

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



Version 1



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



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## Intended Use

The *ipsogen* JAK2 MutaScreen RS Kit is intended for the detection of 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) <math>&gt;18.5 \text{ g.dl}^{-1}</math> (men) or <math>&gt;16.5 \text{ g.dl}^{-1}</math> (women) or Hgb or hematocrit (Hct) <math>&gt;99\text{th}</math> percentile of reference range for age, sex, or altitude of residence or Hgb <math>&gt;17 \text{ g.dl}^{-1}</math> (men) or <math>&gt;15 \text{ g.dl}^{-1}</math> (women) if associated with sustained increase of <math>\geq 2 \text{ g.dl}^{-1}</math> from baseline that cannot be attributed to correction of iron deficiency or Elevated red cell mass <math>&gt;25\%</math> above mean normal predicted value</p> <p>2. Presence of <i>JAK2V617F</i> or similar mutation</p> |
| Minor  | <p>1. Bone marrow trilineage myeloproliferation</p> <p>2. Subnormal serum erythropoietin level</p> <p>3. Endogenous erythroid colony (EEC) growth</p>   |
| Criteria for a diagnosis of essential thrombocythemia (ET) |   |
| Major  | <p>1. Platelet count <math>\geq 450 \times 10^9 \text{ l}^{-1}</math></p> <p>2. Megakaryocyte proliferation with large and mature morphology. No or little granulocyte or erythroid proliferation</p> <p>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)</p> <p>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</p> <p>2. Increased serum lactate dehydrogenase (LDH)</p> <p>3. Anemia</p> <p>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

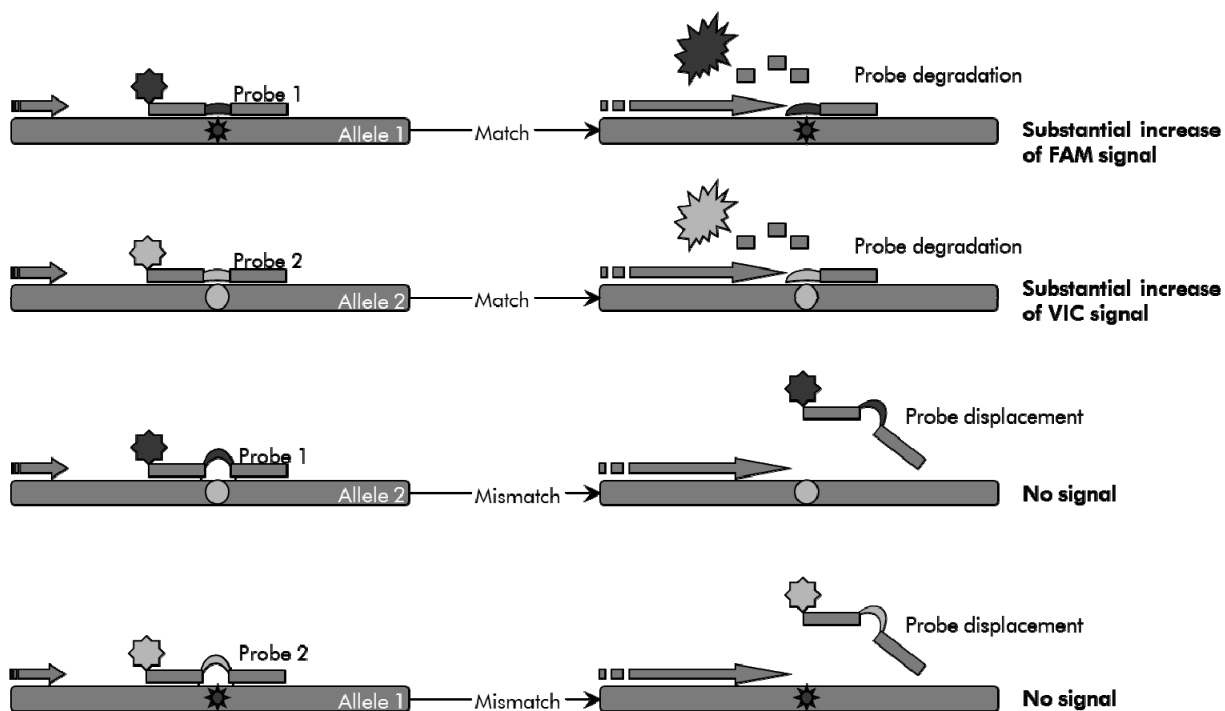
observed in response to some of the new anti-JAK2 targeted drugs in clinical development (10).

## 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), 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* 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* 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 RS Kit uses this technology as illustrated (see Figure 1).



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

# Materials Provided

## Kit contents

|  |            |               |
|--|------------|---------------|
| <b><i>ipsogen JAK2 MutaScreen RS Kit</i></b>             |            | <b>(19)</b>   |
| <b>Catalog no.</b>                                       |            | <b>673123</b> |
| <b>Number of reactions</b>                               |            | <b>19</b>     |
| V617F Positive Control*                                  | MP-VF      | 30 µl         |
| V617F Negative Control†                                  | MN-VF      | 30 µl         |
| Reference Scale M1‡                                      | M1-VF      | 30 µl         |
| Reference Scale M2‡                                      | M2-VF      | 30 µl         |
| Reference Scale M3‡                                      | M3-VF      | 30 µl         |
| Reference Scale M4‡                                      | M4-VF      | 30 µl         |
| Reference Scale M5‡                                      | M5-VF      | 30 µl         |
| Reference Scale M6‡                                      | M6-VF      | 30 µl         |
| Primers and probes mix<br>JAK2 V617F§                    | PPM-VF 10x | 145 µl        |
| <i>ipsogen JAK2 MutaScreen RS Kit Handbook (English)</i> |            | 1             |

\* Positive control: 100% V617F DNA.

† Negative control: 100% wild type DNA; 0% V617F DNA.

‡ Reference scale (genomic DNA dilutions).

§ 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 RS Kit* requires the extraction of genomic DNA. Reagents needed to perform DNA extraction (e.g., QIAGEN® QIAamp® DNA Mini Kit, cat. no.51304) 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 PCR grade water
- Nuclease-free 1x TE buffer, pH 8.0 (e.g., Life Technologies, cat. no. 12090-015)
- Buffer and *Taq* DNA polymerase: The validated reagents are TaqMan Universal PCR Master Mix (Master Mix PCR 2x) (Life Technologies, cat. no. 4304437) and LightCycler TaqMan Master (Master Mix PCR 5x) (Roche, cat. no. 04535286001)
- 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 MDx 5plex HRM<sup>®</sup> or other Rotor-Gene instrument; LightCycler 2.0, or 480; Applied Biosystems 7300 Real-Time PCR System, Applied Biosystems 7500 Real-Time PCR System, ABI PRISM 7000 SDS, ABI PRISM 7700 SDS, or ABI PRISM 7900HT SDS; and associated specific material
- Equipment\* for pulsed-field gel electrophoresis

\* 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.

### 24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

## 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-VF 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, plasmid) 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-VF 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.

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.

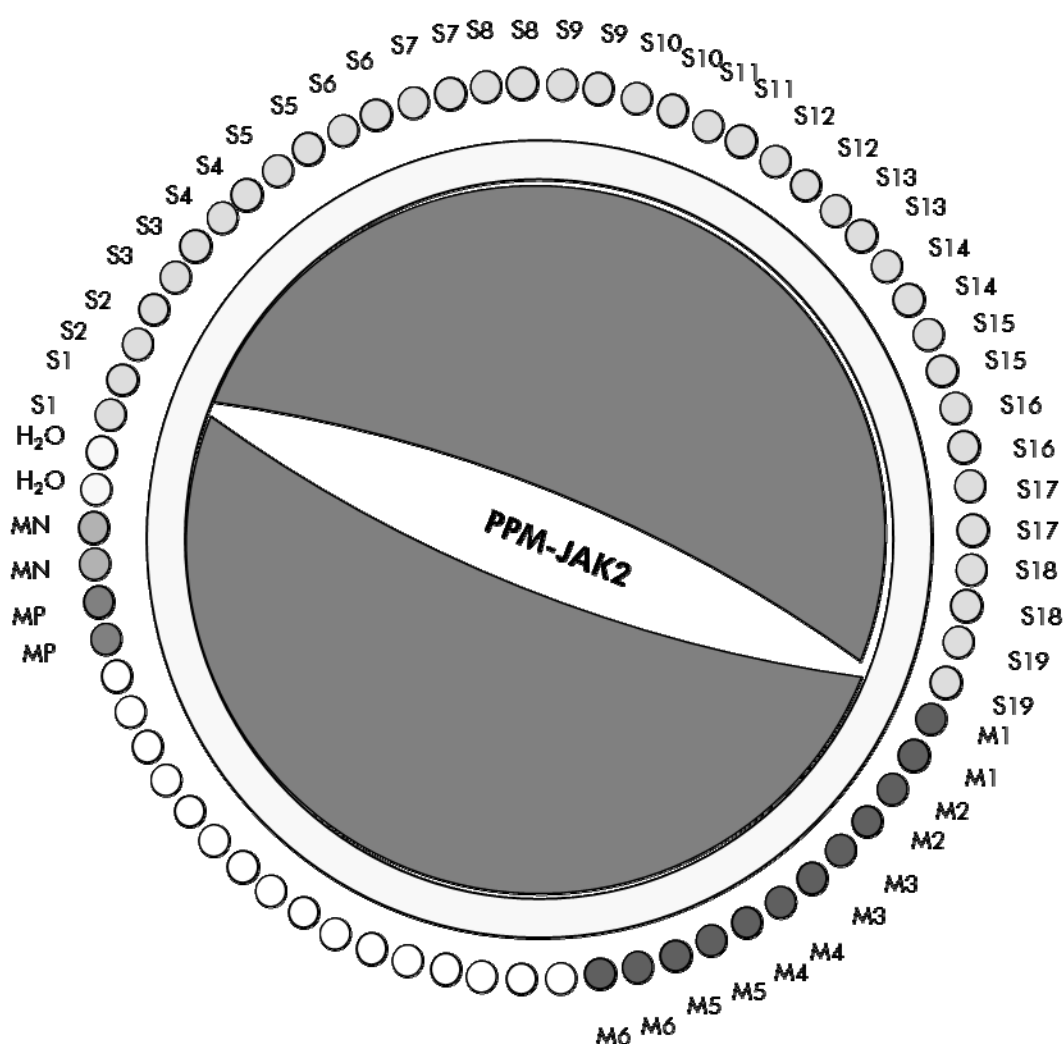
### Protocol: qPCR on Rotor Gene Q instruments with 72-tube rotor

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

**Table 2. Number of reactions for Rotor Gene Q MDx 5plex HRM or Rotor Gene Q 5plex HRM instruments with 72-tube rotor**

| Samples  | Reactions  |
|--|--|
| <b>JAK2 V617F primers and probes mix (PPM-VF) (56 reactions)</b> |  |
| 19 DNA samples   | 19 x 2 reactions   |
| 2 DNA controls   | 2 x 2 reactions (MP-VF, MN-VF, each one tested in duplicate) |
| Reference scale  | 6 x 2 reactions (M1 to M6, 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 RS Kit.** MP-VF: positive control; MN-VF: negative control; M1 to M6: reference scale; 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 minutes before starting the procedure.

