Buffer 1 (AW1) concentrate. Store the reconstituted Wash Buffer 1 (AW1) at room temperature (15–25°C).

Note: Always mix the reconstituted Wash Buffer 1 (AW1) by inverting the bottle several times before starting the procedure.

- **Preparing Wash Buffer 2**

  Using a measuring cylinder, add 30 ml ethanol (96–100%) to the bottle containing 13 ml Wash Buffer 2 (AW2) concentrate. Store the reconstituted Wash Buffer 2 (AW2) at room temperature (15–25°C).

  Note: Always mix the reconstituted Wash Buffer 2 (AW2) by inverting the bottle several times before starting the procedure.

- **Preparing the Elution Buffer**

  One bottle of Elution Buffer (AE) is provided with the kit. To prevent contamination of Elution Buffer (AE), we strongly recommend using pipet tips with aerosol barriers when pipetting Elution Buffer (AE) from the bottle and replacing the cap of the bottle immediately afterwards.

  Equilibrate Elution Buffer (AE) to room temperature (15–25°C).

- **Set a heating block to 56°C for use in step 4.**

Procedure

1. **Pipet 20 µl of QIAGEN Protease (QP) into a lysis tube (LT).**
   Note: Check the expiration date of the reconstituted protease before use.
2. **Add 200 µl of blood sample to the lysis tube (LT).**
3. **Add 200 µl of Lysis Buffer (AL) to the lysis tube (LT), close the lid, and mix using a pulse-vortex for 15 seconds.**
   Note: To ensure efficient lysis, it is essential that the sample and Lysis Buffer (AL) are mixed thoroughly to yield a homogenous solution.

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

   Important: Do not add QIAGEN Protease (QP) directly to Lysis Buffer (AL).
4. **Incubate at 56°C (±1°C) for 10 min (±1 min).**
5. **Centrifuge the lysis tube (LT) for about 5 s at full speed to remove drops from the inside of the lid.**
6. **Add 200 µl ethanol (96–100%) to the lysis tube (LT), close the lid, and mix thoroughly using a pulse-vortex for ≥15 s.**
7. **Centrifuge the lysis tube (LT) for ≥5 seconds at full speed to remove any drops of liquid from the inside of the lid.**

8. **Carefully apply the entire lysate from step 7 to the QIAamp Mini spin column without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.**

   Note: If processing several samples, open only one lysis tube (LT) at a time.

9. **Close the lid of the QIAamp Mini spin column, and centrifuge at approximately 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean wash tube (WT), and discard the tube containing the filtrate.**

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

   Note: If the lysate still does not pass through the membrane during centrifugation, discard the sample and repeat the isolation and purification with new sample material.

10. **Carefully open the QIAamp Mini spin column, and add 500 µl Wash Buffer 1 (AW1) without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.**
11. **Close the lid of the QIAamp Mini spin column, and centrifuge at approximately 6000 x *g* (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean wash tube (WT), and discard the tube containing the filtrate.**
12. **Carefully open the QIAamp Mini spin column, and add 500 µl Wash Buffer 2 (AW2) without wetting the rim. Avoid touching the QIAamp Mini spin column membrane with the pipet tip.**
13. **Close the lid of the QIAamp Mini spin column, and centrifuge at full speed (approximately 20,000 x *g*, or 14,000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean wash tube (WT), and discard the tube containing the filtrate.**
14. **Centrifuge at full speed (approximately 20,000 x *g*, or 14,000 rpm) for 3 min to dry the membrane completely.**
15. **Place the QIAamp Mini spin column in a clean elution tube (ET) and discard the wash tube (WT) containing the filtrate. Carefully open the lid of the QIAamp Mini spin column, and apply 50 to 200 µl Elution Buffer (AE) to the center of the membrane. Close the lid and incubate at room temperature (15–25°C) for 1 minute. Centrifuge at approximately 6000 x *g* (8000 rpm) for 1 minute to elute the DNA.**
16. **Discard used sample tubes, plates and waste according to your local safety regulations.**

Automated gDNA extraction using the QIAsymphony DSP DNA Mini Kit

Automated gDNA extraction must be performed with the QIAsymphony SP in combination with the QIAsymphony DSP DNA Mini Kit (cat. no. 937236) and by following the instructions in the corresponding *QIAsymphony DSP DNA Kit Handbook*.

With the QIAsymphony SP, the QIAsymphony DSP DNA Mini Kit (cat. no. 937236) enables automated DNA purification from human whole blood (using the Blood_200_V7_DSP protocol on the QIAsymphony).

- No pre-treatment is required.
- Transfer 300 µl of whole blood into a micro tube (2.0 ml Type H, Sarstedt, cat. no. 72.694).
- The micro tubes are directly transferred into Tube Insert 3B in the QIAsymphony SP.
- Purification of DNA is performed with magnetic particles.

Important points before starting

- **Important:** Whole blood total volume to be extracted is 300 µl.
- Ensure that you are familiar with operating the QIAsymphony SP. Refer to the user manuals supplied with your instrument for operating instructions.
- Optional maintenance is not mandatory for instrument function, but is highly recommended to reduce risk of contamination.
- Before using a reagent cartridge for the first time, check that 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 QIAsymphony SP.

