

# *ipsogen*<sup>®</sup> JAK2 MutaScreen EZ Kit Handbook



Version 1

**IVD**

Quantitative in vitro diagnostics

For use with Rotor-Gene<sup>®</sup> Q, Applied Biosystems<sup>®</sup>, ABI PRISM<sup>®</sup>,  
and LightCycler<sup>®</sup> instruments



**REF** 673223



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# Contents

<b>Intended Use</b>	<b>4</b>
<b>Summary and Explanation</b>	<b>4</b>
<b>Principle of the Procedure</b>	<b>6</b>
<b>Materials Provided</b>	<b>7</b>
Kit contents	7
<b>Materials Required but Not Provided</b>	<b>8</b>
<b>Warnings and Precautions</b>	<b>9</b>
General precautions	9
<b>Reagent Storage and Handling</b>	<b>9</b>
<b>Procedure</b>	<b>11</b>
Sample DNA preparation	11
Storing nucleic acids	11
Protocols	
■ qPCR on Rotor-Gene Q MDx 5plex HRM or Rotor-Gene Q 5plex HRM instruments with 72-tube rotor	11
■ qPCR on Applied Biosystems 7500 or ABI PRISM 7900HT instruments	21
■ qPCR on the LightCycler 480 instrument	30
<b>Interpretation of Results</b>	<b>38</b>
Graphical representation and quality control criteria	38
Calculation of normalized FAM/VIC ratio and genotyping	39
Troubleshooting guide	40
<b>Quality Control</b>	<b>41</b>
<b>Limitations</b>	<b>42</b>
<b>Performance Characteristics</b>	<b>42</b>
Nonclinical studies	42
Clinical studies	44
<b>References</b>	<b>46</b>
<b>Symbols</b>	<b>47</b>
<b>Contact Information</b>	<b>47</b>
<b>Ordering Information</b>	<b>48</b>

## Intended Use

The *ipsogen* JAK2 MutaScreen EZ Kit is intended for the detection of the JAK2 V617F/G1849T mutation in genomic DNA from subjects with suspected myeloproliferative neoplasm. The absence of JAK2 V617F/G1849T does not exclude the presence of other JAK2 mutations. The test can report false negative results in case of additional mutations located in codons 615 to 619 (1).

**Note:** The kit should be used following the instructions given in this manual, in combination with validated reagents and instruments. Any off-label use of this product and/or modification of the components will void QIAGEN's liability.

## Summary and Explanation

A recurrent somatic mutation, V617F, affecting the Janus tyrosine kinase 2 (JAK2) gene, has been identified in 2005 (2–5), leading to a major breakthrough in the understanding, classification, and diagnosis of myeloproliferative neoplasms (MPN). JAK2 is a critical intracellular signaling molecule for a number of cytokines, including erythropoietin.

The JAK2 V617F mutation is detected in >95% of patients with polycythemia vera (PV), 50–60% of patients with essential thrombocythemia (ET), and in 50% of patients with primary myelofibrosis (PMF). JAK2 V617F has been also detected in some rare cases of chronic myelomonocytic leukemia, myelodysplastic syndrome, systemic mastocytosis, and chronic neutrophilic leukemia, but in 0% of CML (6).

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

**Table 1. WHO criteria for the diagnosis of MPN (adapted from reference 8)**

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

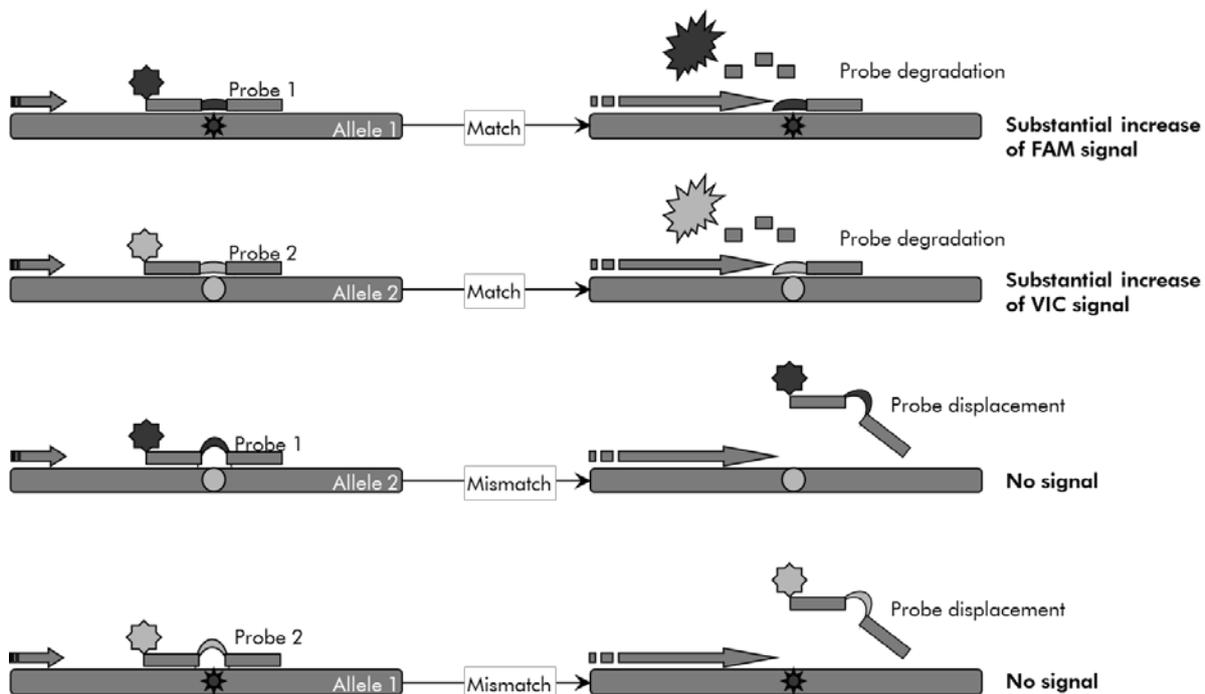
Recently, international experts have proposed criteria for therapeutic trials in PV and ET. Based on data on allograft, alpha-interferon, or hydroxyurea, *JAK2V617F* quantification has been incorporated as a potentially useful tool to monitor treatment response (9). A decrease in *JAK2 V617F* burden has been observed in response to some of the new anti-*JAK2* targeted drugs in clinical development (10).

## Principle of the Procedure

In an allelic discrimination assay, two TaqMan<sup>®</sup> probes are used in a multiplexed assay. One is a perfect match to the allele 2 sequence (e.g., the wild-type allele), and the other one is a perfect match to the allele 1 sequence (e.g., the allele with a mutation). Each probe is labeled with a distinctive fluorescent dye at its 5' end, the reporter, such as FAM<sup>™</sup> or VIC<sup>®</sup>, and contains a non-fluorescent quencher at the 3' end. The probes also contain a minor groove binder (MGB<sup>™</sup>) permitting the use of shorter probes with greater stability and thereby a more accurate allelic discrimination.

During the extension phase of the PCR, the perfectly matched probe is cleaved by the 5'→3' exonuclease activity of *Taq* DNA polymerase, separating the reporter dye from the quencher and thus releasing detectable fluorescence. The probe not perfectly matched will be displaced rather than cleaved by the *Taq* DNA polymerase and no reporter dye is released. The fluorescence signal (VIC or FAM) generated is collected at the end of the PCR (end-point) and immediately indicates the presence of the targeted sequence(s) in the sample (wild-type allele, mutated allele or both) without the requirement of long and laborious post-PCR steps, which also increase the contamination risk. The actual quantity of target sequence is not determined.

The *ipsogen* JAK2 MutaScreen EZ Kit uses this technology as illustrated (see Figure 1).



**Figure 1. TaqMan probe multiplex assay.** The *ipsogen* JAK2 MutaScreen EZ Kit uses this technology for allelic discrimination.

# Materials Provided

## Kit contents

<b>ipsogen JAK2 MutaScreen EZ Kit</b>			<b>(24)</b>
<b>Catalog no.</b>			<b>673223</b>
<b>Number of reactions</b>			<b>24</b>
<b>Box 1: Controls</b>			
V617F Positive Control	PC-VF JAK2		30 $\mu$ l
V617F Negative Control	NC-VF JAK2		30 $\mu$ l
Cut-Off Sample	COS-VF JAK2		30 $\mu$ l
<b>Box 2: qPCR</b>			
Primers and probes mix JAK2 V617F*	PPM-JAK2 10x		145 $\mu$ l
Master Mix for qPCR, 2x	qPCR master mix		720 $\mu$ l
Nuclease-Free Water	H <sub>2</sub> O		1000 $\mu$ l
<b>Service pack</b>			
ipsogen <i>JAK2 MutaScreen EZ Kit Handbook</i> (English)			1

\* Mix of specific reverse and forward primers for the *JAK2* gene, specific V617F FAM probe and wild type VIC probe.

**Note:** Briefly centrifuge tubes before use.

**Note:** Analyzing unknown samples with the *ipsogen JAK2 MutaScreen EZ Kit* requires the extraction of genomic DNA. Reagents needed to perform DNA extraction (e.g., QIAGEN® QIAamp® DNA Blood Maxi Kit, cat. nos. 51192 and 51194) are not provided and must be validated in combination with the kit.

## Materials Required but Not Provided

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

### Reagents

- Nuclease-free 1x TE buffer, pH 8.0
- Reagents for 0.8–1% agarose gel in 0.5x TBE electrophoresis buffer

### Consumables

- Nuclease-free aerosol-resistant sterile PCR pipet tips with hydrophobic filters
- 0.5 ml or 0.2 ml RNase- and DNase-free PCR tubes
- Ice

### Equipment

- Microliter pipets\* dedicated for PCR (1–10  $\mu$ l; 10–100  $\mu$ l; 100–1000  $\mu$ l)
- Benchtop centrifuge\* with rotor for 0.2 ml/0.5 ml reaction tubes (capable of attaining 10,000 rpm)
- Spectrophotometer\* for DNA quantitation
- Real-time PCR instrument:\* Rotor-Gene Q 5plex HRM or other Rotor-Gene instrument; LightCycler 480; Applied Biosystems 7500 Real-Time PCR System or ABI PRISM 7900HT SDS; and associated specific material

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

## Warnings and Precautions

For in vitro diagnostic use

When working with chemicals, always wear a suitable 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](http://www.qiagen.com/safety) where you can find, view, and print the SDS for each QIAGEN kit and kit component.

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

### General precautions

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

This kit is intended for in vitro diagnostic use. Reagents and instructions supplied in this kit have been validated for optimal performance. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data. PPM-JAK2 reagent may be altered if exposed to light. All reagents are formulated specifically for use with this test. For optimal performance of the test, no substitutions should be made.

Use extreme caution to prevent:

- DNase contamination which might cause degradation of the template DNA
- DNA or PCR carryover contamination resulting in false positive signal

We therefore recommend the following.

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

### Reagent Storage and Handling

The kits are shipped on dry ice and must be stored at  $-30^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  upon receipt.

- Minimize exposure to light of the primers and probe mixes (PPM-JAK2 tube).

- Gently mix and centrifuge the tubes before opening.
- Store all kit components in original containers.

These storage conditions apply to both opened and unopened components. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

Expiration dates for each reagent are indicated on the individual component labels. Under correct storage conditions, the product will maintain performance until the expiration date printed on the label.

There are no obvious signs to indicate instability of this product. However, positive and negative controls should be run simultaneously with unknown specimens.

## Procedure

### Sample DNA preparation

Genomic DNA should be obtained either from whole blood, purified peripheral blood lymphocytes, polynuclear cells, or granulocytes. To be able to compare results, we recommend adopting the same cellular fraction and DNA extraction method. DNA extraction should be performed by any home brew or commercial method (e.g., QIAGEN QIAamp DNA Blood Maxi Kit, cat. nos. 51192 and 51194).

DNA quantity is determined by measuring optical density at 260 nm. DNA quality should be assessed by spectrophotometry or gel electrophoresis.

The  $A_{260}/A_{280}$  ratio should be 1.7–1.9. Smaller ratios usually indicate contamination by protein or organic chemicals. Electrophoretic analysis on a 0.8–1% agarose gel should allow visualization of the isolated DNA as a distinct band of about 20 kb. A slight smear is acceptable.

The resultant DNA is diluted to 5 ng/ $\mu$ l in TE buffer. The qPCR reaction is optimized for 25 ng of purified genomic DNA.