2. **Vortex and briefly centrifuge all the tubes (approximately 10 seconds, 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 RS Kit can be used for analysis of 19 samples in duplicate in one experiment (Figure 2).

**Table 3. Preparation of qPCR mix**

| Component                           | Number of reactions ( $\mu$ l) |         | Final concentration |
|-------------------------------------|--------------------------------|---------|---------------------|
|                                     | 1                              | 56+1*   |                     |
| TaqMan Universal PCR Master Mix, 2x | 12.5                           | 712.5   | 1x                  |
| Primers and probes mix, 10x         | 2.5                            | 142.5   | 1x                  |
| Nuclease-free PCR grade water       | 5                              | 285     | –                   |
| Sample (to be added at step 4)      | 5                              | 5 each  | –                   |
| Total volume                        | 25                             | 25 each | –                   |

\* 19 samples; 1 experiment/kit.

4. **Vortex and briefly centrifuge the qPCR mix (approximately 10 seconds, 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.

**Setting the general assay parameters**

**Figures 3, 4**

**Amplification of the DNA**

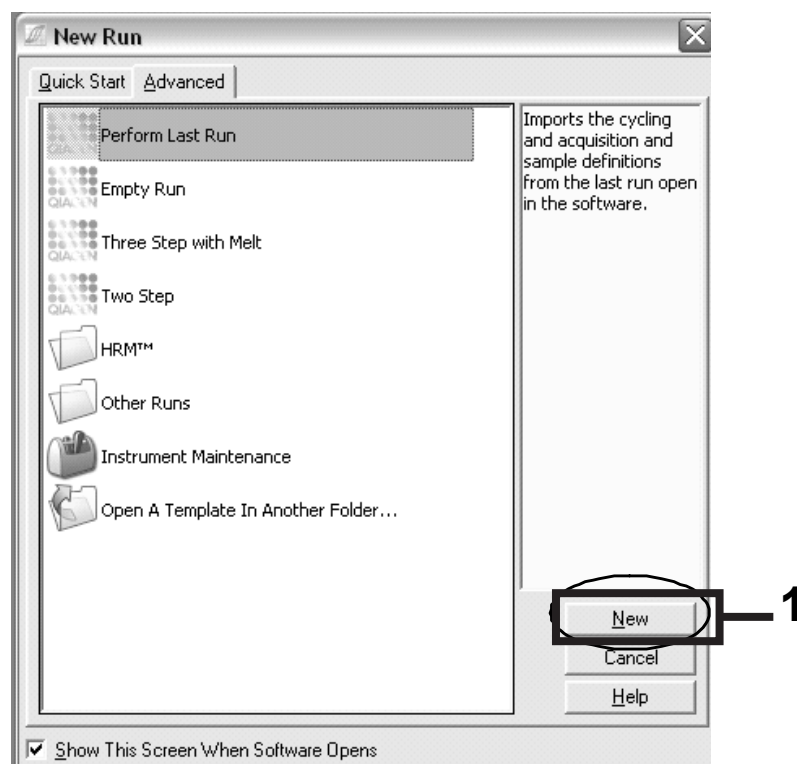
**Figure 5**

**Adjusting the fluorescence channel sensitivity**

**Figure 6**

Please find further information on programming Rotor-Gene Instruments 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. The "New Run" dialog box.**

12. In the “New Run Wizard”, set the volume to 25  $\mu$ l and click “Next”.

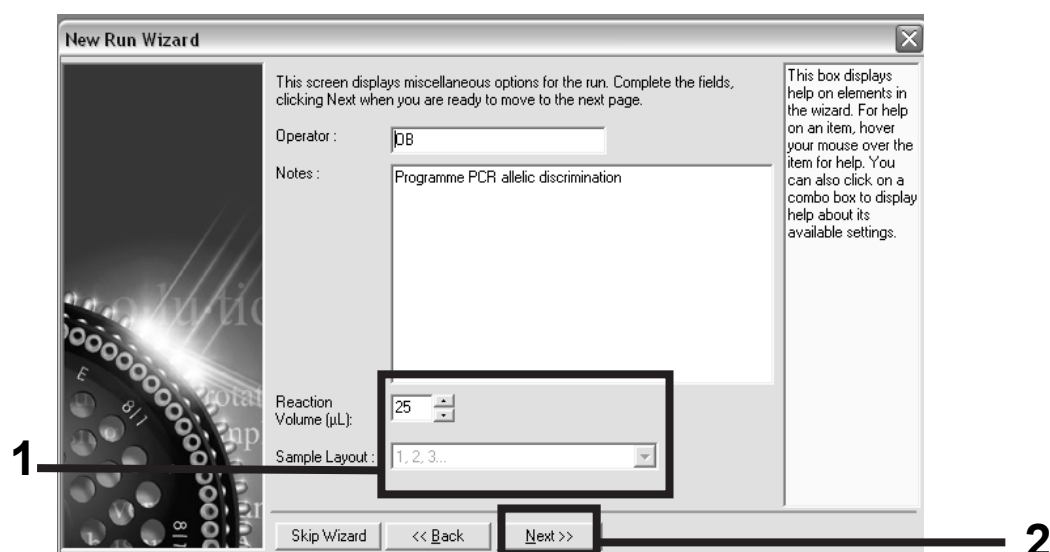


Figure 4. Setting the general assay parameters.

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



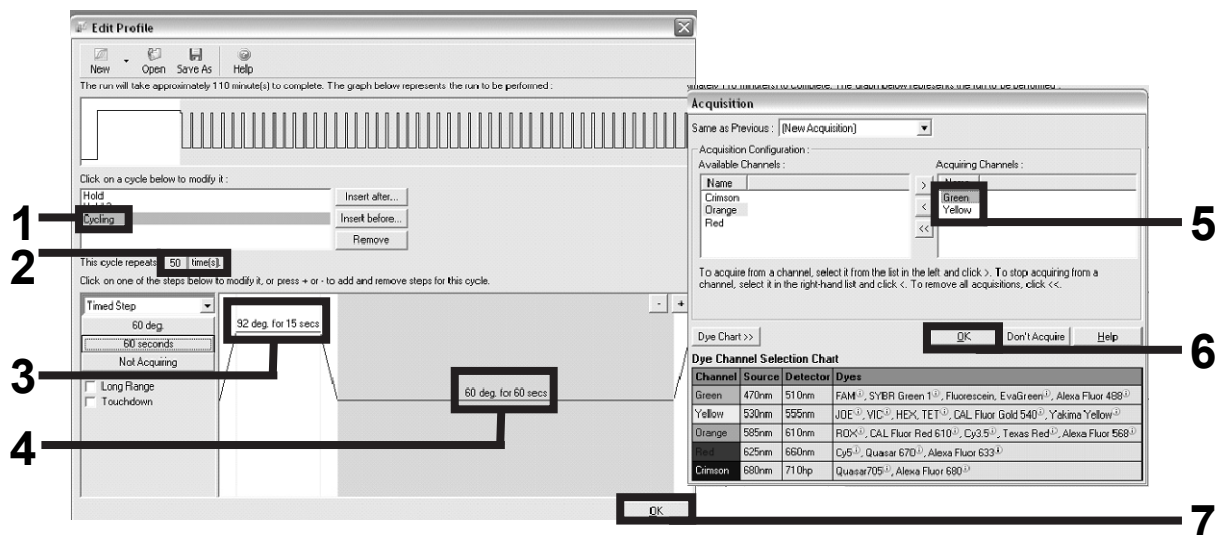


Figure 5. Amplification of the DNA.

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).

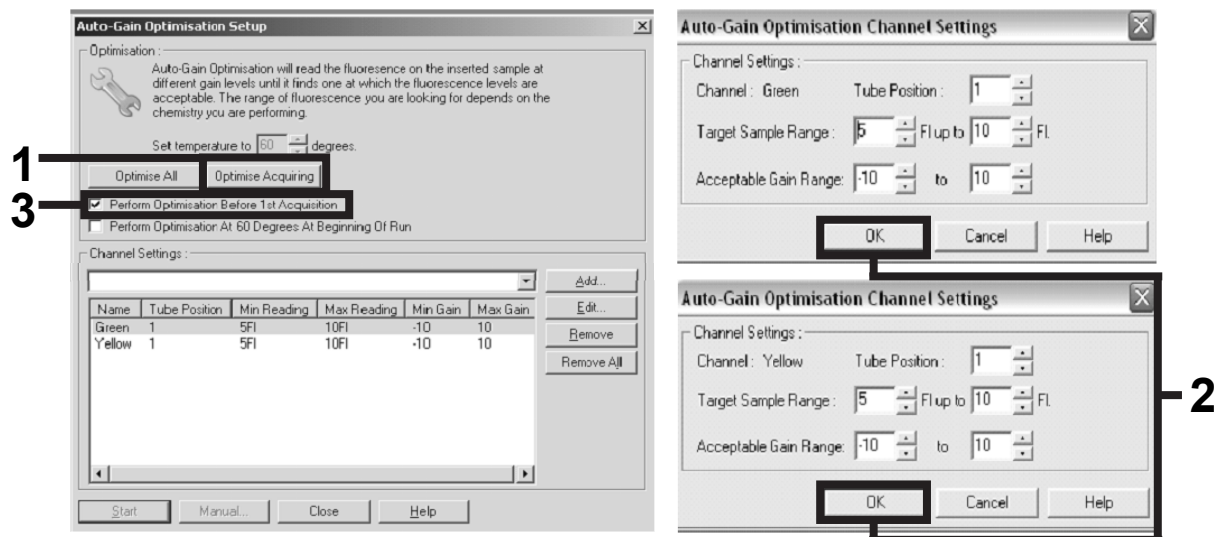


Figure 6. Adjusting the fluorescence channel sensitivity.

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).