Procedure

1. **Close all drawers and the hood.**
2. **Switch on the QIAsymphony 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 QIAsymphony SP.
3. **Log on to the instrument.**
4. **Select the protocol to be run: choose "Select All" button and select "DNA Blood" → Blood_200_V7_DSP for whole blood samples.**
5. **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.**
6. **Load the required elution rack into the "Eluate" drawer.**

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

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

   When using a 96-well plate, make sure that the plate is in the correct orientation, as incorrect placement may cause sample mix-up in downstream analysis.
7. **Load the required reagent cartridge(s) and consumables into the "Reagents and Consumables" drawer.**

   Note: Ensure that pipetting tips are correctly fixed
8. **Perform an inventory scan of the "Reagents and Consumables" drawer.**
9. **Important: Transfer 300 µl of the whole blood sample to be extracted into a micro tube (2.0 ml Type H) and place this into the 3B 2 ml adapter on the tube sample carrier. Load the sample tubes into the "Sample" drawer.**
10. **Using the touchscreen, enter the required information for each batch of samples to be processed:**

- Sample information: change default tube format (choose "Select All" button and select "Sarstedt reference 72.694" from the "Tube Insert" sheet)
- Confirm the selected program: "Blood_200_V7_DSP".
- **Important:** Elution volume and output position: 100 µl for the whole blood protocol.

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

11. Start the run

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

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

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

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

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

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

Note: In general, magnetic particles are not carried over into eluates. If any eluate 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.

13. **Export the QIAsymphony SP result file: this report is generated for each elution plate.**

    - Insert the USB stick into one of the USB ports at the front of the QIAsymphony SP;

    - Click on the "Tools" button;

    - 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 QIAsymphony SP result file**

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

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

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

17. **Clean the QIAsymphony SP.**

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

18. **Close the instrument drawers and switch off the QIAsymphony SP.**

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

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

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

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

Qualification and quantification of DNA

A blank of ATE or AE 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 ≥1.7 as smaller ratios usually indicate protein contamination or the presence of organic chemicals and affect the PCR step.
- DNA quantity is determined by measuring optical density at 260 nm.

  Total amount of DNA purified = concentration x volume of sample in µl.
- If OD_{260}/OD_{280} ratio is below 1.7, and/or if the gDNA concentration is below 10 ng/µL, the sample must not be further processed.

Genomic DNA sample normalization

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

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

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

The JAK2 RGQ PCR Kit must be run on the Rotor-Gene Q 5plex HRM instrument. Take time to familiarize yourself with the Rotor-Gene Q instrument before starting the protocol. See the user manuals for the instrument for details.

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

Samples	Reactions
With the JAK2 MT Reaction Mix	
8 gDNA samples	8 reactions
JAK2 MT Quant Standards	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	
8 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

Sample processing on Rotor-Gene Q 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.