### Storing nucleic acids

For short-term storage of up to 24 hours, we recommend storing purified nucleic acids at 2–8°C. For long-term storage of over 24 hours, we recommend storage at –20°C.

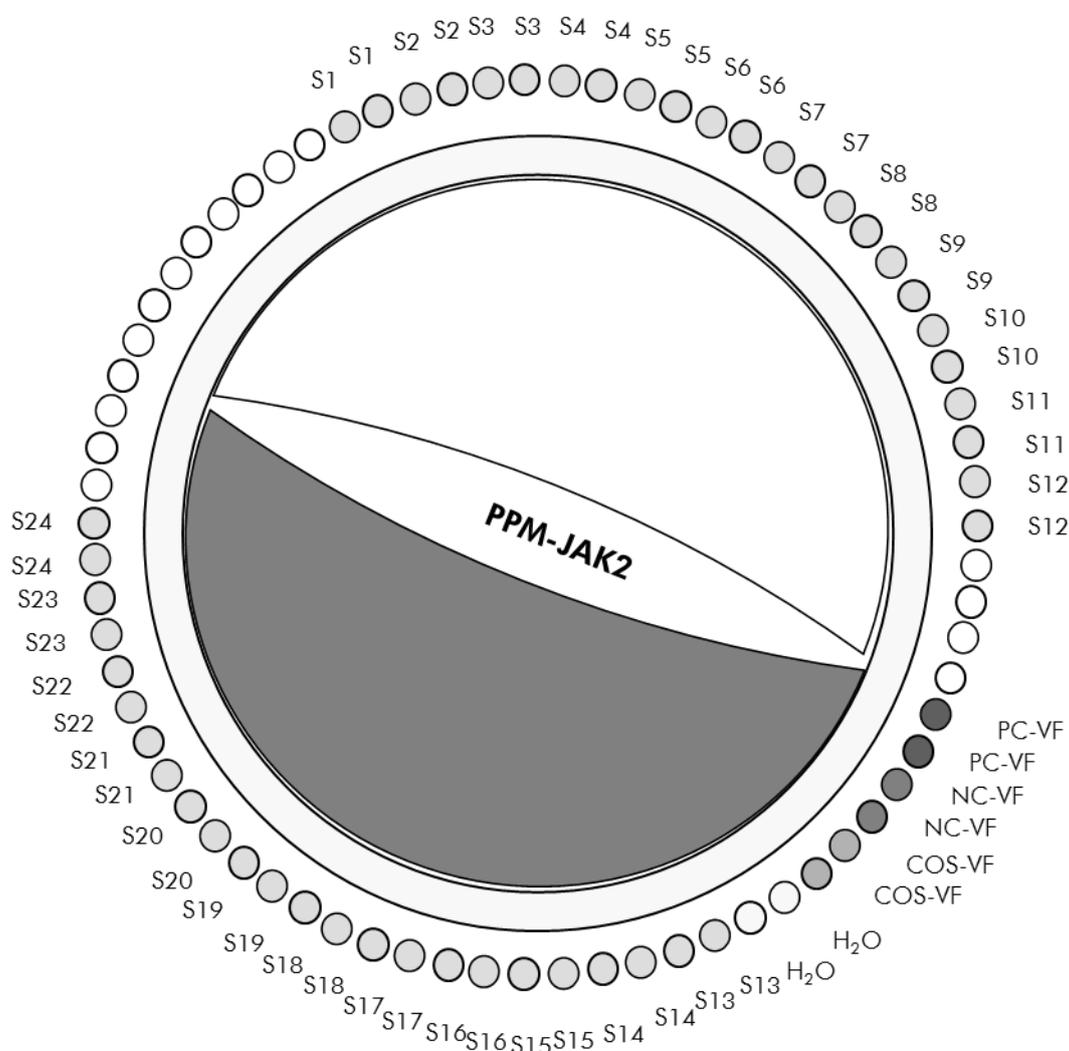
### Protocol: qPCR on Rotor-Gene Q MDx 5plex HRM or Rotor-Gene Q 5plex HRM instruments with 72-tube rotor

Using this instrument, we recommend performing all measurements in duplicate, as indicated in Table 2.

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

Samples	Reactions
<b>With the JAK2 V617F primers and probes mix (PPM-JAK2)</b>	
n DNA samples	n x 2 reactions
3 DNA controls	6 reactions (PC-VF, NC-VF, and COS-VF, each one tested in duplicate)
Water control	2 reactions

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



**Figure 2. Suggested rotor setup for an experiment with the ipsogen JAK2 MutaScreen EZ Kit. PC-VF: positive control; NC-VF: negative control; COS-VF: cut-off sample; S: DNA sample; H<sub>2</sub>O: water control.**

**Note:** Take care to always place a sample to be tested in position 1 of the rotor. Otherwise, during the calibration step, the instrument will not perform calibration, and incorrect fluorescence data will be acquired.

Fill all other positions with empty tubes.

### **qPCR on Rotor-Gene Q instruments with 72-tube rotor**

**Note:** Perform all steps on ice.

#### **Procedure**

**1. Thaw all necessary components and place them on ice.**

Components should be taken out of the freezer approximately 10 min before starting the procedure.

**2. Vortex and briefly centrifuge all the tubes (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**

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

All concentrations are for the final volume of the reaction.

Table 3 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 25  $\mu$ l. A pre-mix can be prepared, according to the number of reactions, using the same primer and probe mix. Extra volumes are included to compensate for pipetting error.

On Rotor-Gene instruments, the *ipsogen* JAK2 MutaScreen EZ Kit can be used for analysis of 24 samples in duplicate in one experiment (Figure 2), 20 samples in duplicate in two experiments, or 15 samples in duplicate in three experiments.

**Table 3. Preparation of qPCR mix**

<b>Component</b>	<b>Number of reactions (<math>\mu</math>l)</b>				<b>Final concentration</b>
	<b>1</b>	<b>56+1*</b>	<b>28+1†</b>	<b>18+1‡</b>	
qPCR master mix, 2x	12.5	712.5	362.5	237.5	1x
Primers and probes mix, 10x	2.5	142.5	72.5	47.5	1x
Nuclease-free PCR grade water	5	285	145	95	–
Sample (to be added at step 6)	5	5 each	5 each	5 each	–
Total volume	25	25 each	25 each	25 each	–

\* 24 samples; one experiment/kit.

† 10 samples; two experiments/kit.

‡ 5 samples; three experiments/kit.

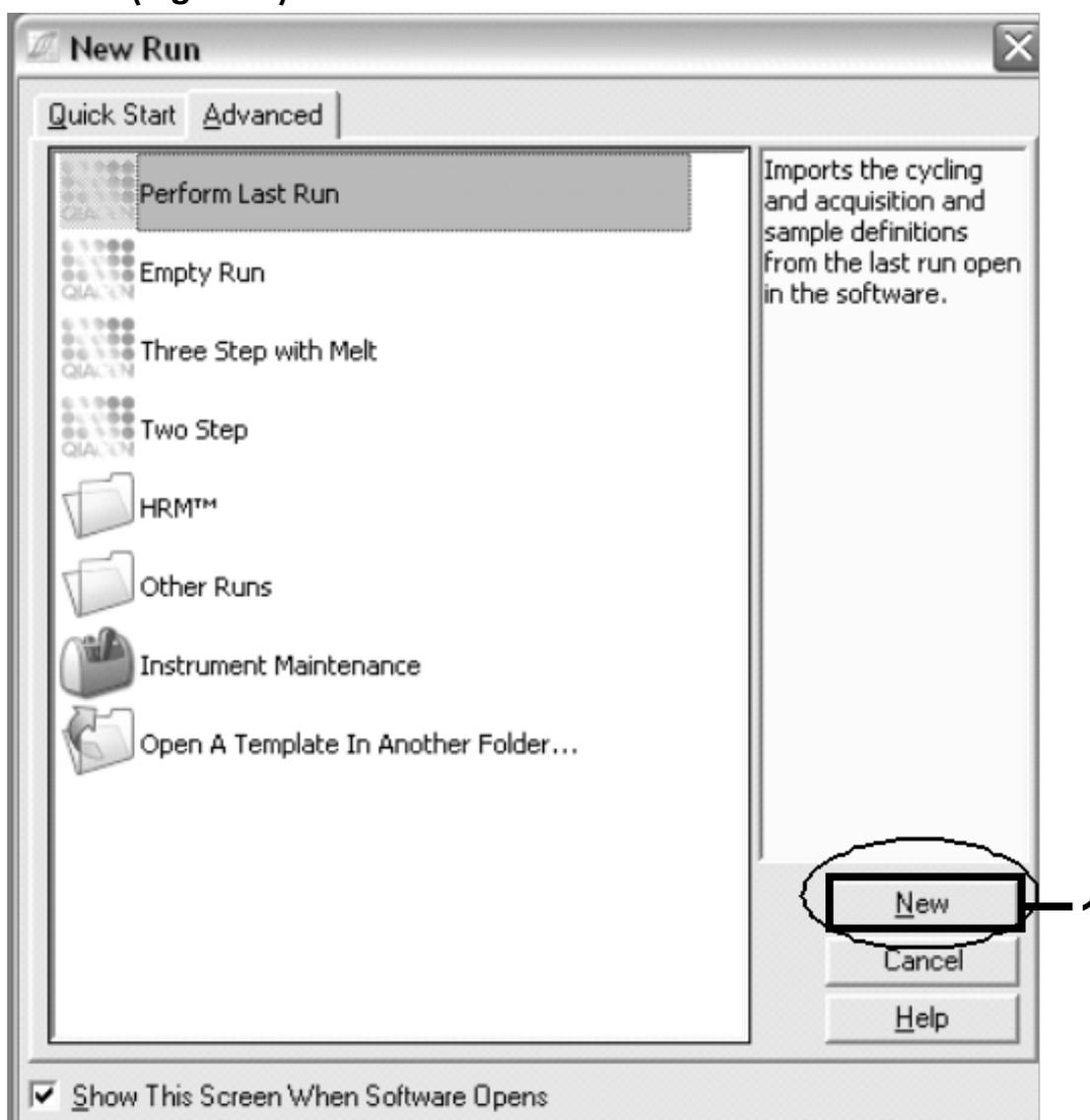
4. **Vortex and briefly centrifuge the qPCR mix (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**
5. **Dispense 20  $\mu$ l of the qPCR pre-mix per tube.**
6. **Add 5  $\mu$ l of the sample DNA material or controls in the corresponding tube (total volume 25  $\mu$ l).**
7. **Mix gently, by pipetting up and down.**
8. **Close the PCR tubes. Place the tubes in the 72-tube rotor according to the manufacturer's recommendations. Fill all other positions with empty tubes.**
9. **Make sure that the locking ring (accessory of the Rotor-Gene Instrument) is placed on top of the rotor to prevent accidental opening of the tubes during the run. Place the rotor in the Rotor-Gene Q instrument according to the manufacturer's recommendations.**

**10. For the detection of JAK2 DNA, create a temperature profile according to the following steps.**

<b>Setting the general assay parameters</b>	<b>Figures 3, 4</b>
<b>Amplification of the DNA</b>	<b>Figure 5</b>
<b>Adjusting the fluorescence channel sensitivity</b>	<b>Figure 6</b>

Further information on programming Rotor-Gene Instruments can be found in the instrument user manual. In the illustrations, the software settings are framed in bold black. Illustrations are included for Rotor-Gene Q Instruments.