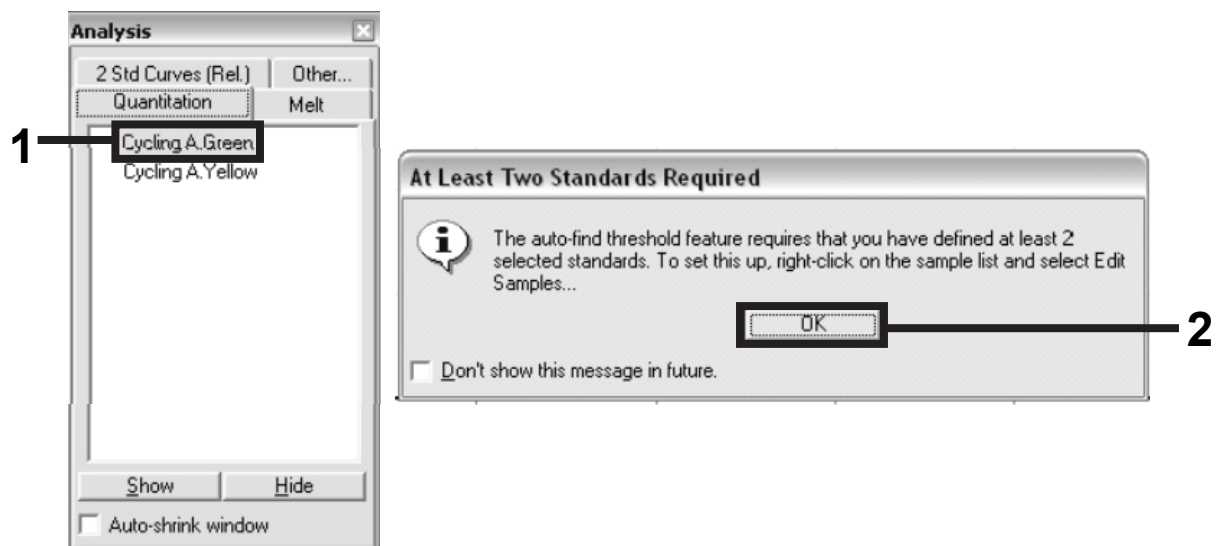


Figure 9. Quantitation: "Cycling A. Green".

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

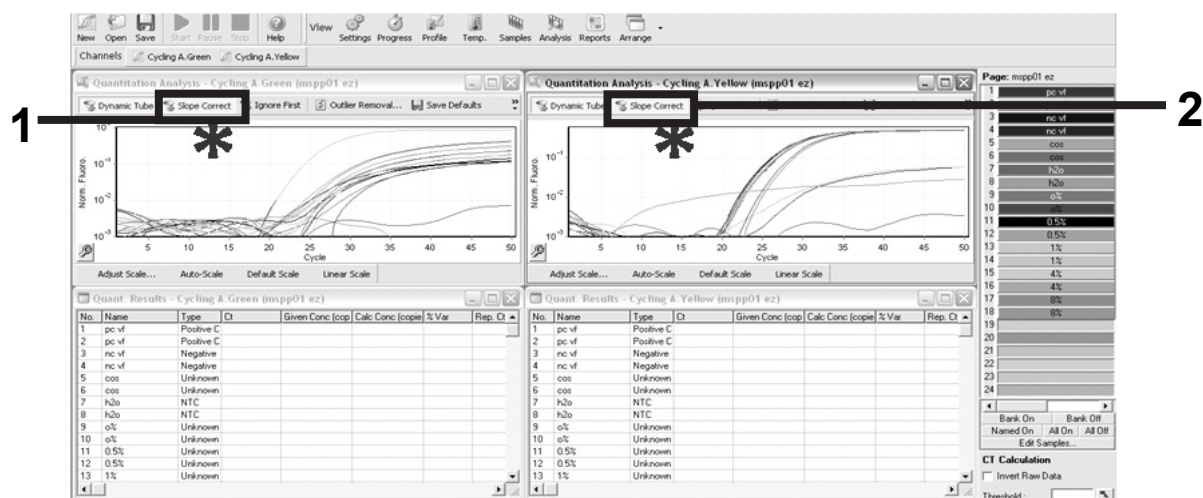


Figure 10. Setting "Slope Correct".

20. To export data, save as an Excel® data sheet. 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. Click "Data", then "Convert", and "Next". Select "Comma" and then click "End". The results will appear as shown in Figure 11.



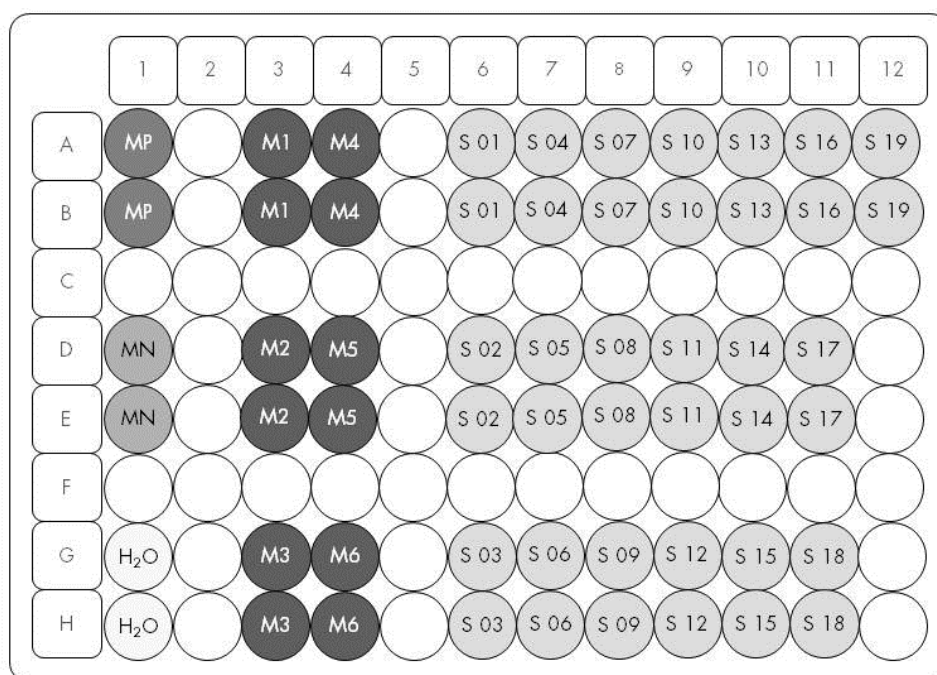
## Protocol: qPCR on Applied Biosystems and ABI PRISM instruments

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

**Table 5. Number of reactions for Applied Biosystems 7300 and 7500, ABI PRISM 7000, ABI PRISM 7700, or ABI PRISM 7900HT instruments**

| Samples  | Reactions  |
|--|--|
| <b>JAK2 V617F primers and probes mix (PPM-VF) (56 reactions)</b> |  |
| 19 DNA samples   | 19 x 2 reactions   |
| 2 DNA controls   | 2 x 2 reactions (MP-VF, MN-VF, each one tested in duplicate) |
| Reference scale  | 6 x 2 reactions (M1 to M6, each one tested in duplicate)     |
| Water control  | 2 reactions  |

### Sample processing on Applied Biosystems 7300 and 7500, ABI PRISM 7000 and 7700, or ABI PRISM 7900HT instruments



**Figure 12. Suggested plate setup for an experiment with the ipsogen JAK2 MutaScreen RS Kit.** MP: positive control; MN: negative control; M1 to M6: reference scale; S: DNA sample; H<sub>2</sub>O: water control.

## qPCR on Applied Biosystems 7300 and 7500, ABI PRISM 7000, ABI PRISM 7700, 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 minutes before starting the procedure.

#### 2. Vortex and briefly centrifuge all the tubes (approximately 10 seconds, 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 7300 and 7500, ABI PRISM 7000, ABI PRISM 7700, or ABI PRISM 7900HT instruments, the *ipsogen* JAK2 MutaScreen RS Kit can be used for analysis of 19 samples in duplicate in one experiment (Figure 12).

**Table 6. Preparation of qPCR mix**

| Component                           | Number of reactions ( $\mu$ l) |         | Final concentration |
|-------------------------------------|--------------------------------|---------|---------------------|
|                                     | 1                              | 56+1*   |                     |
| TaqMan Universal PCR Master Mix, 2x | 12.5                           | 712.5   | 1x                  |
| Primers and probes mix, 10x         | 2.5                            | 142.5   | 1x                  |
| Nuclease-free PCR grade water       | 5                              | 285     | –                   |
| Sample (to be added at step 4)      | 5                              | 5 each  | –                   |
| Total volume                        | 25                             | 25 each | –                   |

\* 19 samples; 1 experiment/kit.

4. **Vortex and briefly centrifuge the qPCR mix (approximately 10 seconds, 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 seconds).**
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 and ABI PRISM instruments**

|                |  |
|----------------|--|
| <b>Hold</b>    | Temperature: 50°C<br>Time: 2 minutes                 |
| <b>Hold 2</b>  | Temperature: 95°C<br>Time: 10 minutes                |
| <b>Cycling</b> | 50 times<br>92°C for 15 seconds<br>60°C for 1 minute |

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

For programming details of the Applied Biosystems 7300 and 7500, ABI PRISM 7000, ABI PRISM 7700, or 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.

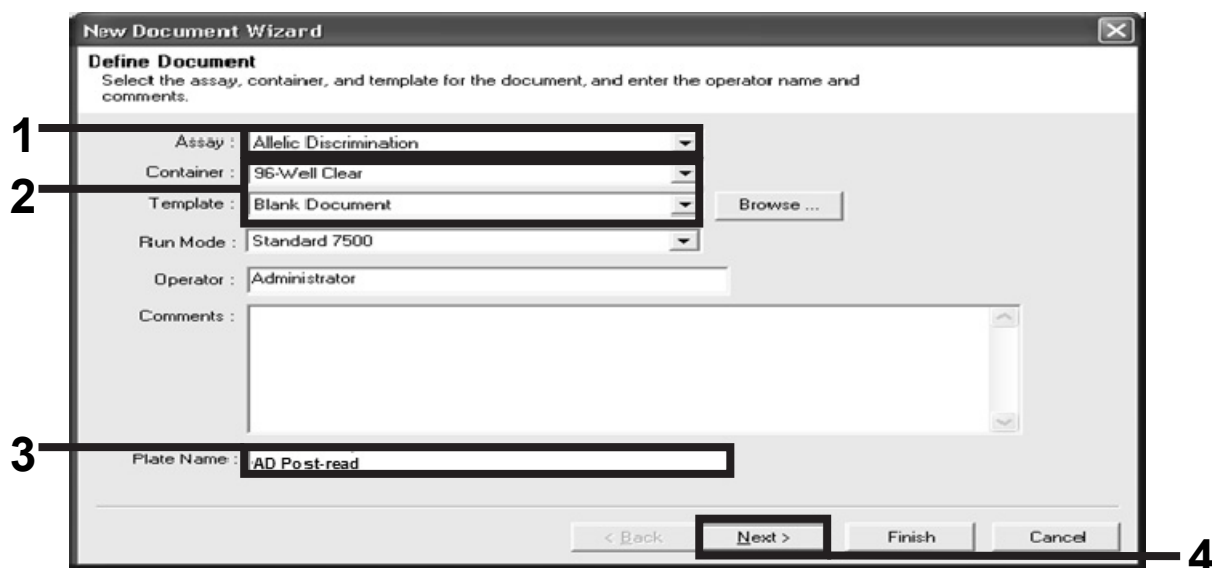


Figure 13. Pre-settings for creating a new post-read run (New Document Wizard).