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

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

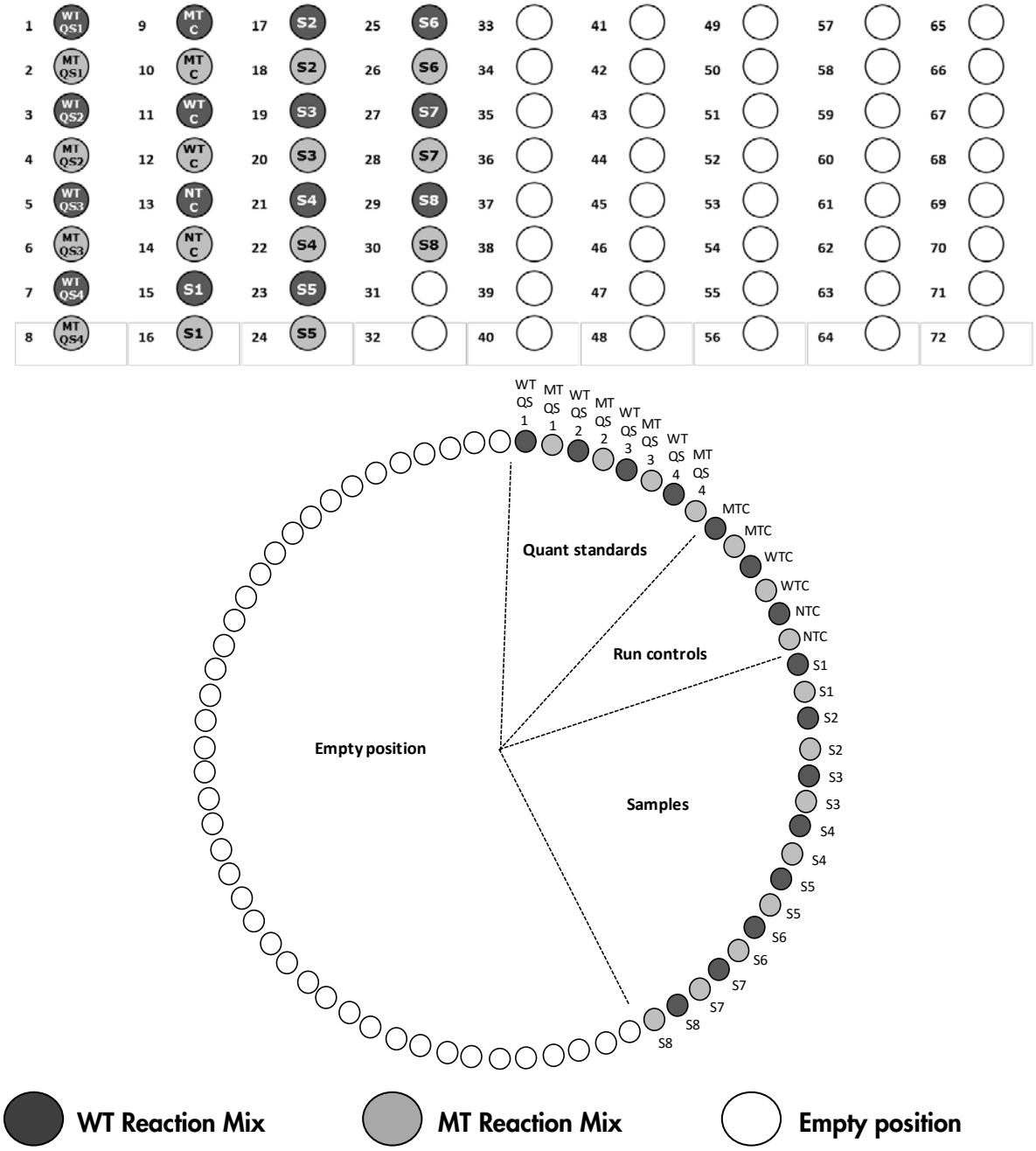

Figure 3. Suggested plate and rotor setup for an experiment with the JAK2 RGQ PCR Kit. WTC: JAK2 WT Control; **MTC:** JAK2 MT Control; **WT-QS:** JAK2 wild-type Quant standards; **MT-QS:** JAK2 MT Quant Standards; **S:** gDNA sample; **NTC:** No template control (water). All of the remaining positions should be filled with empty tubes.

Things to do before starting

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

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

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

Procedure

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

   All concentrations are for the final volume of the reaction.

   Tables 2 and 3 describe the pipetting scheme for the preparation of one Mutant (MT) and one Wild Type (WT) reagent mixes, calculated to achieve final reaction volumes of 25 µl. Extra volumes are included to compensate for pipetting error and allow for 8 samples plus controls.

Table 2. 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
Taq 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 to compensate pipetting error.

Table 3. 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
Taq 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 to compensate pipetting error.

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

  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 non-specific template or reaction mix contamination, and therefore false-positive results.

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

2. Prepare Rotor-Gene Q 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 3, page 22, with empty capped strip tubes placed into all unused positions.

  Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions as shown in Figure 3, page 22.

- Attach the locking ring.
- Load the Rotor-Gene Q instrument with the rotor and locking ring, and close the instrument lid.

- Program the Rotor-Gene Q instrument with the thermal cycling program as indicated in Table 4.

Table 4. Temperature profile

Mode of analysis	Quantitation
Hold	Temperature: 95°C Time: 10 min
Cycling	45 times 95°C for 15 s 60°C for 1 min With acquisition of FAM™ fluorescence in channel Green: single and acquisition of HEX fluorescence in channel Yellow: single

3. **Click "Gain Optimisation" in the "New Run Wizard" dialog box to open the "Auto-Gain Optimisation Setup" dialog. Set parameters for FAM and HEX channel according to Table 5.**

Table 5. Auto-Gain Optimization settings

Optimize before first acquisition	Yes	
Optimize at beginning of run	No	
Temperature	60°C for 1 min	
Channel	Green	Yellow
Tube position	1	1
Target sample minimum	5	5
Target sample maximum	10	10
Gain minimum	–10	–10
Gain maximum	10	10

4. **Start the thermal cycling program.**
5. **Create WT and MT subsets with FAM channel.**
6. **Create an IC subset with HEX channel.**
7. **Fill the "Edit samples" window.**
8. **Once the thermal cycling has ended, carry out the following (see also Table 6, page 27):**

Target analysis: WT + MT analysis: FAM fluorescence

- Select "Analysis" and double click on "Cycling A. Green"
- Select "Dynamic Tube", "Slope Correct"
- Check that "Outlier Removal" is set to 10% (corresponding to the NTC threshold)
- Define the threshold at 0.03
- Set the graph to "Linear Scale"

IC analysis: HEX fluorescence

- Select "Analysis" and "Cycling A. Yellow"
- Select "Dynamic Tube"
- Check that "Slope Correct" is not selected
- Check that "Outlier Removal" is set to 0% (corresponding to the NTC threshold)
- Define the threshold at 0.05
- Set the graph to "Linear Scale"

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

Table 6. Analysis parameters

Channel	Green	Yellow
Threshold	0.03	0.05
Left threshold	1	1
Dynamic tube	Yes	Yes
Ignore first xx cycles	0	0
Slope correction	Yes	No
NTC percentage (threshold)	0.10	0.00
Minimum reaction efficiency (threshold)	–1	–1
Algorithm (digital filter)	1 (light)	1 (light)

Interpretation of Results

Data analysis principle

Data for the threshold cycle (C_T) values can be exported from the qPCR instrument and pasted into an Excel® file for analysis. These C_T values can then be plotted to obtain a standard curve for both the WT and MT standards using the following equation and Table 7, page 28.