**11. Start the Rotor-Gene software. In the “New Run” dialog box, click “New” (Figure 3).**



**Figure 3. The “New Run” dialog box.**

12. Open the “New Run Wizard” dialog box. Select 25 for the PCR reaction volume and click “Next” (Figure 4).

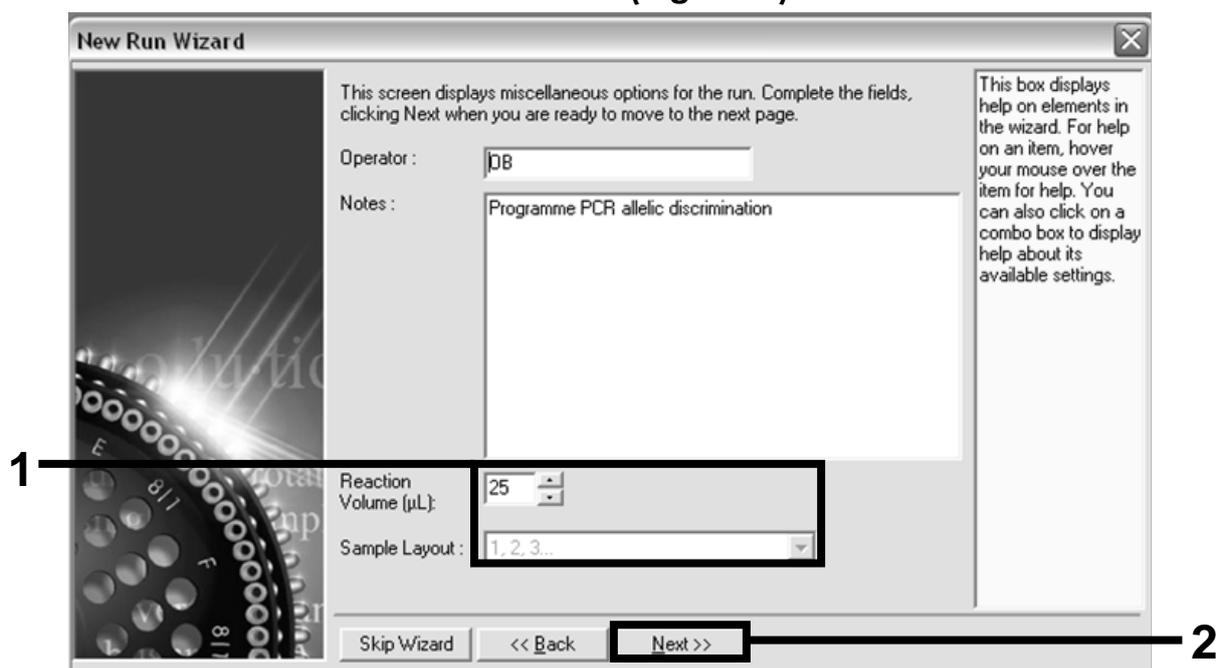


Figure 4. Setting the general assay parameters.

13. Click the “Edit Profile” button in the next “New Run Wizard” dialog box, and program the temperature profile as shown in Table 4 and Figure 5. Be sure to add the last acquiring step at 60°C, at each cycle, for both channels Green (FAM) and Yellow (VIC).

Table 4. Temperature profile

<b>Hold</b>	Temperature: 50°C Time: 2 min
<b>Hold 2</b>	Temperature: 95°C Time: 10 min
<b>Cycling</b>	50 times 92°C for 15 s 60°C for 1 min; single Acquisition of FAM fluorescence in channel Cycling A Green Acquisition of VIC fluorescence in channel Cycling A Yellow

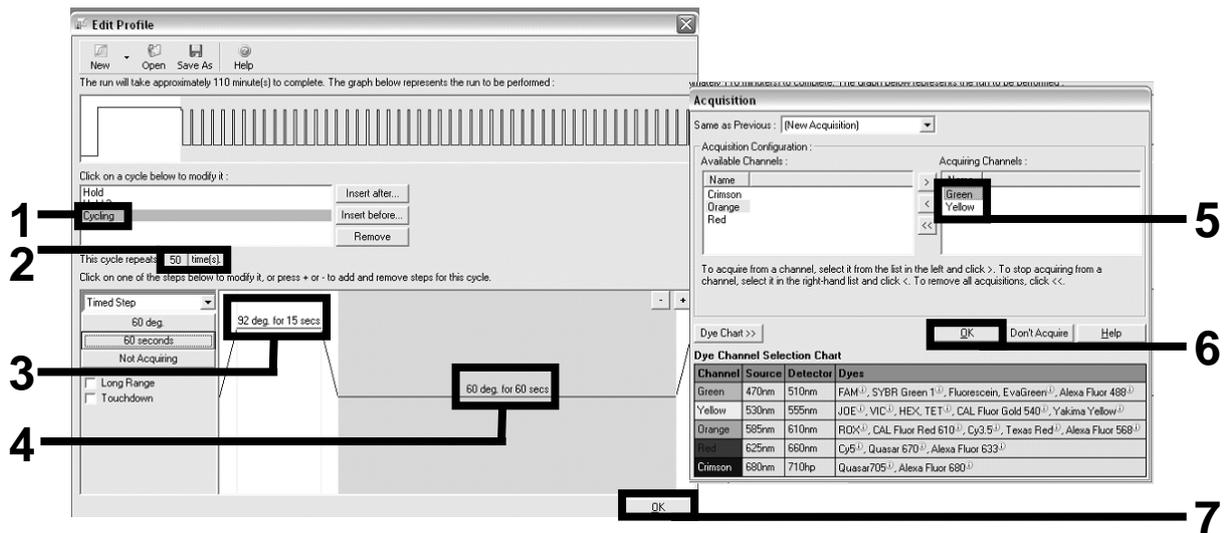


Figure 5. Amplification of the DNA.

14. The detection range of the fluorescence channels has to be determined according to the fluorescence intensities in the PCR tubes. Click "Gain Optimisation" in the "New Run Wizard" dialog box to open the "Auto-Gain Optimisation Setup" dialog box. Click "Optimise Acquiring" (Figure 6), and then click "OK" in the "Auto-Gain Optimisation Channel Settings" dialog boxes for each channel (Green and Yellow, Figure 6). Make sure that the "Perform Optimisation Before 1st Acquisition" box is checked for each channel (Figure 6).

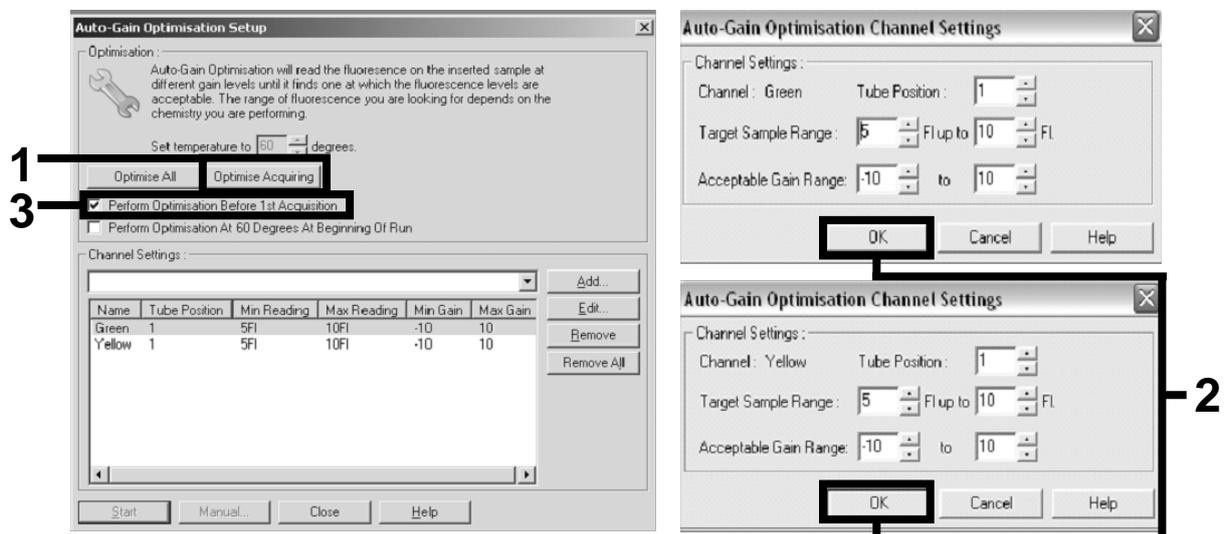


Figure 6. Adjusting the fluorescence channel sensitivity.

15. The gain values determined by the channel calibration are saved automatically and are listed in the last menu window of the programming procedure. Click "Start Run" to run the program.

16. Enter the rotor setup in the Rotor-Gene software (Figure 7).

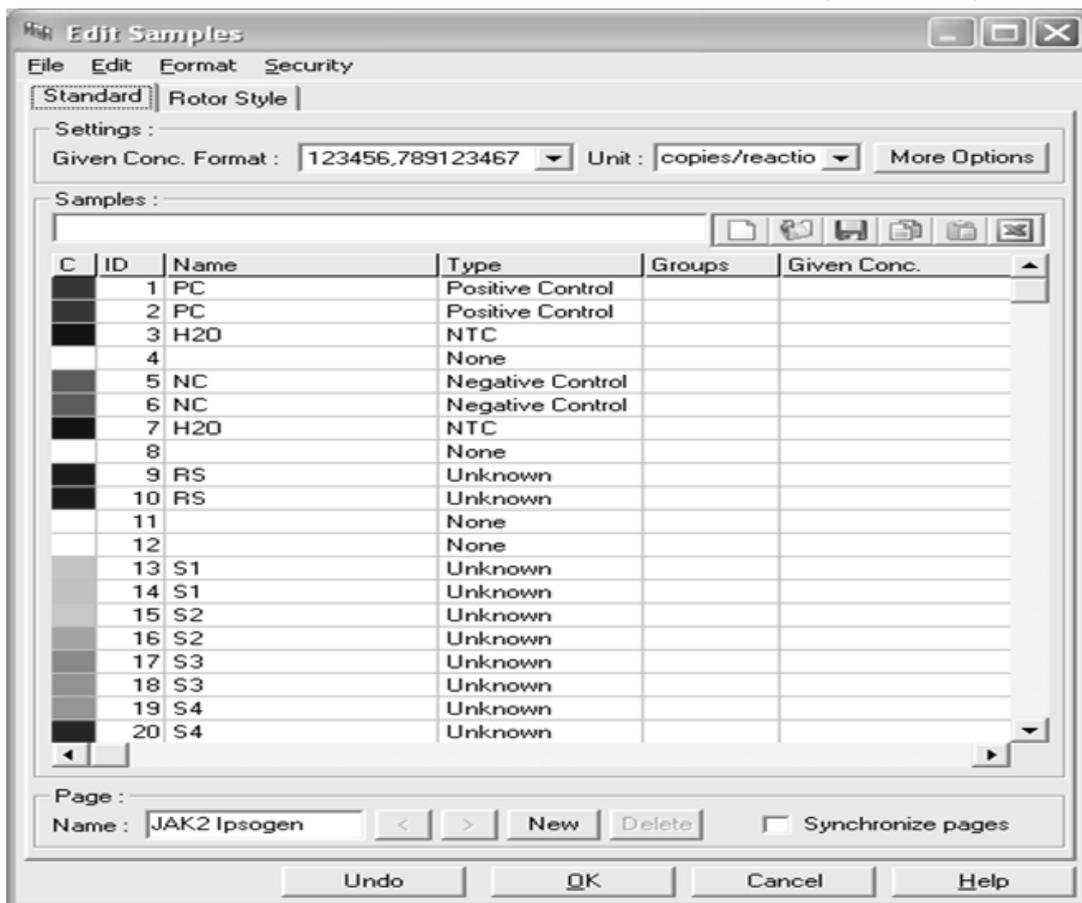


Figure 7. Rotor-Gene setup: "Edit Samples".

End point analysis procedure for Rotor-Gene Q 5plex HRM instrument setting

17. After the PCR program has ended, click "Analysis" in the toolbar (Figure 8).



Figure 8. Analysis.

18. In the "Analysis" dialog box, double-click "Cycling A. Green" and then "OK" (Figure 9). Repeat for "Cycling A. Yellow".