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).

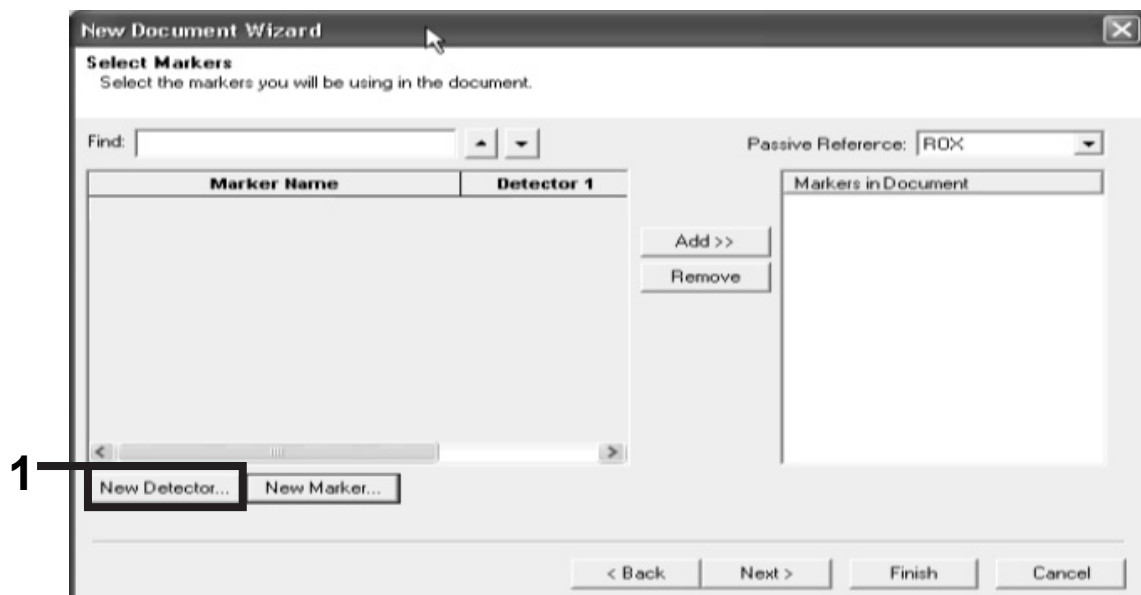


Figure 14. The "Markers in Document" panel does not contain a suitable marker for your application.



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).

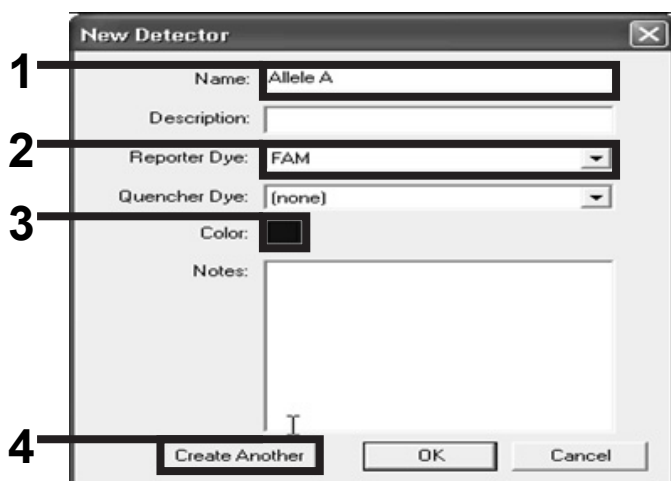


Figure 15. Creating detectors.

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).

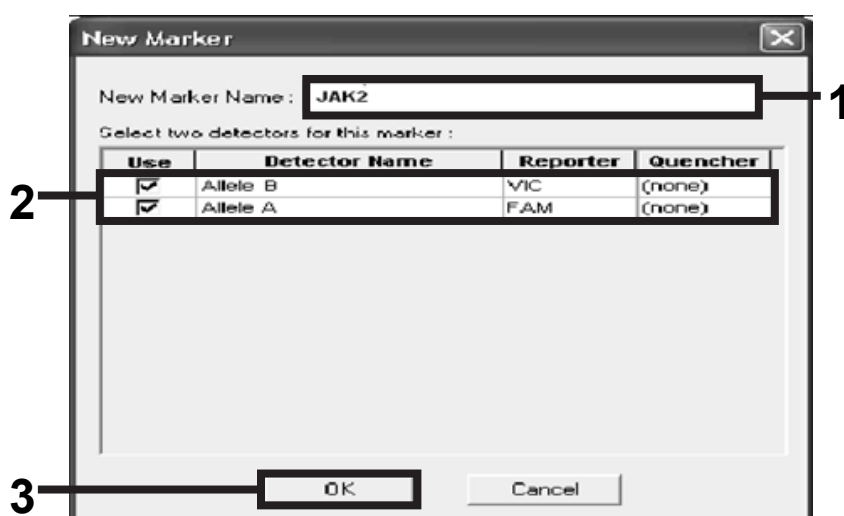


Figure 16. Creating markers.

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”.

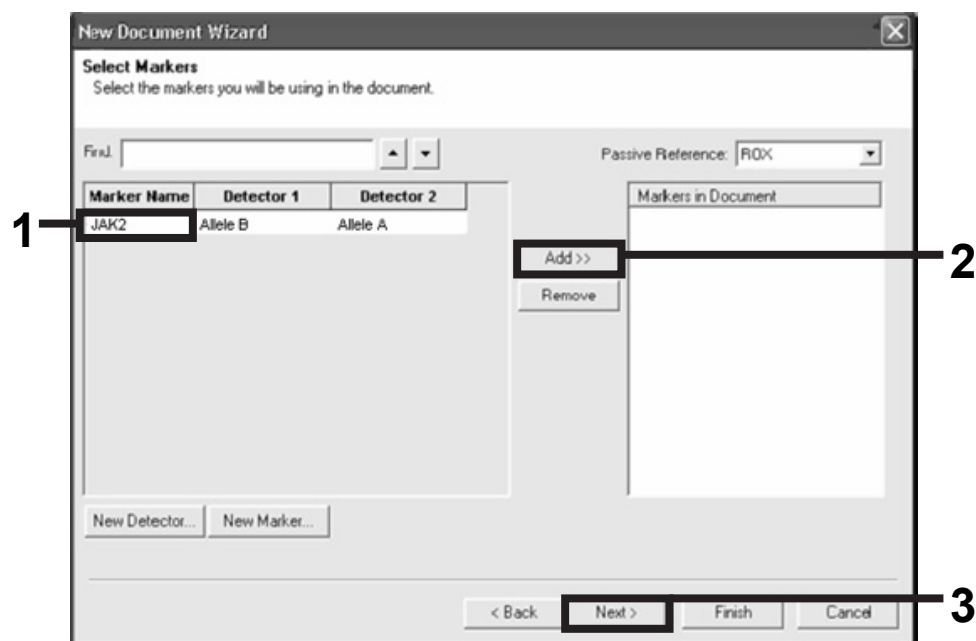


Figure 17. Selecting markers.

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 seconds at 60°C. During this run, the instrument collects FAM and VIC fluorescence in each well (Figure 18).

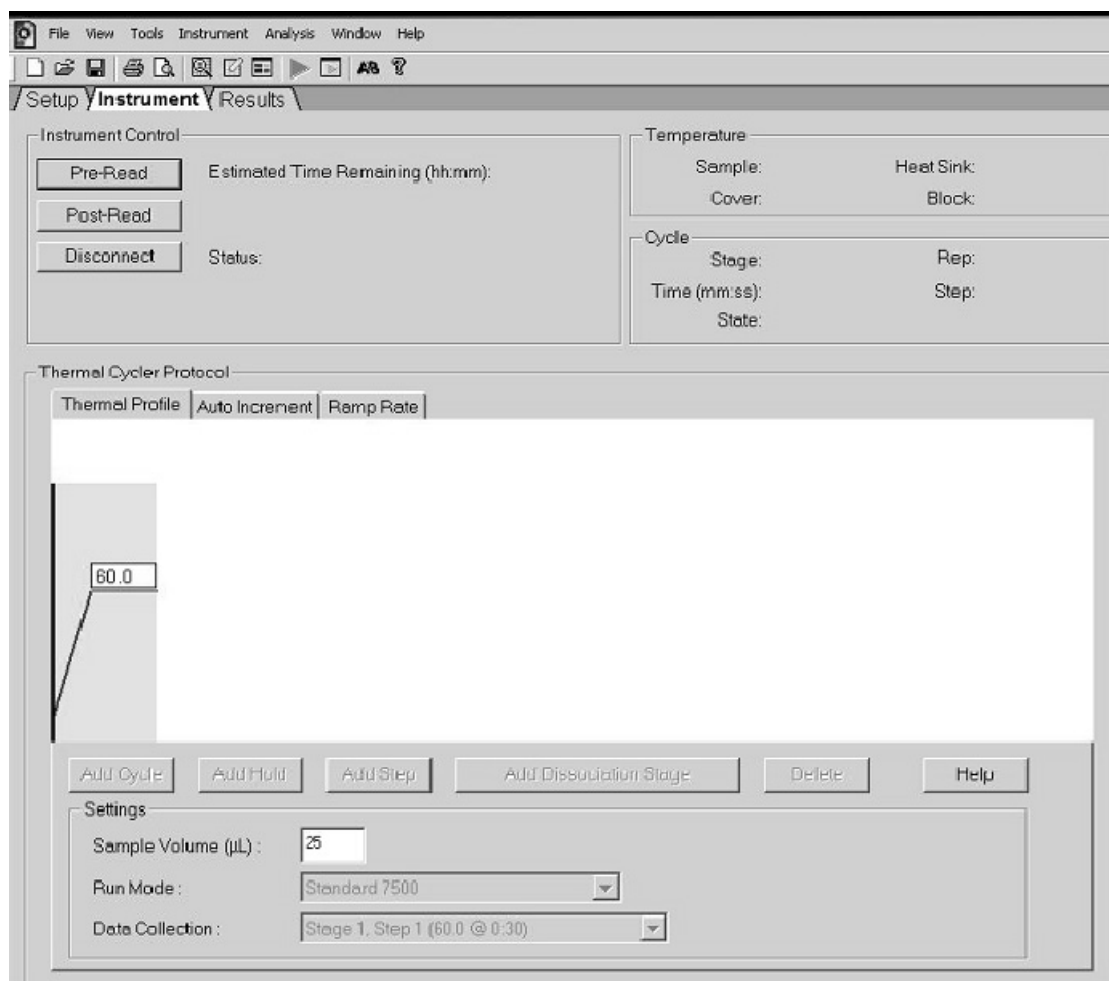


Figure 18. Post-read run.