$y = C_T$; $x = \log_{10} CN$

where CN= gene copy number in the 5 µl sample

Table 7. Quantitative data for the WT and MT Quant Standards

Standard	Copy number (CN)	$\log_{10}$ CN
JAK2 MT Quant Standard 1	5 x 10^1 V617F	1.7
JAK2 MT Quant Standard 2	5 x 10^2 V617F	2.7
JAK2 MT Quant Standard 3	5 x 10^3 V617F	3.7
JAK2 MT Quant Standard 4	5 x 10^4 V617F	4.7
JAK2 WT Quant Standard 1	5 x 10^1 WT	1.7
JAK2 WT Quant Standard 2	5 x 10^2 WT	2.7
JAK2 WT Quant Standard 3	5 x 10^3 WT	3.7
JAK2 WT Quant Standard 4	5 x 10^4 WT	4.7

Standard curve and quality criteria

Figures 4 and 6 (pages 29 and 30) show examples of results obtained with JAK2 RGQ PCR Kit and Figures 5 and 7 (pages 29 and 30) provide examples of the theoretical curve calculated using the 4 standard dilutions of JAK2 MT Quant and JAK2 WT Quant.

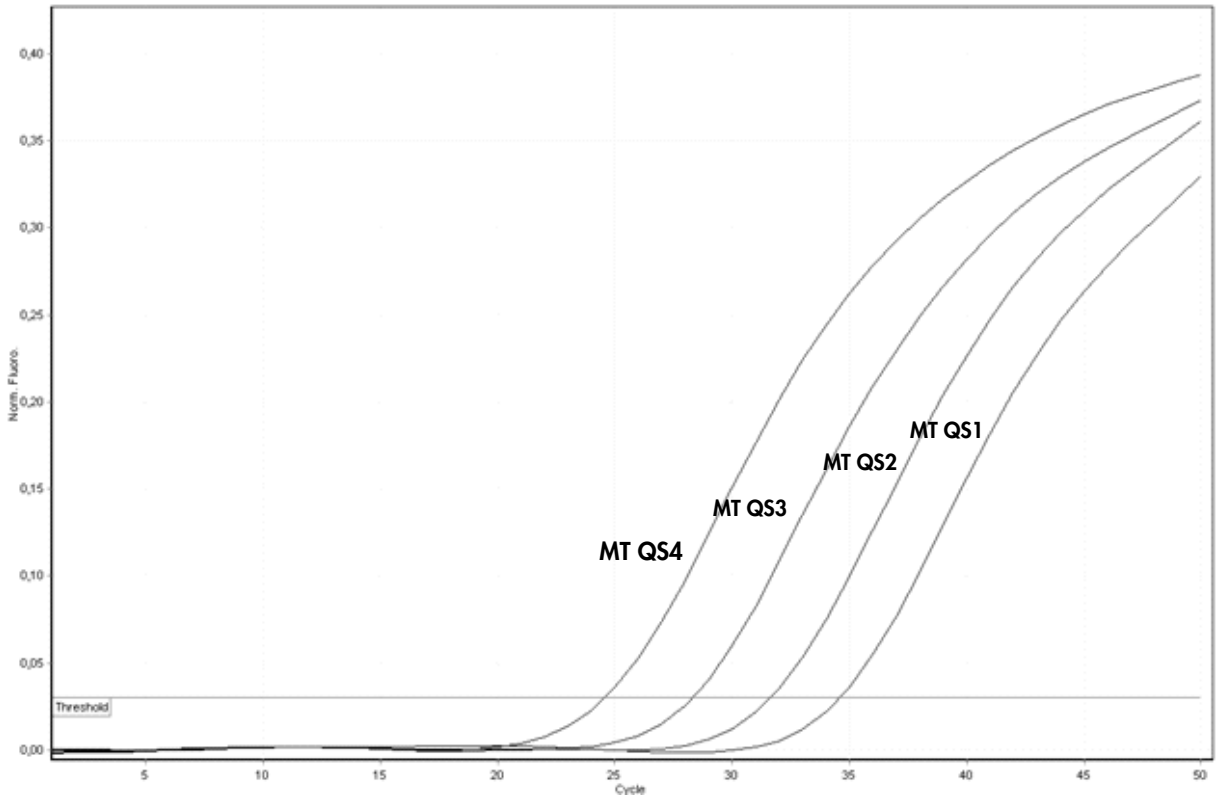

Figure 4. Amplification plot of 5 x 10^1, 5 x 10^2, 5 x 10^3, and 5 x 10^4 copies of the JAK2 V617F plasmid (controls MT QS1, MT QS2, MT QS3 and MT QS4, respectively).