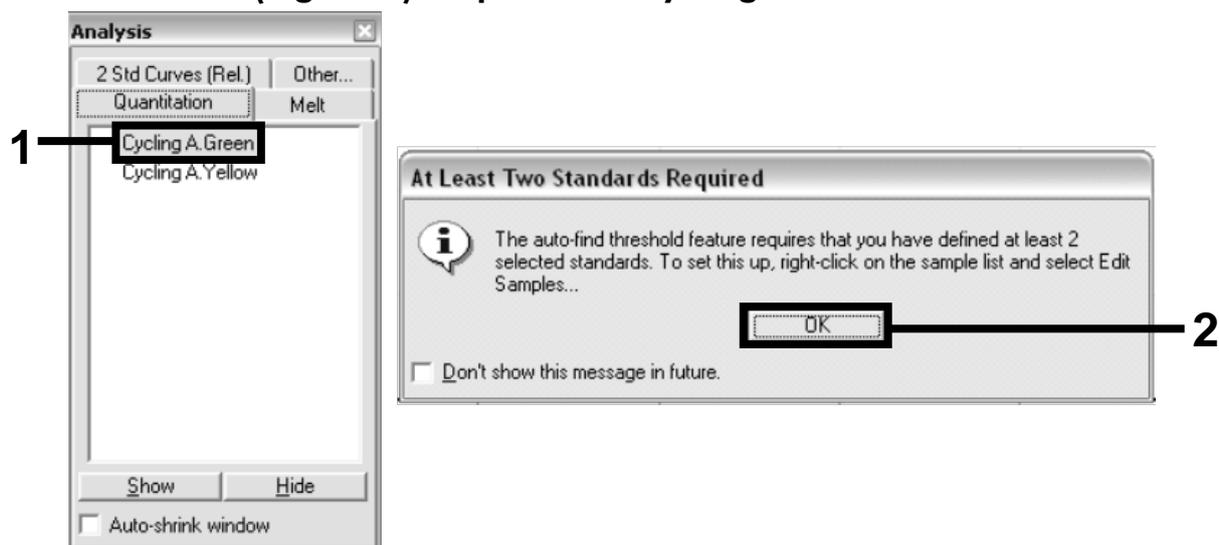


Figure 9. Quantitation: "Cycling A. Green".

19. A new window appears (Figure 10). Click "Slope Correct" in both panels, as shown in Figure 10.

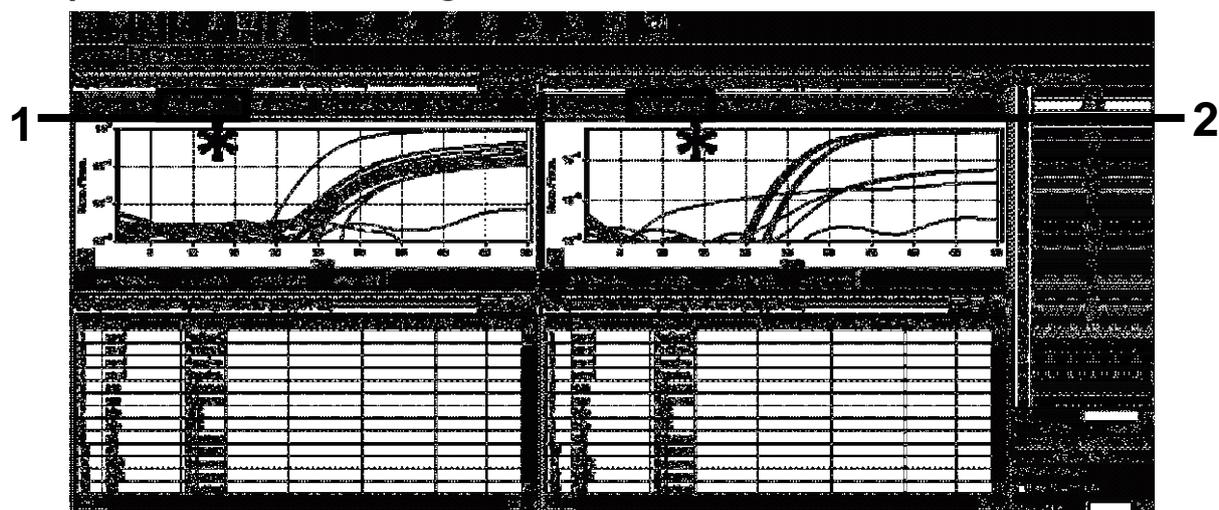


Figure 10. Setting "Slope Correct".

20. To export data, save as an Excel® data sheet. Select "File/Save As/Excel data sheet", and click "OK". Give a name to the export file, and save the text file (\*.txt).
21. Open the text file in Excel and select column A. Select "Data/Convert and Next". Select "Comma" and then click "End". The results will appear as shown in Figure 11.



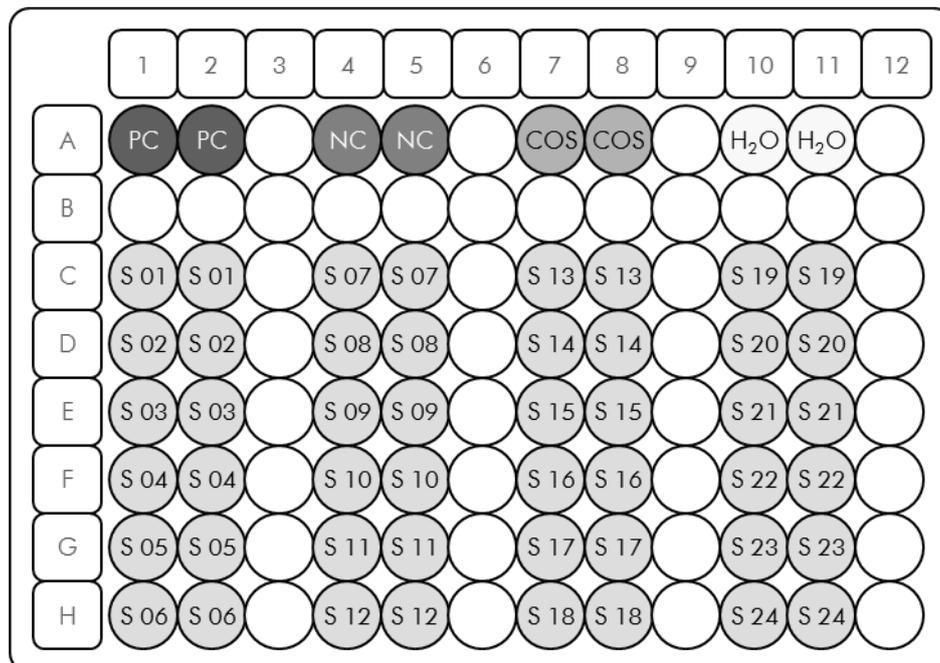
## Protocol: qPCR on Applied Biosystems 7500 or ABI PRISM 7900HT instruments

Using 96-well plate qPCR equipment, we recommend performing all measurements in duplicate as indicated in Table 5.

**Table 5. Number of reactions for Applied Biosystems 7500 or ABI PRISM 7900HT instruments**

Samples	Reactions
<b>With the JAK2 V617F primers and probes mix (PPM-JAK2)</b>	
n DNA samples	n x 2 reactions
3 DNA controls	6 reactions (PC-VF, NC-VF, and COS-VF, each one tested in duplicate)
Water control	2 reactions

### Sample processing on Applied Biosystems 7500 or ABI PRISM 7900HT instruments



**Figure 12. Suggested plate setup for an experiment with the *ipsogen* JAK2 MutaScreen EZ Kit.** PC: positive control; NC: negative control; COS: cut-off sample; S: DNA sample; H<sub>2</sub>O: water control.

## qPCR on Applied Biosystems 7500 or ABI PRISM 7900HT instruments

**Note:** Perform all steps on ice.

### Procedure

**1. Thaw all necessary components and place them on ice.**

Components should be taken out of the freezer approximately 10 min before starting the procedure.

**2. Vortex and briefly centrifuge all the tubes (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**

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

All concentrations are for the final volume of the reaction.

Table 6 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 25  $\mu$ l. A pre-mix can be prepared, according to the number of reactions, using the same primer and probe mix. Extra volumes are included to compensate for pipetting error.

On the Applied Biosystems 7500 or ABI PRISM 7900HT, the *ipsogen* JAK2 MutaScreen EZ Kit can be used for analysis of 24 samples in duplicate in one experiment (Figure 12), 20 samples in duplicate in two experiments, or 15 samples in duplicate in three experiments.

**Table 6. Preparation of qPCR mix**

<b>Component</b>	<b>Number of reactions (<math>\mu</math>l)</b>				<b>Final concentration</b>
	<b>1</b>	<b>56+1*</b>	<b>28+1<sup>†</sup></b>	<b>18+1<sup>‡</sup></b>	
TaqMan Universal PCR Master Mix, 2x	12.5	712.5	362.5	237.5	1x
Primers and probes mix, 10x	2.5	142.5	72.5	47.5	1x
Nuclease-free PCR grade water	5	285	145	95	–
Sample (to be added at step 6)	5	5 each	5 each	5 each	–
Total volume	25	25 each	25 each	25 each	–

\* 24 samples; one experiment/kit.

<sup>†</sup> 10 samples; two experiments/kit.

<sup>‡</sup> 5 samples; three experiments/kit.

4. **Vortex and briefly centrifuge the qPCR mix (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**
5. **Dispense 20  $\mu$ l of the qPCR pre-mix per well.**
6. **Add 5  $\mu$ l of the sample DNA material or controls in the corresponding well (total volume 25  $\mu$ l).**
7. **Mix gently, by pipetting up and down.**
8. **Close the plate and briefly centrifuge (300 x g, approximately 10 s).**
9. **Place the plate in the thermal cycler according to the manufacturer's recommendations.**
10. **Program the thermal cycler with the thermal cycling program as indicated in Table 7, and start the run.**

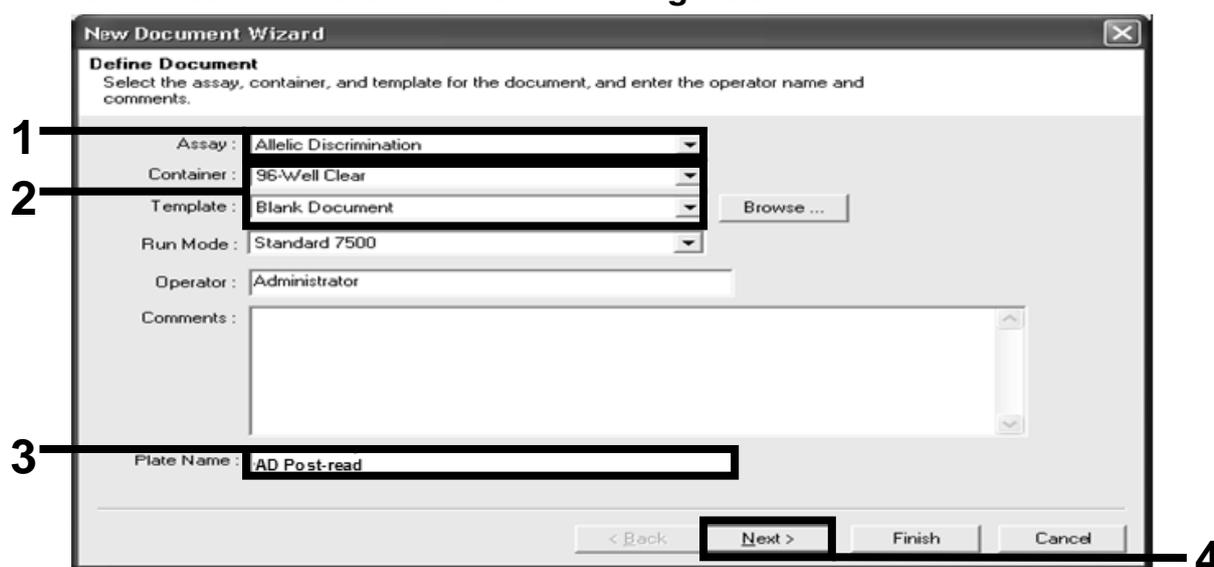
**Table 7. Temperature profile for Applied Biosystems 7500 and ABI PRISM 7900 HT instruments**

<b>Hold</b>	Temperature: 50°C Time: 2 min
<b>Hold 2</b>	Temperature: 95°C Time: 10 min
<b>Cycling</b>	50 times 92°C for 15 s 60°C for 1 min; single

**Post-read run analysis procedure for Applied Biosystems and ABI PRISM instruments**

For programming details of the Applied Biosystems 7500 and ABI PRISM 7900HT instruments, refer to the instrument user guide. For a better overview, the software settings are framed in bold black.

11. After the run is finished, select **“Start/Program”**, and then select **“File/New”**.
12. In the **“New Document Wizard”** dialog box, click the **“Assay”** drop-down list, and select **“Allelic Discrimination”** (Figure 13).
13. Accept the default settings for the **“Container”** and **“Template”** fields (**“96-Well Clear”** and **“Blank Document”**, Figure 13). In the **“Plate Name”** field, type **AD Post-read** (Figure 13), and then click **“Next>”** to access the **“Select Markers”** dialog box.