**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.**

|    |           |             |        |         |              |          |          |             |              |              |               |             |
|----|-----------|-------------|--------|---------|--------------|----------|----------|-------------|--------------|--------------|---------------|-------------|
| 12 | Comments: |             |        |         | VIC Sample 1 |          |          |             | FAM Sample 1 |              |               |             |
| 13 | SDS v1.2  |             |        |         |              |          |          |             |              |              |               |             |
| 14 |           |             |        |         |              |          |          |             |              |              |               |             |
| 15 | Well      | Sample Name | Marker | Task    | Passive Ref  | Allele X | Allele Y | Allele X Rn | Allele Y Rn  | Call         | Quality Value | Method      |
| 16 | A1        | sample 1    | VIC    | Unknown | 247.897      | JAK2-VIC | JAK2-FAM | 2.184       | 6.221        | Undetermined | 100.00        | Manual Call |
| 17 | A2        | sample 1    | VIC    | Unknown | 295.565      | JAK2-VIC | JAK2-FAM | 2.451       | 6.805        | Undetermined | 100.00        | Manual Call |
| 18 | A3        | sample 2    | VIC    | Unknown | 351.338      | JAK2-VIC | JAK2-FAM | 2.595       | 6.2          | Undetermined | 100.00        | Manual Call |
| 19 | A4        | sample 2    | VIC    | Unknown | 379.909      | JAK2-VIC | JAK2-FAM | 2.553       | 6.01         | Undetermined | 100.00        | Manual Call |
| 20 | A5        | sample 3    | VIC    | Unknown | 372.895      | JAK2-VIC | JAK2-FAM | 2.913       | 5.329        | Undetermined | 100.00        | Manual Call |
| 21 | A6        | sample 3    | VIC    | Unknown | 359.717      | JAK2-VIC | JAK2-FAM | 2.806       | 5.278        | Undetermined | 100.00        | Manual Call |
| 22 | A7        | sample wt   | VIC    | Unknown | 343.536      | JAK2-VIC | JAK2-FAM | 2.569       | 1.948        | Undetermined | 100.00        | Manual Call |
| 23 | A8        | sample wt   | VIC    | Unknown | 277.677      | JAK2-VIC | JAK2-FAM | 2.684       | 2.015        | Undetermined | 100.00        | Manual Call |
| 24 | A9        | C-          | VIC    | Unknown | 330.943      | JAK2-VIC | JAK2-FAM | 2.623       | 1.967        | Undetermined | 100.00        | Manual Call |
| 25 | A10       | C-          | VIC    | Unknown | 314.623      | JAK2-VIC | JAK2-FAM | 2.672       | 2.013        | Undetermined | 100.00        | Manual Call |
| 26 | A11       | C-          | VIC    | Unknown | 269.500      | JAK2-VIC | JAK2-FAM | 2.82        | 1.892        | Undetermined | 100.00        | Manual Call |
| 27 | A12       | C+          | VIC    | Unknown | 211.520      | JAK2-VIC | JAK2-FAM | 1.249       | 6.14         | Undetermined | 100.00        | Manual Call |
| 28 | B1        | C+          | VIC    | Unknown | 270.623      | JAK2-VIC | JAK2-FAM | 1.346       | 6.894        | Undetermined | 100.00        | Manual Call |
| 29 | B2        | C+          | VIC    | Unknown | 365.112      | JAK2-VIC | JAK2-FAM | 1.265       | 6.528        | Undetermined | 100.00        | Manual Call |
| 30 | B3        | ER          | VIC    | Unknown | 372.150      | JAK2-VIC | JAK2-FAM | 2.214       | 2.03         | Undetermined | 100.00        | Manual Call |
| 31 | B4        | ER          | VIC    | Unknown | 404.145      | JAK2-VIC | JAK2-FAM | 2.419       | 2.295        | Undetermined | 100.00        | Manual Call |
| 32 | B5        | ER          | VIC    | Unknown | 410.977      | JAK2-VIC | JAK2-FAM | 2.681       | 2.52         | Undetermined | 100.00        | Manual Call |
| 33 | B6        | H2O         | VIC    | Unknown | 395.431      | JAK2-VIC | JAK2-FAM | 0.655       | 1.346        | Undetermined | 100.00        | Manual Call |
| 34 | B7        | H2O         | VIC    | Unknown | 415.223      | JAK2-VIC | JAK2-FAM | 0.727       | 1.241        | Undetermined | 100.00        | Manual Call |
| 35 | 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.**

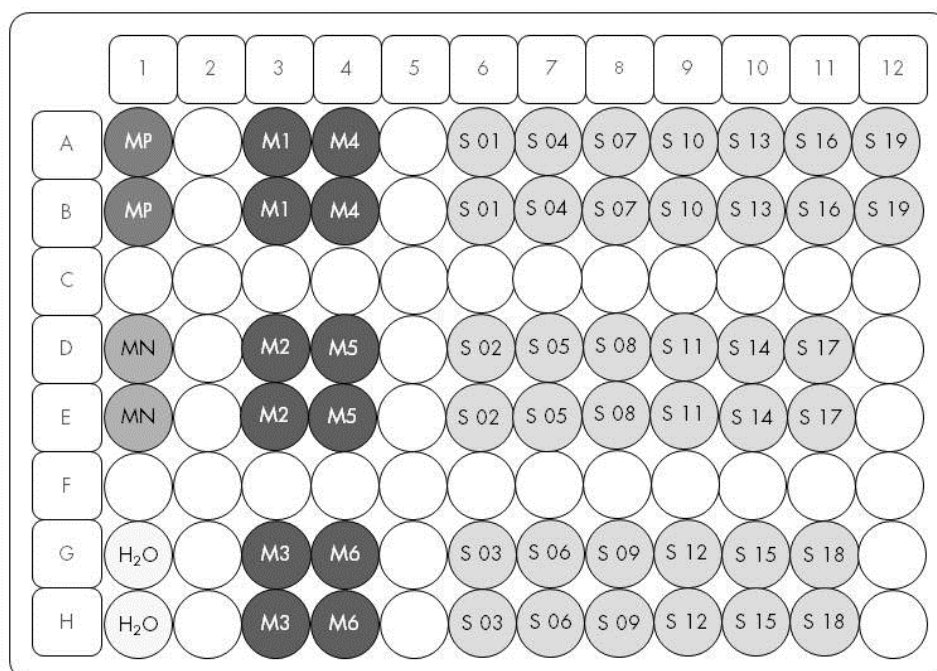
## Protocol: qPCR on the LightCycler 480 instrument

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>JAK2 V617F primers and probes mix (PPM-VF) (56 reactions)</b> |  |
| 19 DNA samples   | 19 x 2 reactions   |
| 2 DNA controls   | 2 x 2 reactions (MP-VF, MN-VF, each one tested in duplicate) |
| Reference scale  | 6 x 2 reactions (M1 to M6, 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 RS Kit.** MP: positive control; MN: negative control; M1 to M6: reference scale; 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 minutes before starting the procedure.

**2. Vortex and briefly centrifuge all the tubes (approximately 10 seconds, 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 Applied Biosystems 7300 and 7500, ABI PRISM 7000, ABI PRISM 7700, or ABI PRISM 7900HT instruments, the *ipsogen* JAK2 MutaScreen RS Kit can be used for analysis of 19 samples in duplicate in one experiment (Figure 20).

**Table 9. Preparation of qPCR mix**

| Component                           | Number of reactions ( $\mu$ l) |         | Final concentration |
|-------------------------------------|--------------------------------|---------|---------------------|
|                                     | 1                              | 56+1*   |                     |
| TaqMan Universal PCR Master Mix, 2x | 12.5                           | 712.5   | 1x                  |
| Primers and probes mix, 10x         | 2.5                            | 142.5   | 1x                  |
| Nuclease-free PCR grade water       | 5                              | 285     | –                   |
| Sample (to be added at step 4)      | 5                              | 5 each  | –                   |
| Total volume                        | 25                             | 25 each | –                   |

\* 19 samples; 1 experiment/kit.

4. Vortex and briefly centrifuge the qPCR mix (approximately 10 seconds, 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 seconds).
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.