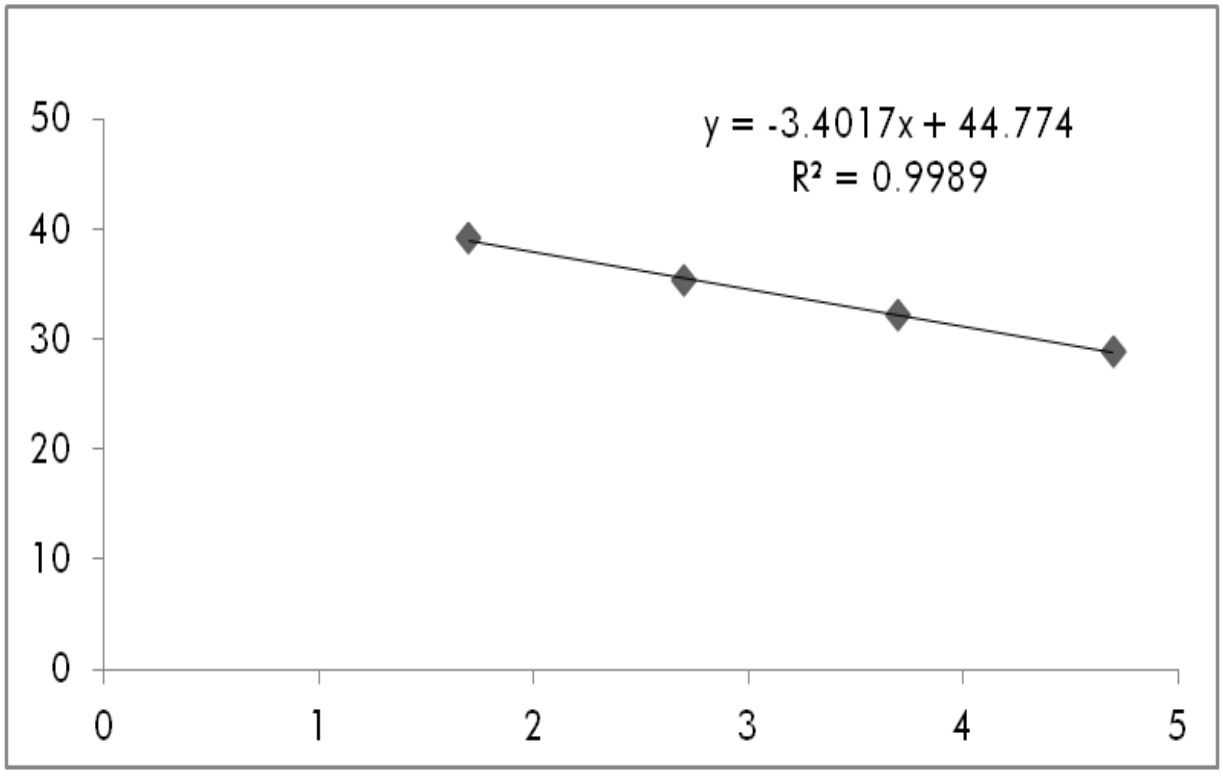

Figure 5. Standard curve for JAK2 V617F plasmid.

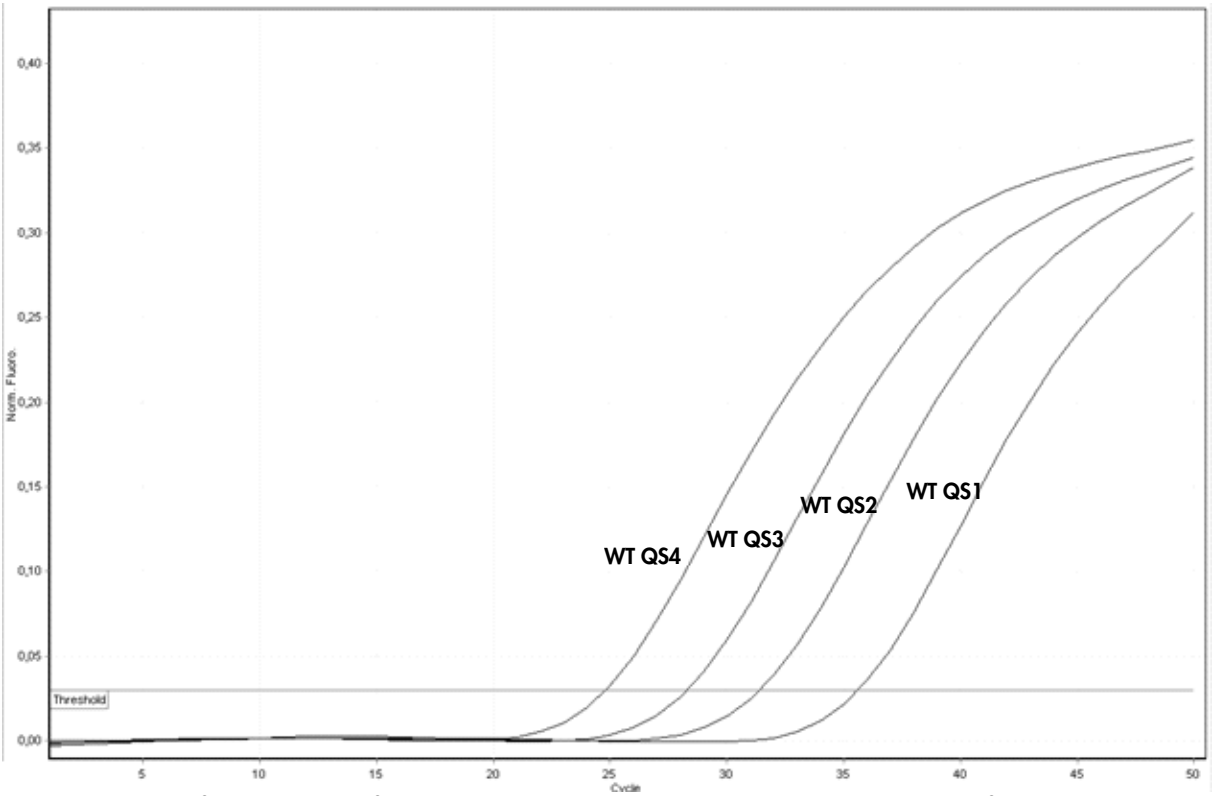

Figure 6. Amplification plot of 5 x 10^1, 5 x 10^2, 5 x 10^3, and 5 x 10^4 copies of the JAK2 wild-type plasmid (controls WT QS1, WT QS2, WT QS3 and WT QS4, respectively).