**Figure 13. Pre-settings for creating a new post-read run (New Document Wizard).**

14. If the “Markers in Document” panel in the “Select Markers” dialog box contains a suitable marker for your application, proceed with step 18. If not, then continue with step 15.
15. Create detectors and markers as follows. Click “New Detector” (Figure 14).

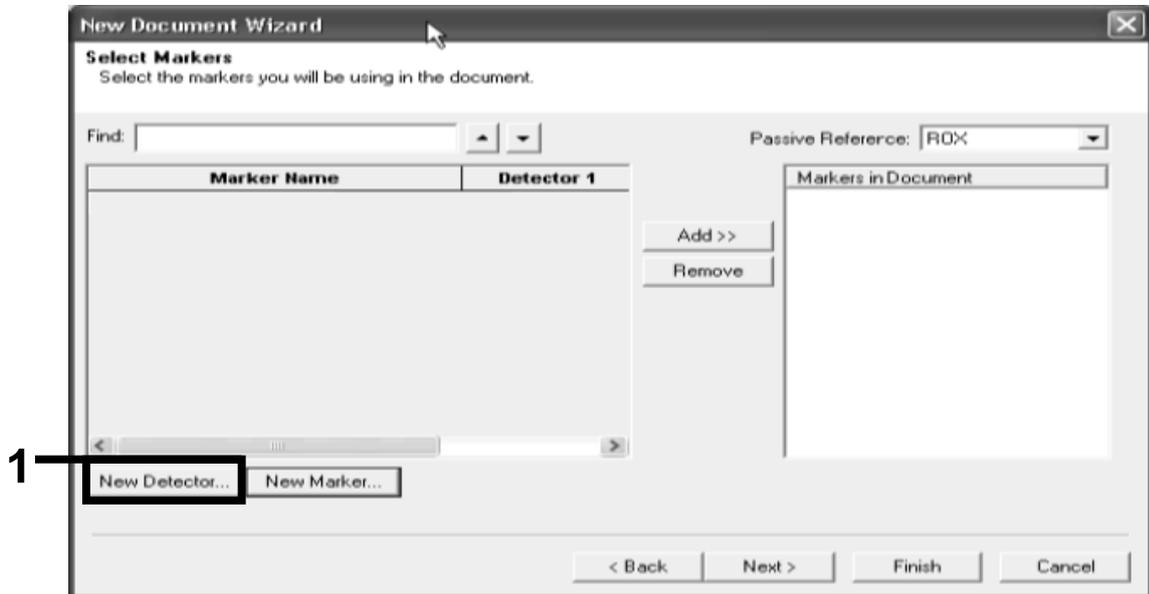


Figure 14. The “Markers in Document” panel does not contain a suitable marker for your application.

16. In the “New Detector” dialog box, type *Allele A* in the “Name” field (Figure 15). Leave the “Reporter Dye” set to “FAM”. Click the “Color” button, select a color, and then click “OK” (Figure 15). Click “Create Another” (Figure 15).

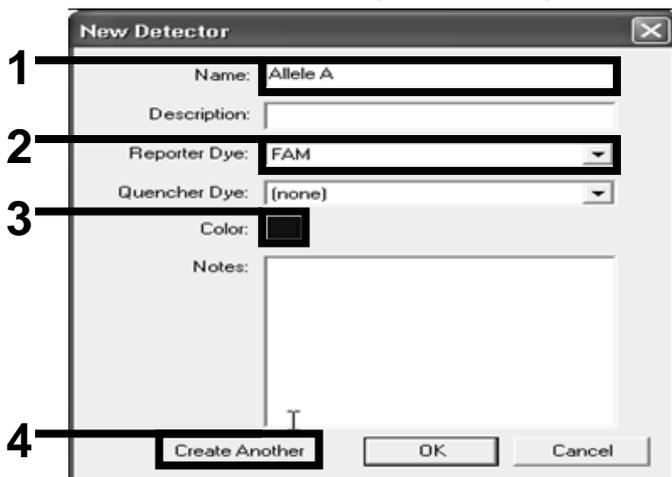


Figure 15. Creating detectors.

17. In the next "New Detector" dialog box, type *Allele B* in the "Name" field. Select "VIC" in the "Reporter Dye" field. Click the "Color" button, select a color, and then click "OK".
18. Click "New Marker" in the "Select Markers" dialog box (see Figure 14).
19. In the "New Marker" dialog box, type *JAK2* in the "New Marker Name" field (Figure 16). Select the "Allele A" and "Allele B" detectors as created in steps 16 and 17 (or already defined), and click "OK" (Figure 16).

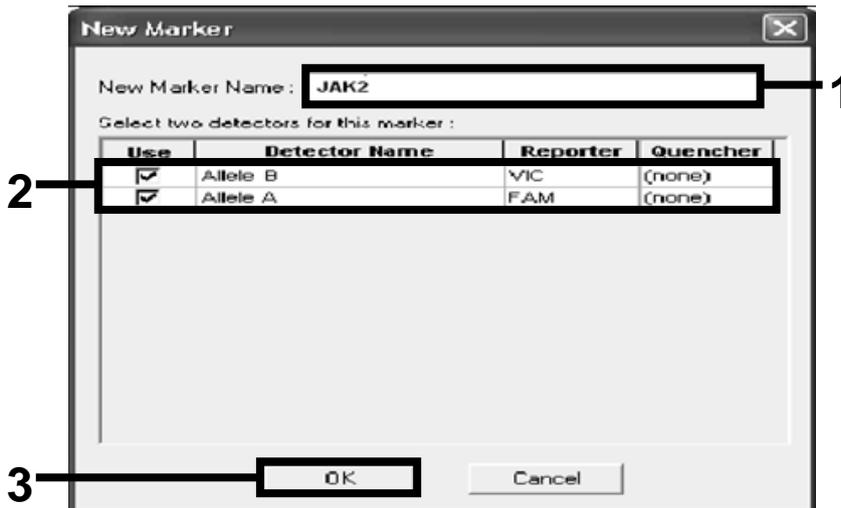


Figure 16. Creating markers.

20. In the “Select Markers” dialog box, select “JAK2”, as created above, or a suitable predefined marker, and then click “Add>>” (Figure 17).

**Note:** To remove a marker, select it and then click “Remove”.

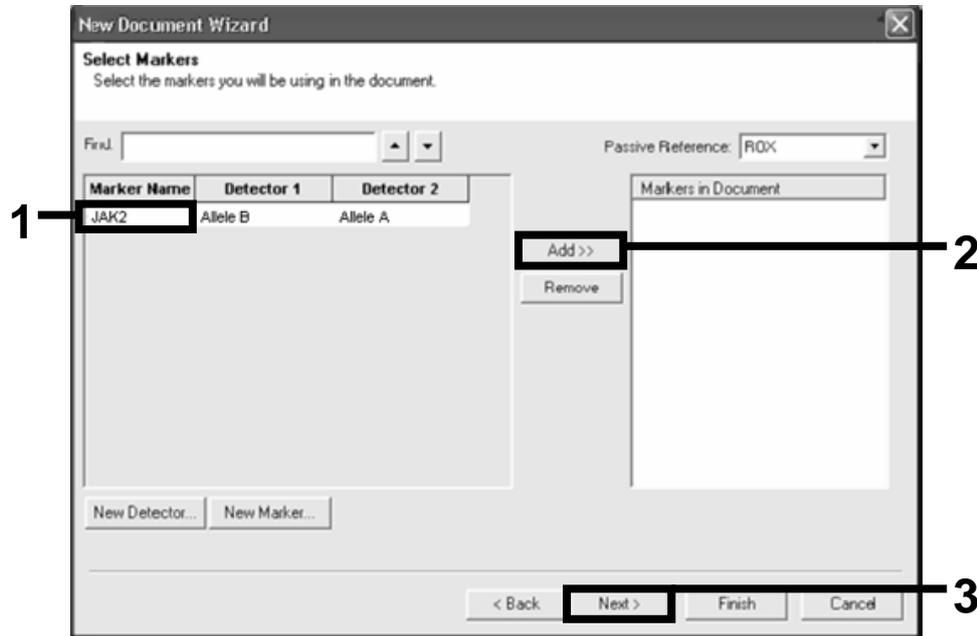


Figure 17. Selecting markers.

21. Click “Next>”.

22. In the “Setup Sample Plate” dialog box, click and drag to select the marker for wells that contain samples. Click “Finish”.

23. Select the “Instrument” tab, and change the sample volume to 25  $\mu$ l.

24. Select “File/Save” and then click “Save” to retain the name you assigned when you created the plate.

25. Load the reaction plate into the instrument according to the manufacturer’s recommendations

## 26. Start the post-read run. Click “Post-Read”.

The instrument will perform a run of 1 cycle for 60 s at 60°C. During this run, the instrument collects FAM and VIC fluorescence in each well (Figure 18).

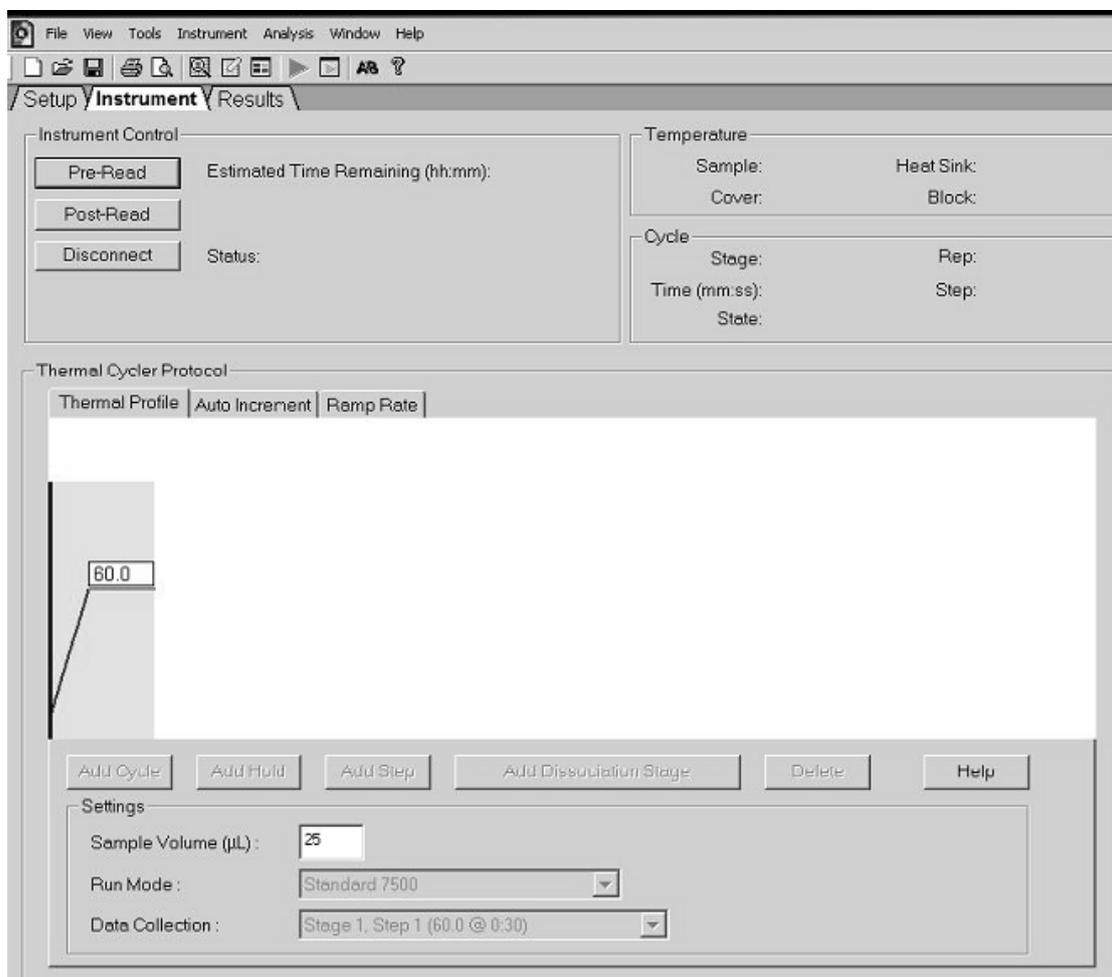


Figure 18. Post-read run.

27. Select “File/Export” and then click “Results” to export the results to an Excel file. The results will appear as shown in Figure 19.