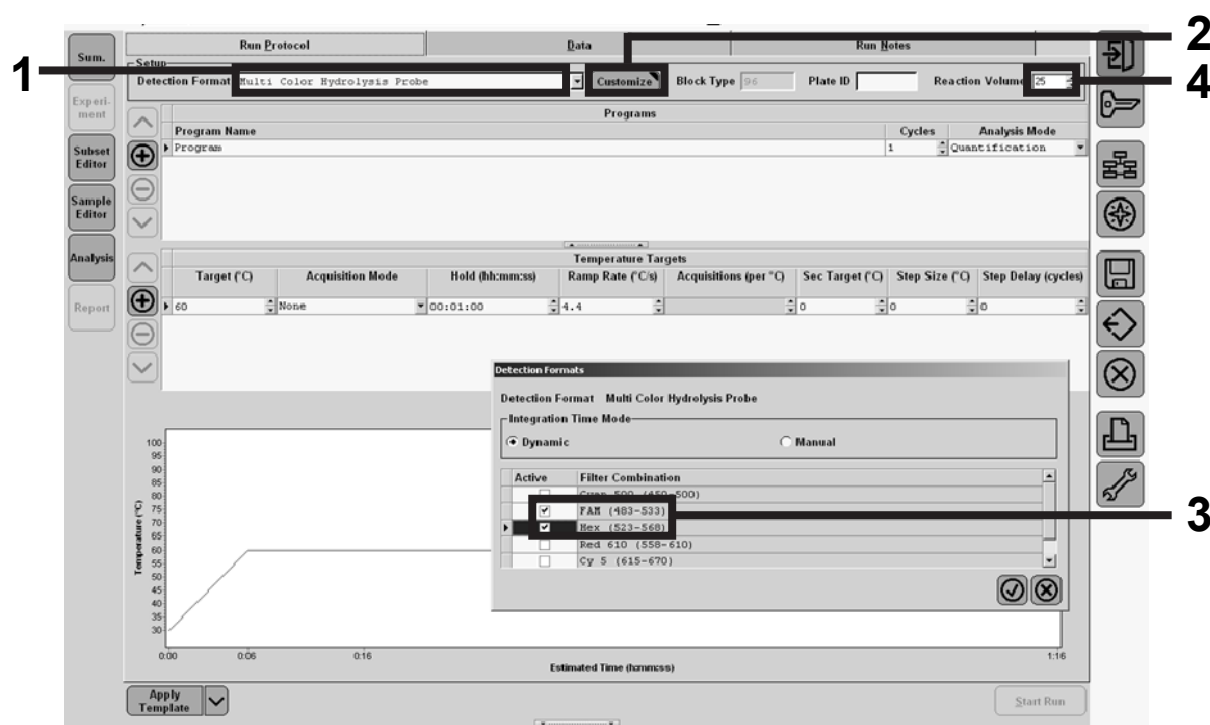


Figure 21. LightCycler 480 I: Setting the detection format.

- 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.

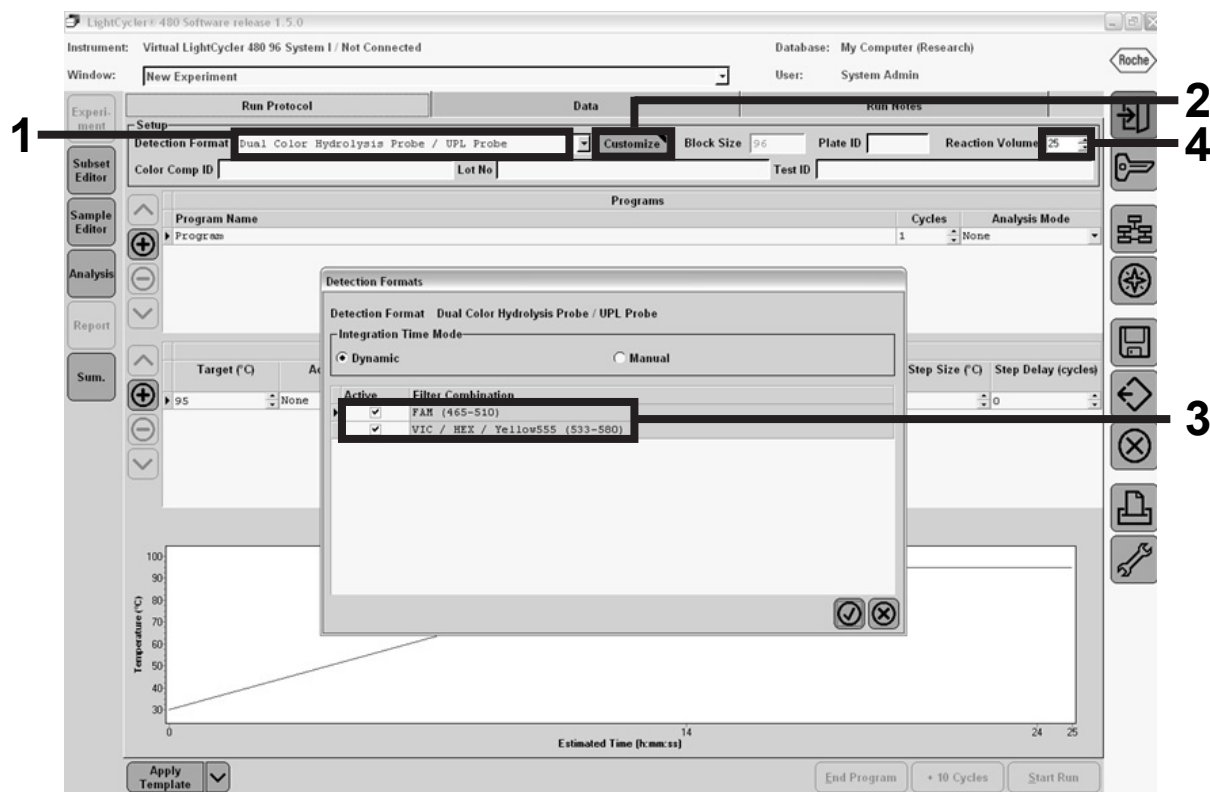


Figure 22. LightCycler 480 II: Setting the detection format.



**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.

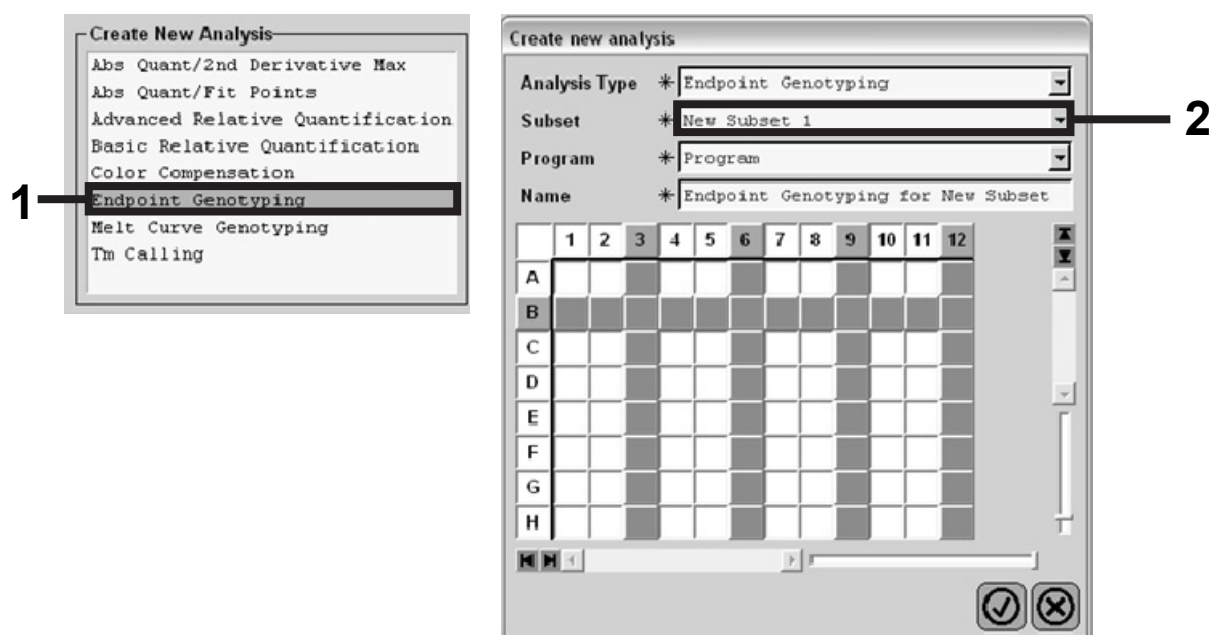
**Table 10. Temperature profile for the LightCycler 480 instrument**

|                |  |
|----------------|--|
| <b>Hold</b>    | Temperature: 50°C<br>Time: 2 minutes                                 |
| <b>Hold 2</b>  | Temperature: 95°C<br>Time: 10 minutes                                |
| <b>Cycling</b> | 50 times<br>92°C for 15 seconds; single<br>60°C for 1 minute; single |
| <b>Hold 3</b>  | 60°C for 1 minute; 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).