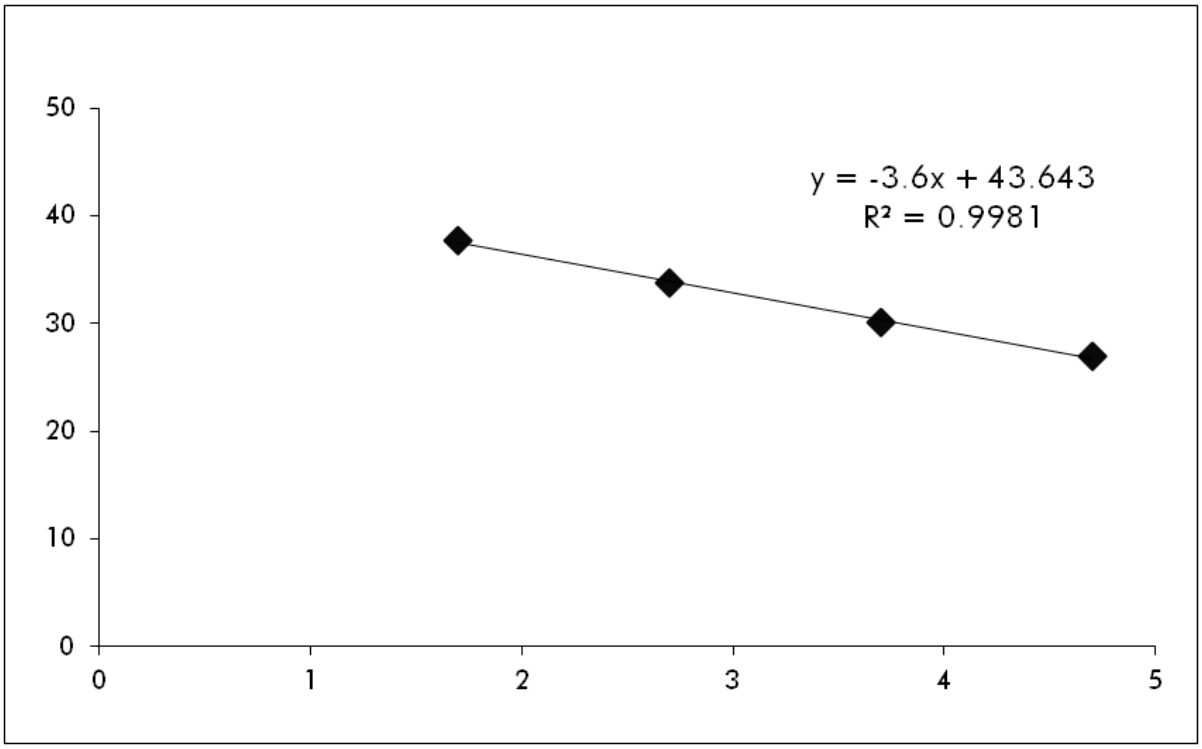

Figure 7. Standard curve for JAK2 wild-type plasmid.

The standard curves can be used to calculate the mutant and wild-type $\log_{10}$ copy numbers in the unknown samples.

The mutant standard curve equation should be used to transform raw C_T values (obtained with MT Reaction Mix) for the unknown and control samples, into JAK2 mutant copy numbers (CN_{Mutant}) by using the following formula.

$$\log_{10} CN_{Mutant} = \frac{(C_{TMutant} - \text{standard curve intercept}_{Mutant})}{\text{standard curve slope}_{Mutant}}$$

The wild-type standard curve equation should be used to transform raw C_T values (obtained with WT Reaction Mix) for the unknown and control samples, into JAK2 wild-type copy numbers (CN_{WT}) by using the following formula.

$$\log_{10} CN_{WT} = \frac{(C_{TWT} - \text{standard curve intercept}_{WT})}{\text{standard curve slope}_{WT}}$$

Results are relative to 50 ng of total gDNA and should be expressed as the percentage of JAK2 V617F (see page 33).

Acceptance criteria

At the end of the run, the run release should be checked.