Well	Sample Name	Marker	Task	Passive Ref	Allele X	Allele Y	Allele X Rn	Allele Y Rn	Call	Quality Value	Method
Comments:				VIC Sample 1				FAM Sample 1			
SDS v1.2											
A1	sample 1	VIC	Unknown	247.897	JAK2-VIC	JAK2-FAM	2.184	6.221	Undetermined	100.00	Manual Call
A2	sample 1	VIC	Unknown	295.565	JAK2-VIC	JAK2-FAM	2.451	6.805	Undetermined	100.00	Manual Call
A3	sample 2	VIC	Unknown	351.338	JAK2-VIC	JAK2-FAM	2.595	6.2	Undetermined	100.00	Manual Call
A4	sample 2	VIC	Unknown	379.909	JAK2-VIC	JAK2-FAM	2.553	6.01	Undetermined	100.00	Manual Call
A5	sample 3	VIC	Unknown	372.895	JAK2-VIC	JAK2-FAM	2.913	5.329	Undetermined	100.00	Manual Call
A6	sample 3	VIC	Unknown	359.717	JAK2-VIC	JAK2-FAM	2.806	5.278	Undetermined	100.00	Manual Call
A7	sample wt	VIC	Unknown	343.536	JAK2-VIC	JAK2-FAM	2.569	1.948	Undetermined	100.00	Manual Call
A8	sample wt	VIC	Unknown	277.677	JAK2-VIC	JAK2-FAM	2.684	2.015	Undetermined	100.00	Manual Call
A9	C-	VIC	Unknown	330.943	JAK2-VIC	JAK2-FAM	2.623	1.967	Undetermined	100.00	Manual Call
A10	C-	VIC	Unknown	314.623	JAK2-VIC	JAK2-FAM	2.672	2.013	Undetermined	100.00	Manual Call
A11	C-	VIC	Unknown	269.500	JAK2-VIC	JAK2-FAM	2.82	1.892	Undetermined	100.00	Manual Call
A12	C+	VIC	Unknown	211.520	JAK2-VIC	JAK2-FAM	1.249	6.14	Undetermined	100.00	Manual Call
B1	C+	VIC	Unknown	270.623	JAK2-VIC	JAK2-FAM	1.346	6.894	Undetermined	100.00	Manual Call
B2	C+	VIC	Unknown	365.112	JAK2-VIC	JAK2-FAM	1.265	6.528	Undetermined	100.00	Manual Call
B3	ER	VIC	Unknown	372.150	JAK2-VIC	JAK2-FAM	2.214	2.03	Undetermined	100.00	Manual Call
B4	ER	VIC	Unknown	404.145	JAK2-VIC	JAK2-FAM	2.419	2.295	Undetermined	100.00	Manual Call
B5	ER	VIC	Unknown	410.977	JAK2-VIC	JAK2-FAM	2.681	2.52	Undetermined	100.00	Manual Call
B6	H2O	VIC	Unknown	395.431	JAK2-VIC	JAK2-FAM	0.655	1.346	Undetermined	100.00	Manual Call
B7	H2O	VIC	Unknown	415.223	JAK2-VIC	JAK2-FAM	0.727	1.241	Undetermined	100.00	Manual Call
B8	H2O	VIC	Unknown	366.885	JAK2-VIC	JAK2-FAM	0.606	1.277	Undetermined	100.00	Manual Call

Figure 19. Example of results, shown in an Excel file.

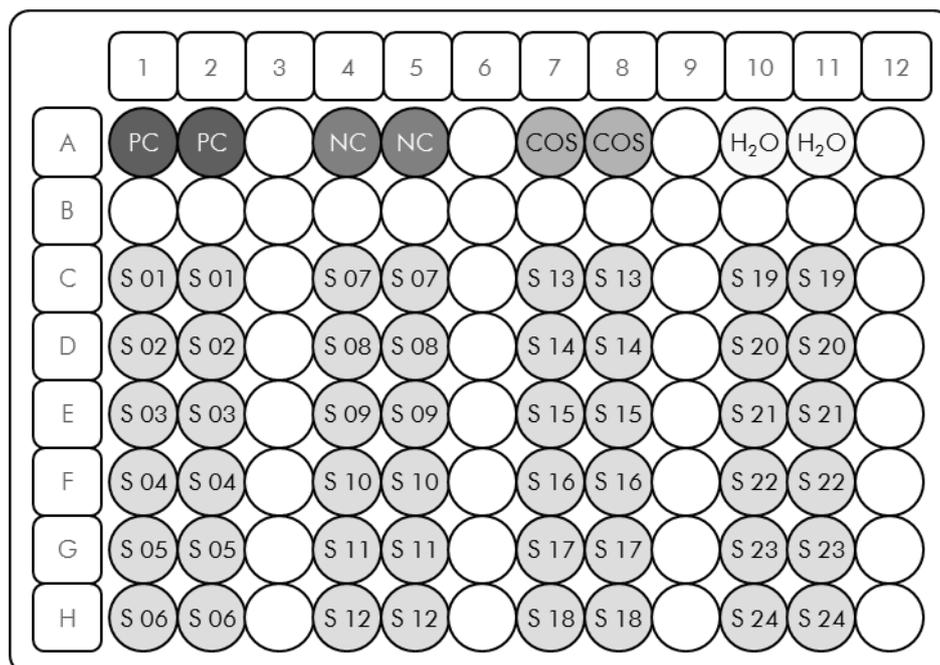
## Protocol: qPCR on the LightCycler 480 instrument

Using 96-well plate qPCR equipment, we recommend performing all measurements in duplicate as indicated in Table 8.

**Table 8. Number of reactions for the LightCycler 480 instrument**

Samples	Reactions
<b>With the JAK2 V617F primers and probes mix (PPM-JAK2)</b>	
n DNA samples	n x 2 reactions
3 DNA controls	6 reactions (PC-VF, NC-VF, and COS-VF, each one tested in duplicate)
Water control	2 reactions

### Sample processing on the LightCycler 480 instrument



**Figure 20. Suggested plate setup for an experiment with the *ipsogen* JAK2 MutaScreen EZ Kit.** PC: positive control; NC: negative control; COS: cut-off sample; S: DNA sample; H<sub>2</sub>O: water control.

## qPCR on the LightCycler 480 instrument

**Note:** Perform all steps on ice.

### Procedure

**1. Thaw all necessary components and place them on ice.**

Components should be taken out of the freezer approximately 10 min before starting the procedure.

**2. Vortex and briefly centrifuge all the tubes (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**

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

All concentrations are for the final volume of the reaction.

Table 9 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 25  $\mu$ l. A pre-mix can be prepared, according to the number of reactions, using the same primer and probe mix. Extra volumes are included to compensate for pipetting error.

On the LightCycler 480 instrument, the *ipsogen* JAK2 MutaScreen EZ Kit can be used for analysis of 24 samples in duplicate in one experiment (Figure 20), 20 samples in duplicate in two experiments, or 15 samples in duplicate in three experiments.

**Table 9. Preparation of qPCR mix**

<b>Component</b>	<b>Number of reactions (<math>\mu</math>l)</b>				<b>Final concentration</b>
	<b>1</b>	<b>56+1*</b>	<b>28+1<sup>†</sup></b>	<b>18+1<sup>‡</sup></b>	
TaqMan Universal PCR Master Mix, 2x	12.5	712.5	362.5	237.5	1x
Primers and probes mix, 10x	2.5	142.5	72.5	47.5	1x
Nuclease-free PCR grade water	5	285	145	95	–
Sample (to be added at step 6)	5	5 each	5 each	5 each	–
Total volume	25	25 each	25 each	25 each	–

\* 24 samples; one experiment/kit.

<sup>†</sup> 10 samples; two experiments/kit.

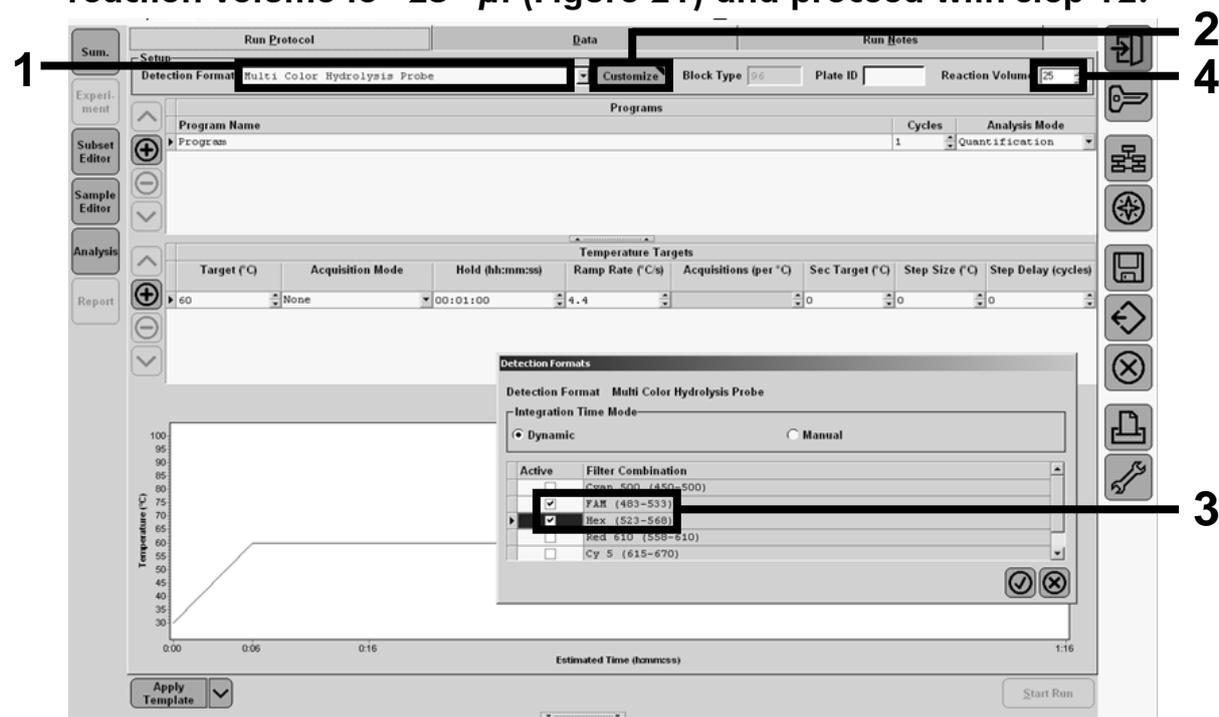
<sup>‡</sup> 5 samples; three experiments/kit.

4. **Vortex and briefly centrifuge the qPCR mix (approximately 10 s, 10,000 rpm, to collect the liquid in the bottom of the tube).**
5. **Dispense 20  $\mu$ l of the qPCR pre-mix per well.**
6. **Add 5  $\mu$ l of the sample DNA material or controls in the corresponding well (total volume 25  $\mu$ l).**
7. **Mix gently, by pipetting up and down.**
8. **Close the plate and briefly centrifuge (300 x g, approximately 10 s).**
9. **Place the plate in the thermal cycler according to the manufacturer's recommendations.**
10. **On the home page, select "New Experiment".**

**11. For the LightCycler 480 I, follow step 11a. For the LightCycler 480 II, follow step 11b.**

For programming details of the LightCycler 480 instrument, refer to the instrument user guide. For a better overview, the software settings are framed in bold black.

**11a. LightCycler 480 I: Select “Multi Color Hydrolysis Probe”, click “Customize”, and then check that the channels “FAM (483–533)” and “Hex (533–568)” (i.e., VIC) are selected (Figure 21). Set the reaction volume to “25”  $\mu$ l (Figure 21) and proceed with step 12.**



**Figure 21. LightCycler 480 I: Setting the detection format.**

11b. LightCycler 480 II: Select “Dual Color Hydrolysis Probe”, click “Customize”, and then check that the channels “FAM (465–510)” and “VIC / HEX / (533–580)” are selected (Figure 22). Set the reaction volume to “25”  $\mu$ l (Figure 22) and proceed with step 12.