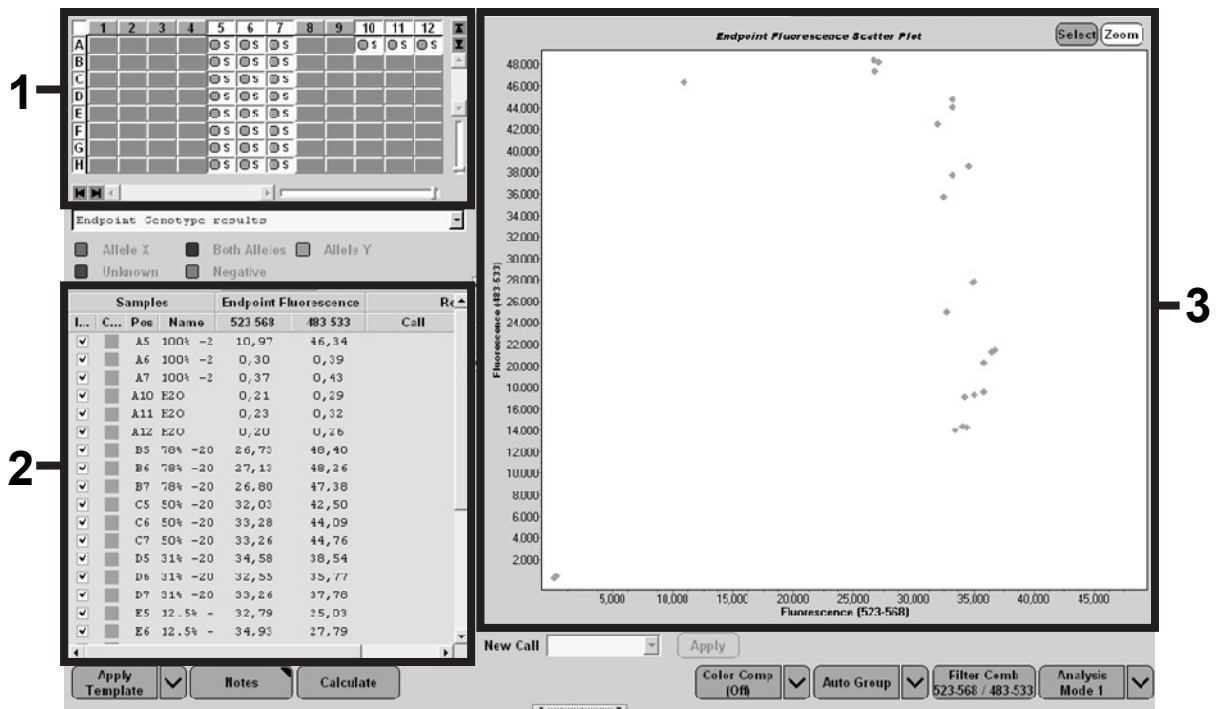
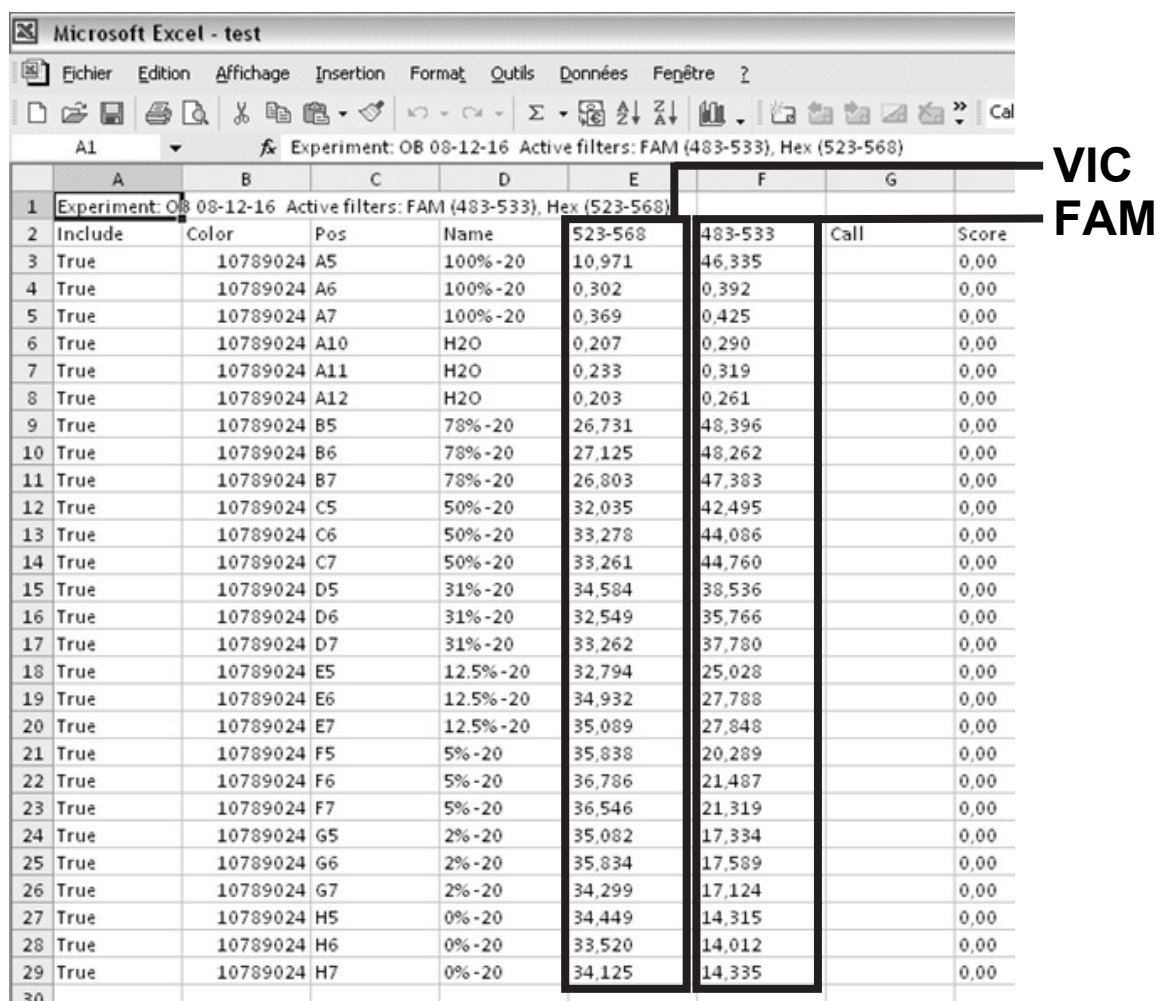


Figure 25. Data summary.

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  |
| 3  | True   | 10789024 | A5  | 100%-20  | 10,971  | 46,335  | Score |
| 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.

## Protocol: qPCR on LightCycler 2.0 instrument

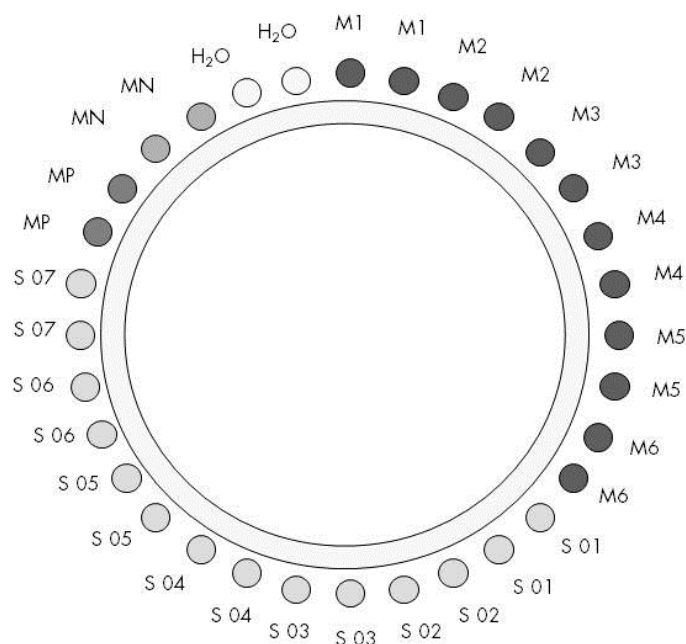
**Note:** Because of particular technological requirements, LightCycler 2.0 experiments must be performed using specific reagents. We recommend the use of the LightCycler TaqMan Master. Follow the manufacturer's instructions to prepare the Master Mix 5x.

Using a 32-capillary rotor, we recommend performing all measurements in duplicate as indicated in Table 11.

**Table 11. Number of reactions for LightCycler 2.0 instrument**

| Samples  | Reactions  |
|--|--|
| <b>JAK2 V617F primers and probes mix (PPM-VF) (32 reactions)</b> |  |
| 7 DNA samples  | 7 x 2 reactions  |
| 2 DNA controls   | 2 x 2 reactions (MP-VF, MN-VF, each one tested in duplicate) |
| Reference scale  | 6 x 2 reactions (M1 to M6, each one tested in duplicate)     |
| Water control  | 2 reactions  |

## Sample processing on LightCycler 2.0 instrument



**Figure 27. Suggested rotor setup for an experiment with the ipsogen JAK2**

**MutaScreen RS Kit.** MP: positive control; MN: negative control; M1 to M6: reference scale; S: DNA sample; H<sub>2</sub>O: water control.

## qPCR on LightCycler 2.0 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 minutes before starting the procedure.

**2. Vortex and briefly centrifuge all the tubes (approximately 10 seconds, 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 12 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 20  $\mu$ 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 2.0 instrument, the *ipsogen* JAK2 MutaScreen RS Kit can be used for analysis of 7 samples in duplicate in one experiment (Figure 27).

**Table 12. Preparation of qPCR mix for LightCycler 2.0 instrument**

| Component                         | Number of reactions ( $\mu$ l) |         | Final concentration |
|-----------------------------------|--------------------------------|---------|---------------------|
|                                   | 1                              | 32+1*   |                     |
| LightCycler TaqMan Master Mix, 5x | 4                              | 132     | 1x                  |
| Primers and probes mix, 10x       | 2                              | 66      | 1x                  |
| Nuclease-free PCR grade water     | 9                              | 297     | –                   |
| Sample (to be added at step 4)    | 5                              | 5 each  | –                   |
| Total volume                      | 20                             | 20 each | –                   |

\* 14 samples; 2 experiments/kit.

4. Vortex and briefly centrifuge the qPCR mix (approximately 10 seconds, 10,000 rpm, to collect the liquid in the bottom of the tube).
5. Dispense 15  $\mu$ l of the qPCR pre-mix per capillary.
6. Add 5  $\mu$ l of the sample DNA material or controls in the corresponding capillary (total volume 20  $\mu$ l).
7. Mix gently, by pipetting up and down.
8. Place the capillaries in the adapter provided with the instrument and briefly centrifuge (700 x g, approximately 10 seconds).
9. Load the samples in the thermal cycler according to the manufacturer's recommendations.
10. Program the thermal cycler (Figure 28) with the program as indicated in Table 13.

For programming details of the LightCycler 2.0 instrument, refer to the instrument user guide. For a better overview, the software settings are framed in bold black.

**Note:** Make sure that the setting is for Quantification and single acquisition of FAM fluorescence and single acquisition of VIC fluorescence in both the amplification/cycling step and the final hold at 60°C.