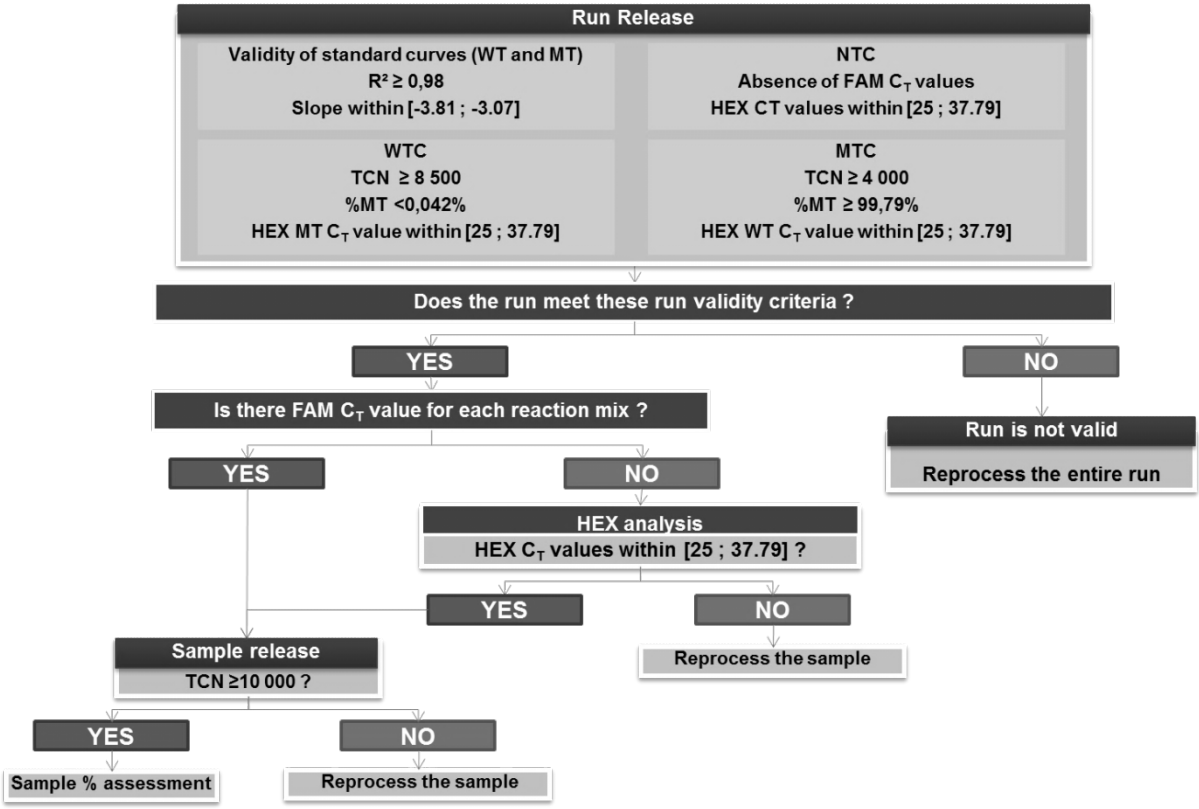

Figure 8: Acceptance criteria workflow

Validity of standards curves (JAK2 WT and MT Quant Standards)

As the JAK2 MT Quant and JAK2 WT Quant Standards are 10-fold dilutions, the theoretical slope of the curve is –3.32. A slope between –3.07 and –3.81 is acceptable as long as R^2 is ≥0.98.

Positive and negative controls

The JAK2 MT Control should give a JAK2 V617F percentage that is equal or higher than 99.79%.

The total copy number of this MT Control should be equal or higher than 4000.

The JAK2 WT Control should give a JAK2 V617F percentage that is lower than 0.042%.

The total copy number of this WT Control should be equal or higher than 8500.

Regarding the IC specifications (Internal Control), HEX MT C_T values for the WT Control should be included [25; 37.79].

HEX WT C_T values for the MT Control should be included [25; 37.79].

If these controls fail to function correctly, please see the “Troubleshooting Guide” on page 34 to find a solution.

Water controls

Negative controls should give zero copy number for both JAK2 V617F and JAK2 wild-type detection.

HEX C_T values should also be observed within the range [25; 37.79] in both reaction mixes.

A positive water control results from a cross-contamination; please see the "Troubleshooting Guide" on page 34 to find a solution.

Samples

If all run release criteria are valid, sample release criteria should be checked. At least one C_T value (FAM and/or HEX) must be observed in each assay (WT and MT) to release a sample.

If no target amplification occurs in an assay, the corresponding internal control must be within the defined range for C_T [25; 37.79] to ensure the reaction took place under good conditions.

If all these previous specifications are met, the total copy number and the percentage of JAK2 mutations can be determined by using the following formula.

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

The sample total copy number should be equal or higher than 10,000 copies to consider the results as valid.

If amplification is observed in the FAM channel but not in the HEX channel, please see the "Troubleshooting Guide" on page 34 to find a solution.

Expression of the results

According to the percentage of JAK2 mutations observed, determine the genotype of the unknown samples using the data contained in Table 8 below.

Table 8: Interpretation of genotyping results

Standard	Interpretation
JAK2 V617F (%) ≥0.042	The JAK2 V617F mutation is detected
JAK2 V617F (%) <0.042	The JAK2 V617F mutation is not detected

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol in this handbook or sample and assay technologies (for contact information, see "Contact Information", page 39).

For troubleshooting information relating to extraction kits QIAamp DSP DNA Blood Mini Kit (cat. no. 61104), QIAamp DNA Blood Mini Kit (cat. no. 51104), or QIAsymphony DNA DSP Mini Kit (cat. no. 937236), please refer to the respective handbooks.

	Comments and suggestions
Automated extraction on QIAsymphony	
Sample flagged as "unclear"	This can be due to a pause during the extraction run. If the extraction run was completed, proceed to the OD ratio and concentration measurement step. If not, repeat the extraction run.
Sample flagged as "unprocessed"	This refers to an initial sample volume error. Verify the blood volume by pipetting. If the volume is too low, increase so the sample is 300 µl and restart the run.
Sample flagged as "invalid"	An error occurred during the extraction run. Repeat the extraction step for this sample.