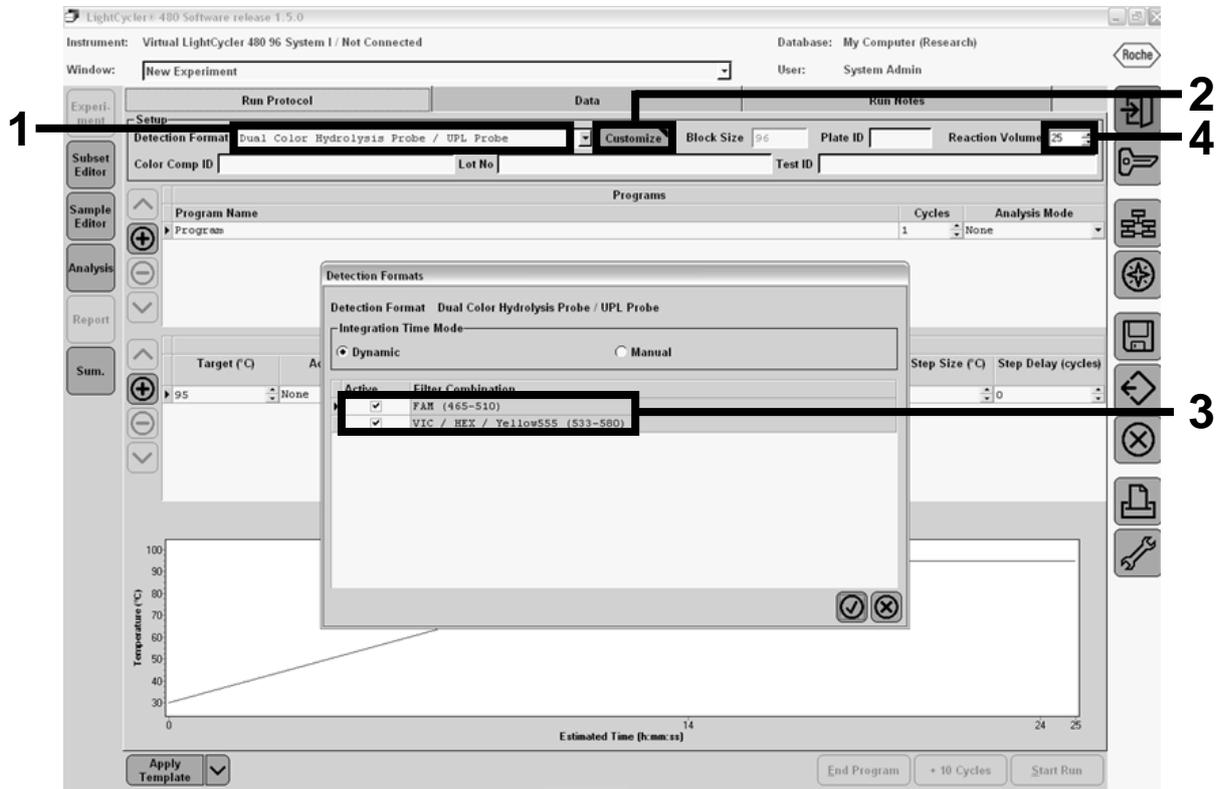


Figure 22. LightCycler 480 II: Setting the detection format.

12. Program the thermal cycler with the thermal cycling program as indicated in Table 10, and start the run.

**Note:** When describing the plate setup on the instrument, select “Endpt Geno” in the “Step 1 : select workflow” section.

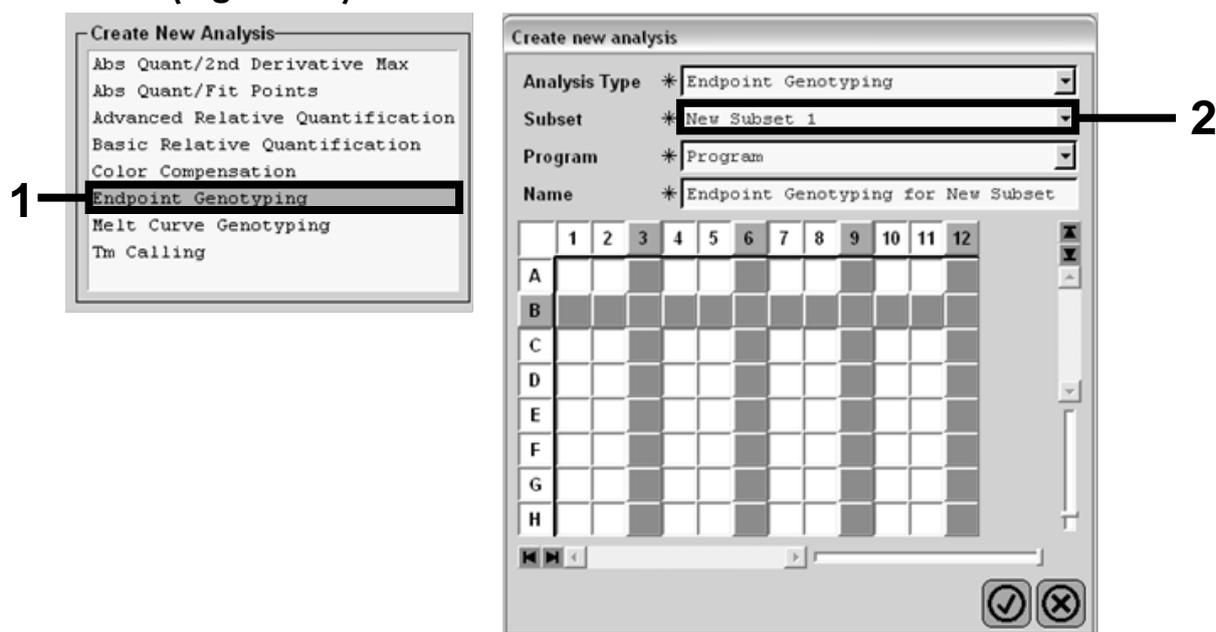
**Table 10. Temperature profile for the LightCycler 480 instrument**

<b>Hold</b>	Temperature: 50°C Time: 2 min
<b>Hold 2</b>	Temperature: 95°C Time: 10 min
<b>Cycling</b>	50 times 92°C for 15 s; single 60°C for 1 min; single
<b>Hold 3</b>	60°C for 1 min; single

**End-point analysis procedure for the LightCycler 480 instrument**

13. After the run is finished, click “Analysis”.

14. In the “Create New Analysis” dialog box, select “Endpoint Genotyping”, and then select the subset to analyze in the “Subset” menu (Figure 23).



**Figure 23. Selecting analysis type and subset to analyze.**

15. In the next window, select “Hex” (i.e., VIC) fluorescence for “Allele X” and “FAM” fluorescence for “Allele Y” (Figure 24).



Figure 24. Selecting fluorescence for “Allele X” and “Allele Y”.

16. The next window (Figure 25) shows plate setup (1, upper left), fluorescence results for each sample (2, bottom left), and the scatter plot with allelic discrimination (3, right; FAM and VIC fluorescence measured at the 50th PCR cycle).

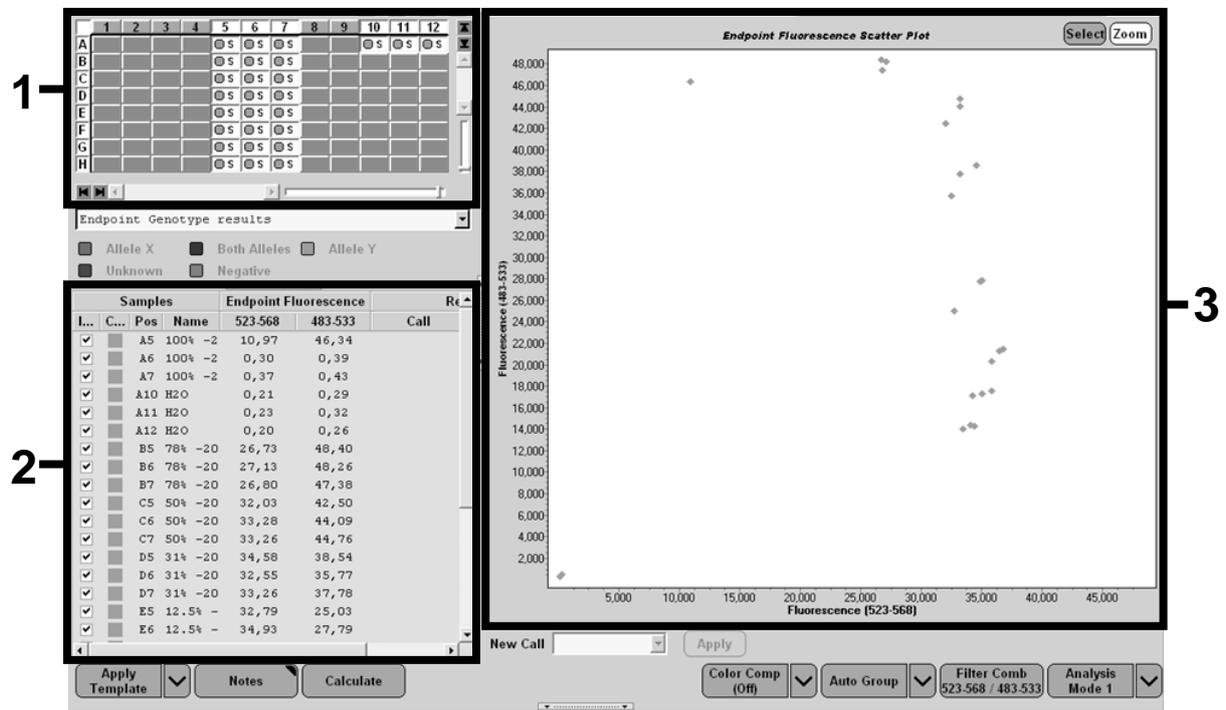


Figure 25. Data summary.

17. To export data, right-click on the sample results template, and then select “Export Table”. The file will be saved in a text (.txt) file format.

18. To view and analyze results, open the file using Excel. The results will appear as shown in Figure 26.

	A	B	C	D	E	F	G	
1	Experiment: OB 08-12-16 Active filters: FAM (483-533), Hex (523-568)							
2	Include	Color	Pos	Name	523-568	483-533	Call	Score
3	True	10789024	A5	100%-20	10,971	46,335		0,00
4	True	10789024	A6	100%-20	0,302	0,392		0,00
5	True	10789024	A7	100%-20	0,369	0,425		0,00
6	True	10789024	A10	H2O	0,207	0,290		0,00
7	True	10789024	A11	H2O	0,233	0,319		0,00
8	True	10789024	A12	H2O	0,203	0,261		0,00
9	True	10789024	B5	78%-20	26,731	48,396		0,00
10	True	10789024	B6	78%-20	27,125	48,262		0,00
11	True	10789024	B7	78%-20	26,803	47,383		0,00
12	True	10789024	C5	50%-20	32,035	42,495		0,00
13	True	10789024	C6	50%-20	33,278	44,086		0,00
14	True	10789024	C7	50%-20	33,261	44,760		0,00
15	True	10789024	D5	31%-20	34,584	38,536		0,00
16	True	10789024	D6	31%-20	32,549	35,766		0,00
17	True	10789024	D7	31%-20	33,262	37,780		0,00
18	True	10789024	E5	12.5%-20	32,794	25,028		0,00
19	True	10789024	E6	12.5%-20	34,932	27,788		0,00
20	True	10789024	E7	12.5%-20	35,089	27,848		0,00
21	True	10789024	F5	5%-20	35,838	20,289		0,00
22	True	10789024	F6	5%-20	36,786	21,487		0,00
23	True	10789024	F7	5%-20	36,546	21,319		0,00
24	True	10789024	G5	2%-20	35,082	17,334		0,00
25	True	10789024	G6	2%-20	35,834	17,589		0,00
26	True	10789024	G7	2%-20	34,299	17,124		0,00
27	True	10789024	H5	0%-20	34,449	14,315		0,00
28	True	10789024	H6	0%-20	33,520	14,012		0,00
29	True	10789024	H7	0%-20	34,125	14,335		0,00
30								

Figure 26. Example of results, shown in an Excel file.