	Comments and suggestions
Cooling block temperature error	An error message at the end of the run about the cooling temperature means that samples stayed at room temperature from the end of the extraction run. If the samples stayed at room temperature for <12 hours, gDNA quality should not be altered and gDNA can be quantified. If this was >12 hours, gDNA samples may have been degraded. If this is the case, repeat the extraction.
Elution plate removal error	At the end of the run, an error message may appear if the elution plate was removed without checking the relevant operation on screen. This can be rectified by clicking on the relevant box.

General handling for JAK2 mutation status assessment using the 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 <1.7, perform a new DNA extraction.
b) Check the DNA concentration	The 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 pipets before repeating the qPCR step.

	Comments and suggestions
Run control fails on a JAK2 MT Quant and JAK2 WT Quant standard	
a) Vial inversion b) Inversion during distribution c) Cross contamination d) Standard partial degradation e) PCR reagents partially degraded f) Non-specific amplification	Check the pipetting scheme and the set-up of the reaction. Store kit contents at –30 to –15°C and keep the reaction mixes protected from light. Avoid repeated freezing and thawing.
No or low signal for one standard	
a) Distribution issue b) Use of the same reaction mix for WT and MT QS	Check the pipetting scheme and the set-up of the reaction. Repeat the PCR run.
No Template Control (NTC) of H_2O is positive	
a) Cross contamination b) Reagent contamination c) Strip tube inversion d) Probe degradation	Replace all critical reagents. Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carry-over contamination. Keep reaction mixes protected from light. Check for false positives on the fluorescence curves.
No signal, even in standard controls	
Pipetting error or omitted reagents	Check pipetting scheme and the set-up of the reaction. Repeat the PCR run.

	Comments and suggestions
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.
WT Control is positive, but MT Control is not positive enough	
Carry-over contamination	Replace all critical reagents. Repeat the experiment with new aliquots of all reagents. Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carry-over contamination. Make sure tips are changed in between pipetting different reagents.
WT Control or MT Control signal using reciprocal reaction mixes	
a) Cross contamination b) Reagent contamination c) Tube inversion	Replace all critical reagents. Repeat the experiment with new aliquots of all reagents. Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carry-over contamination. Check pipetting scheme and the setup of the reaction.
Inverted detection of the positive control	
a) Cross contamination b) Distribution inversion of the reaction mix in the tube or premix	Check pipetting scheme and the setup of the reaction.

	Comments and suggestions
No signal for a sample or control, even for the internal control	
a) Reaction mix not added b) Reaction mix degraded	Check pipetting scheme and the set-up of the reaction. If the internal control is not amplified, the reaction mix was not added or is degraded. Repeat the qPCR step with a new reaction mix.
Amplification of the sample (FAM amplification), but not of the internal control (HEX amplification)	
	The FAM channel analysis (corresponding to the target test) has priority over the HEX channel analysis (corresponding to the internal control test). The presence of a FAM C_T is first checked in each reaction mix, if a FAM C_T value is detected, the HEX C_T will not be checked. If a FAM C_T value cannot be detected, the HEX C_T will be checked.

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

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of JAK2 RGQ PCR Kit is tested against predetermined specifications to ensure consistent product quality.

References

1. Jovanovich, J. et al. (2013) Establishing optimal quantitative-polymerase chain reaction assays for routine diagnosis and tracking of minimal residual disease in JAK2V617F associated myeloproliferative neoplasms: A joint European LeukemiaNet/MPN&MPNr-EuroNet (COST action BM0902) study. Leukemia **27**, 2032.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Description
	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Protect from light
	Consult instructions for use
	Caution

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Ordering Information

Product	Contents	Cat. no.
JAK2 RGQ PCR Kit (24)	For 24 reactions: Wild-type JAK2 Control, JAK2 V617F Control, JAK2 WT Quant Standards, JAK2 MT Quant Standards, JAK2 WT Reaction Mix, JAK2 MT Reaction Mix, *Taq* DNA polymerase, TE buffer for dilution, water for NTC	673613
Rotor-Gene Q		
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt (HRM) analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9001580
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and HRM analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9002032
Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and HRM analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9001650
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cycler and HRM analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9002033

Product	Contents	Cat. no.
Loading Block 72 x 0.1ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
QIAamp DNA Blood Mini Kit	For 50 preps: QIAamp Mini Spin Columns, QIAGEN Protease, Reagents, Buffers, Collection Tubes (2 ml)	51104
QIAamp DNA DSP Blood Mini Kit	For 50 preps: QIAamp Mini Spin Columns, Buffers, Reagents, Tubes, VacConnectors	61104
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 QIAsymphony SP/AS instruments	997024
Elution Microtubes CL (24 x 96)	Nonsterile polypropylene tubes (0.85 ml maximum capacity, less than 0.7 ml storage capacity, 0.4 ml elution capacity); 2304 in racks of 96; includes cap strips	19588
RNase A (17,500 U)	2.5 ml (100 mg/ml; 7000 units/ml, solution)	19101
Buffer ATL (200 ml)	200 ml Tissue Lysis Buffer for 1000 preparations	19076

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