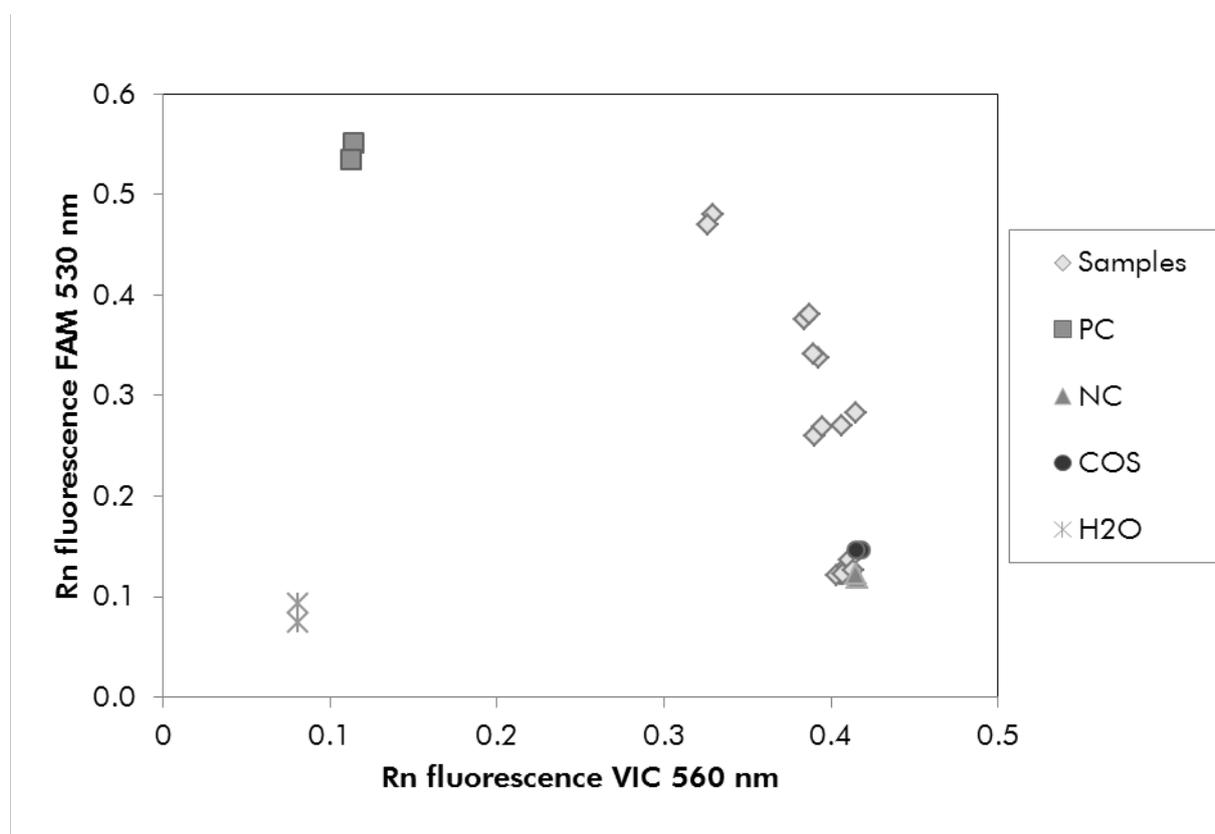
## Interpretation of Results

Extract exported data from the Analyze Export File generated by the system, and check the fluorescence levels (these must be consistent between duplicates).

Prepare a graphical representation (scatter plot) of fluorescence data. The x axis is VIC fluorescence; the y axis is FAM fluorescence.

## Graphical representation and quality control criteria

An example of a scatter plot is shown in Figure 27.



**Figure 27. Scatter plot of a representative allelic discrimination experiment.**

Instruments: Rotor-Gene Q, Applied Biosystems, ABI PRISM, and LightCycler 480.

Samples should be located on the arc connecting the negative controls (NC) to the positive controls (PC).

Improper positioning of any control may indicate an experimental error.

- Positive controls should be located in the upper left.
- Negative controls should be located in the bottom right.
  - Poor positioning of a negative control may indicate contamination.
- The cut-off sample (COS) should appear above the negative controls.

- Water controls (H<sub>2</sub>O) should be located at the bottom left.
- Poor positioning of a water control (higher than NC for FAM measurement or higher than PC for VIC) may indicate contamination.

**Note:** Any improperly positioned control or sample on the scatter plot should be discarded, and the experiment should be performed again with a new DNA aliquot.

## Calculation of normalized FAM/VIC ratio and genotyping

Calculate the FAM/VIC ratios for all the samples. Calculate the FAM/VIC ratios for the positive control (PC), the cut-off sample (COS), and the negative control (NC). The ratios must be consistent between duplicates. Calculate the average ratio of all duplicates.

Calculate the normalized ratio (NRatio) for the cut-off sample (COS) and for all the samples:

$$\text{NRatio}_{\text{Sample}} = \frac{\text{Ratio}_{\text{Sample}}}{\text{Ratio}_{\text{NC}}}$$

**Note:** The gray zone (GZ) of a test is defined as an area of values where the discriminatory performance is insufficiently accurate. A value in the gray zone indicates that the target marker cannot be scored as either present or absent. The gray zone must be calculated for each experiment.

Calculate the gray zone, or the incertitude area, around the normalized ratio of the COS (NRatio<sub>COS</sub>):

$$\text{GZ: } [(\text{NRatio}_{\text{COS}} \times 0.94); (\text{NRatio}_{\text{COS}} \times 1.06)]$$

Compare the normalized ratio of each sample to the NRatio<sub>COS</sub> GZ. Interpretation of results is outlined in Table 11.

**Table 11. Interpretation of genotyping results using normalized ratios**

Results	Interpretation
$\text{NRatio}_{\text{Sample}} > \text{NRatio}_{\text{COS}} \times 1.06$	JAK2 V617F is detected
$\text{NRatio}_{\text{Sample}} < \text{NRatio}_{\text{COS}} \times 0.94$	JAK2 V617F is not detected
$\text{NRatio}_{\text{Sample}}$ within $\text{NRatio}_{\text{COS}}$ GZ	Result inconclusive

## 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](http://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 protocols in this handbook or sample and assay technologies (for contact information, see "Contact Information", page 47).

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### Comments and suggestions

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#### Positive control signal negative

- |  |  |
|--|--|
| a) Pipetting error                         | Check pipetting scheme and the setup of the reaction.<br><br>Repeat the PCR run.   |
| b) Inappropriate storage of kit components | Store the <i>ipsogen</i> JAK2 MutaScreen EZ Kit at $-30$ to $-15^{\circ}\text{C}$ and keep primers and probes mix (PPM) protected from light. See "Reagent Storage and Handling", page 9.<br><br>Avoid repeated freezing and thawing.<br><br>Aliquot reagents for storage. |

#### Negative controls are positive

- |                     |  |
|---------------------|--|
| Cross-contamination | Replace all critical reagents.<br><br>Repeat the experiment with new aliquots of all reagents.<br><br>Always handle samples, kit components, and consumables in accordance with commonly accepted practices to prevent carry-over contamination. |
|---------------------|--|

#### No signal, even in positive controls

- |   |  |
|---|--|
| a) Pipetting error or omitted reagents  | Check pipetting scheme and the setup of the reaction.<br><br>Repeat the PCR run. |
| b) Inhibitory effects of the sample material, caused by insufficient purification | Repeat the DNA preparation.  |

## Comments and suggestions

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- |  |  |
|--|--|
| c) LightCycler: Incorrect detection channel chosen | Set Channel Setting to F1/F2 or 530 nm/640 nm.   |
| d) LightCycler: No data acquisition programmed     | Check the cycle programs.<br>Select acquisition mode "single" at the end of each annealing segment of the PCR program. |

### Absent or low signal in samples but positive controls okay

- |                                       |   |
|---------------------------------------|---|
| Poor DNA quality or low concentration | Always check the DNA quality and concentration before starting. |
|---------------------------------------|---|

### LightCycler: Fluorescence intensity too low

- |  |  |
|--|--|
| a) Inappropriate storage of kit components | Store the <i>ipsogen</i> JAK2 MutaScreen EZ Kit at -30 to -15°C and keep primers and probes mix (PPM) protected from light. See "Reagent Storage and Handling", page 9.<br><br>Avoid repeated freezing and thawing.<br><br>Aliquot reagents for storage. |
| b) Very low initial amount of target DNA   | Increase the amount of sample DNA.<br><br><b>Note:</b> Depending of the chosen method of DNA preparation, inhibitory effects may occur.  |

### LightCycler: Fluorescence intensity varies

- |                    |  |
|--------------------|--|
| a) Pipetting error | Variability caused by so-called "pipetting error" can be reduced by analyzing data in the F1/F2 or 530 nm/640 nm mode. |
|--------------------|--|

## Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *ipsogen* JAK2 MutaScreen EZ Kit is tested against predetermined specifications to ensure consistent product quality. Certificates of Analysis are available upon request at [www.qiagen.com/support/](http://www.qiagen.com/support/).

## Limitations

All reagents may exclusively be used in in vitro diagnostics. The product is to be used by personnel specially instructed and trained in the in vitro diagnostics procedures only.

Strict compliance with the user manual is required for optimal PCR results.

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

Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings. It is the user's responsibility to validate system performance for any procedures in their laboratory that are not covered by the QIAGEN performance studies.

## Performance Characteristics

Performance studies were performed on an Applied Biosystems 7500 Real-Time PCR System.

## Nonclinical studies

Nonclinical studies were conducted to establish the analytical performance of the *ipsogen* JAK2 MutaScreen EZ Kit.

### Precision near the cut-off limit

Five samples corresponding to low mutation levels were measured 40 times using three batches of the *ipsogen* JAK2 MutaScreen EZ Kit. Results are summarized in Table 12.

**Table 12. Precision data for nonclinical studies**

Sample	Replicates	Mutation detected	Result inconclusive	Mutation not detected
0%	40	0	0	40
0.5%	40	0	0	40
1%	40	0	5	35
4%	40	40	0	0
8%	40	40	0	0

## Input limits

The recommended input of genomic DNA is 25 ng. Differing amounts of input DNA were tested to determine if the amount of genomic DNA could affect the normalized ratio, leading to inconclusive results. Results are summarized in Table 13.

**Table 13. Effect of amount of input genomic DNA**

Sample	Input (ng)	Replicates	Mutation detected	Result inconclusive	Mutation not detected
0%	2.5	3	0	0	3
	10	3	0	0	3
	25	3	0	0	3
	100	3	0	0	3
	250	3	0	0	3
<b>Total 0%</b>		<b>15</b>	<b>0</b>	<b>0</b>	<b>15</b>

Table continued on next page

**Table 13. Continued**

<b>Sample</b>	<b>Input (ng)</b>	<b>Replicates</b>	<b>Mutation detected</b>	<b>Result inconclusive</b>	<b>Mutation not detected</b>
1%	2.5	3	0	0	3
	10	3	0	0	3
	25	3	0	0	3
	100	3	0	1	2
	250	3	0	2	1
<b>Total 1%</b>		<b>15</b>	<b>0</b>	<b>3</b>	<b>12</b>
4%	2.5	3	2	1	0
	10	3	3	0	0
	25	3	3	0	0
	100	3	3	0	0
	250	3	3	0	0
<b>Total 4%</b>		<b>15</b>	<b>14</b>	<b>1</b>	<b>0</b>
100%	2.5	3	3	0	0
	10	3	3	0	0
	25	3	3	0	0
	100	3	3	0	0
	250	3	3	0	0
<b>Total 100%</b>		<b>15</b>	<b>15</b>	<b>0</b>	<b>0</b>

## Clinical studies

DNA samples from 98 subjects, previously characterized with an independent method, were analyzed together with 9 DNA samples from healthy donors using the *ipsogen* JAK2 MutaScreen EZ Kit. Results are summarized in Table 14.

**Table 14. Results for previously characterized samples using the *ipsogen* JAK2 MutaScreen EZ Kit**

<b>Sample</b>	<b>Replicates</b>	<b>Mutation detected</b>	<b>Result inconclusive</b>	<b>Mutation not detected</b>	<b>Test failure</b>
JAK2 wild type	50	0	1	49	0
0% JAK2 V617F <Sample ≤ 2%	9	2	1	6	0
JAK2 V617F >5%	48	47	0	0	1

For 98% of expected negative samples the mutation was not detected.

For 100% of expected positive samples the mutation was detected.

When equivocal results are excluded (i.e., samples below 2% or inconclusive results), overall agreement is 100%.

## References

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## Symbols

The following symbols may appear on the packaging and labeling:



Contains reagents sufficient for <N> reactions



Use by



In vitro diagnostic medical device



Catalog number



Lot number



Material number



Global Trade Item Number



Temperature limitation



Manufacturer



Consult instructions for use

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<b>Rotor-Gene Q MDx — for IVD-validated real-time PCR analysis in clinical applications</b>		
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9002032
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