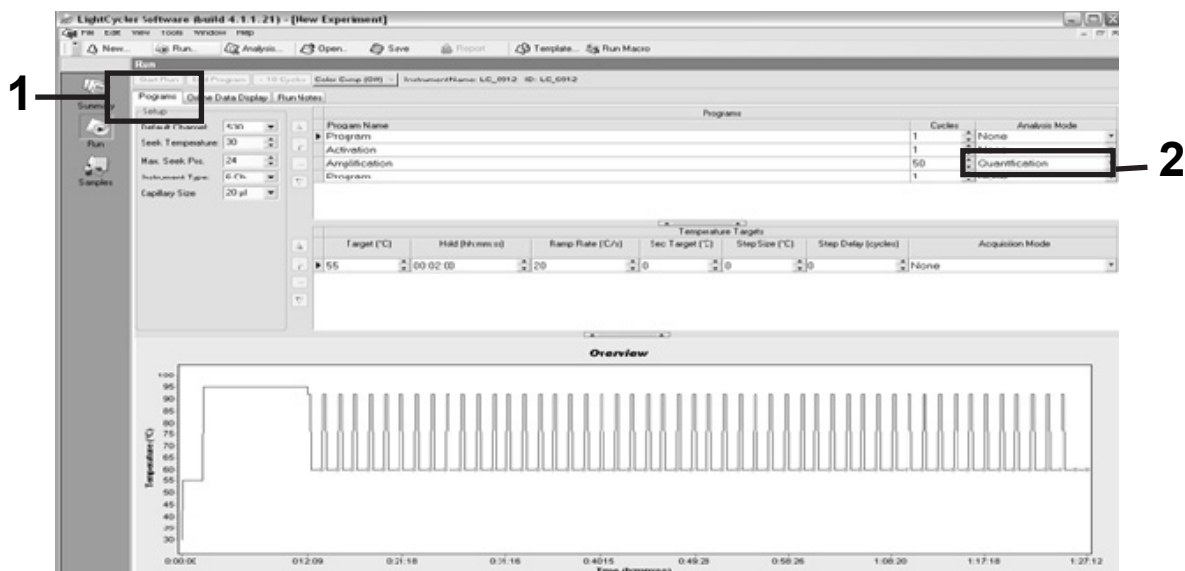


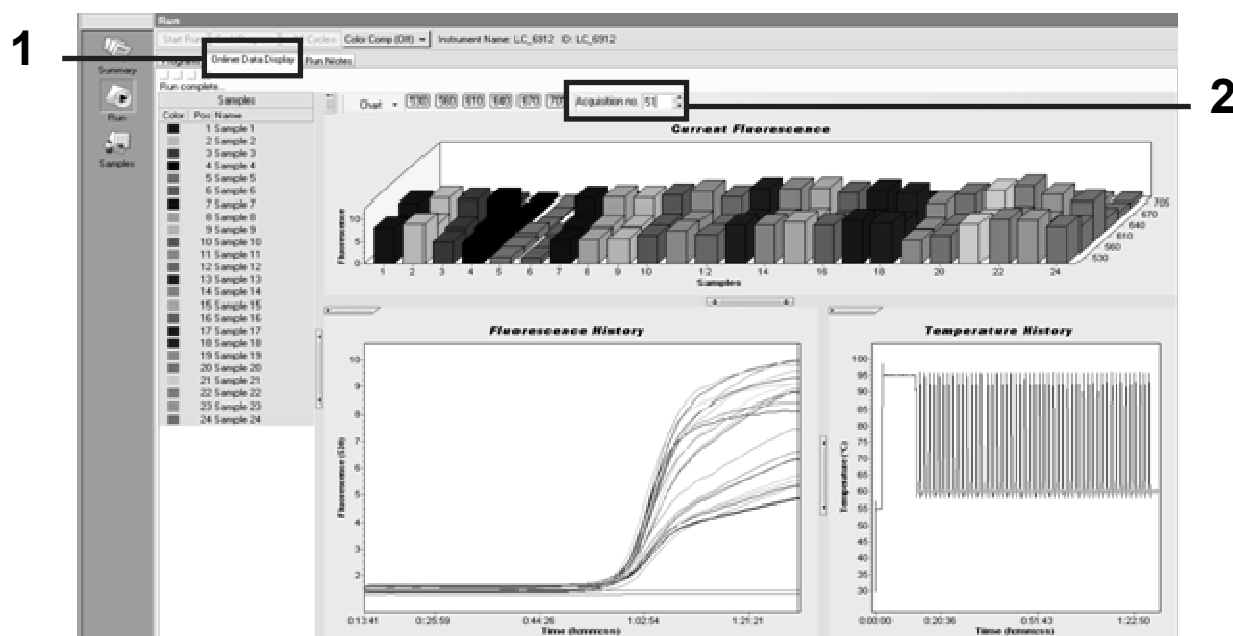
Figure 28. Programming screen for LightCycler 2.0.

**Table 13. Temperature profile for LightCycler 2.0 instrument**

|                |   |
|----------------|---|
| <b>Hold</b>    | Temperature: 55°C<br>Time: 2 minutes<br>Ramp: 20                        |
| <b>Hold 2</b>  | Temperature: 95°C<br>Time: 10 minutes<br>Ramp: 20                       |
| <b>Cycling</b> | 50 times<br>92°C for 15 seconds; ramp: 20<br>60°C for 1 minute; ramp 20 |
| <b>Hold 3</b>  | 60°C for 1 minute; ramp 20  |

## End-point analysis procedure for LightCycler 2.0 instrument

11. At the end of the amplification run, click the tab for “Online Data Display” (Figure 29). Open the display menu on the top left of the “Current Fluorescence” window, then write 51 in “Acquisition no.” .



**Figure 29. Results and history in Online Data Display.**

12. Right-click near the “Current Fluorescence” graph and select “Export”.

13. Click the “Excel” box on the “Export chart” dialog box (Figure 30). Enter a name in the “Filename” dialog field. Select an export destination for the result file with the ... button. Click “Export”.

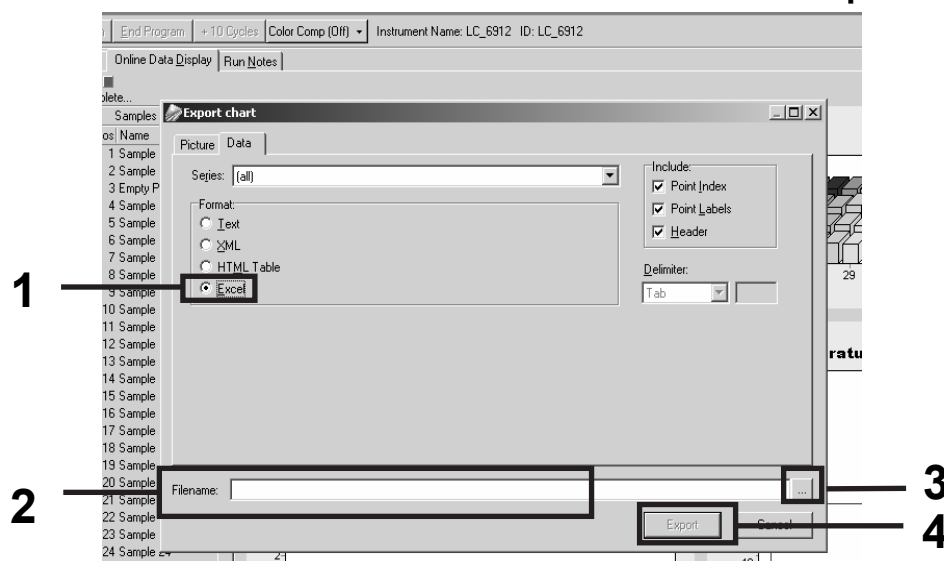


Figure 30. Selecting the export format and data file destination.

14. To view and analyze results, open the file in Excel. The results for LightCycler 2.0 will appear as shown.

| Position |        |                     |    |        |                     |    |        |                     |     |        |   |   |
|----------|--------|---------------------|----|--------|---------------------|----|--------|---------------------|-----|--------|---|---|
| I        | J      | K                   | L  | M      | N                   | O  | P      | Q                   | R   | S      | T | U |
| X        | Bar    | Text                | X  | Bar    | Text                | X  | Bar    | Text                | Bar |        |   |   |
| 1        | 2,9709 | 1: Sample 1 (610)   | 1  | 8,2734 | 1: Sample 1 (560)   | 1  | 6,6361 | 1: Sample 1 (530)   | 1   | 4,9943 |   |   |
| 2        | 3,0182 | 2: Sample 2 (610)   | 2  | 8,4428 | 2: Sample 2 (560)   | 2  | 6,7659 | 2: Sample 2 (530)   | 2   | 5,0767 |   |   |
| 3        | 2,9496 | 3: Sample 3 (610)   |    |        | 3: Sample 3 (560)   | 3  | 6,5568 | 3: Sample 3 (530)   | 3   | 4,9699 |   |   |
| 4        | 2,9526 | 4: Sample 4 (610)   | 4  | 8,2887 | 4: Sample 4 (560)   | 4  | 6,6163 | 4: Sample 4 (530)   | 4   | 4,9119 |   |   |
| 5        | 2,9450 | 5: Sample 5 (610)   | 5  | 8,2689 | 5: Sample 5 (560)   | 5  | 6,6209 | 5: Sample 5 (530)   | 5   | 4,9638 |   |   |
| 6        | 2,9969 | 6: Sample 6 (610)   | 6  | 8,4184 | 6: Sample 6 (560)   | 6  | 6,7674 | 6: Sample 6 (530)   | 6   | 5,1209 |   |   |
| 7        | 3,0045 | 7: Sample 7 (610)   | 7  | 8,4520 | 7: Sample 7 (560)   | 7  | 6,7506 | 7: Sample 7 (530)   | 7   | 5,0507 |   |   |
| 8        | 3,2822 | 8: Sample 8 (610)   | 8  | 9,1936 | 8: Sample 8 (560)   | 8  | 7,3960 | 8: Sample 8 (530)   | 8   | 5,5314 |   |   |
| 9        | 3,0274 | 9: Sample 9 (610)   | 9  | 8,5557 | 9: Sample 9 (560)   | 9  | 6,8437 | 9: Sample 9 (530)   | 9   | 5,0843 |   |   |
| 10       | 2,8336 | 10: Sample 10 (610) | 10 | 7,9713 | 10: Sample 10 (560) | 10 | 6,3905 | 10: Sample 10 (530) | 10  | 4,7883 |   |   |
| 11       | 2,8275 | 11: Sample 11 (610) | 11 | 7,9774 | 11: Sample 11 (560) | 11 | 6,3874 | 11: Sample 11 (530) | 11  | 4,7669 |   |   |
| 12       | 2,8351 | 12: Sample 12 (610) | 12 | 8,0171 | 12: Sample 12 (560) | 12 | 6,4118 | 12: Sample 12 (530) | 12  | 4,7944 |   |   |
| 13       | 2,9511 | 13: Sample 13 (610) | 13 | 8,3726 | 13: Sample 13 (560) | 13 | 6,6957 | 13: Sample 13 (530) | 13  | 4,9699 |   |   |
| 14       | 2,8367 | 14: Sample 14 (610) | 14 | 8,0217 | 14: Sample 14 (560) | 14 | 6,4439 | 14: Sample 14 (530) | 14  | 4,7654 |   |   |
| 15       | 2,9908 | 15: Sample 15 (610) | 15 | 8,4337 | 15: Sample 15 (560) | 15 | 6,7445 | 15: Sample 15 (530) | 15  | 5,0523 |   |   |
| 16       | 2,8885 | 16: Sample 16 (610) | 16 | 8,1498 | 16: Sample 16 (560) | 16 | 6,5568 | 16: Sample 16 (530) | 16  | 4,9577 |   |   |
| 17       | 3,0152 | 17: Sample 17 (610) | 17 | 8,4901 | 17: Sample 17 (560) | 17 | 6,8193 | 17: Sample 17 (530) | 17  | 5,1225 |   |   |
| VIC      |        |                     |    |        |                     |    | FAM    |                     |     |        |   |   |

Figure 31. Example of LightCycler 2.0 results, shown in an Excel file.



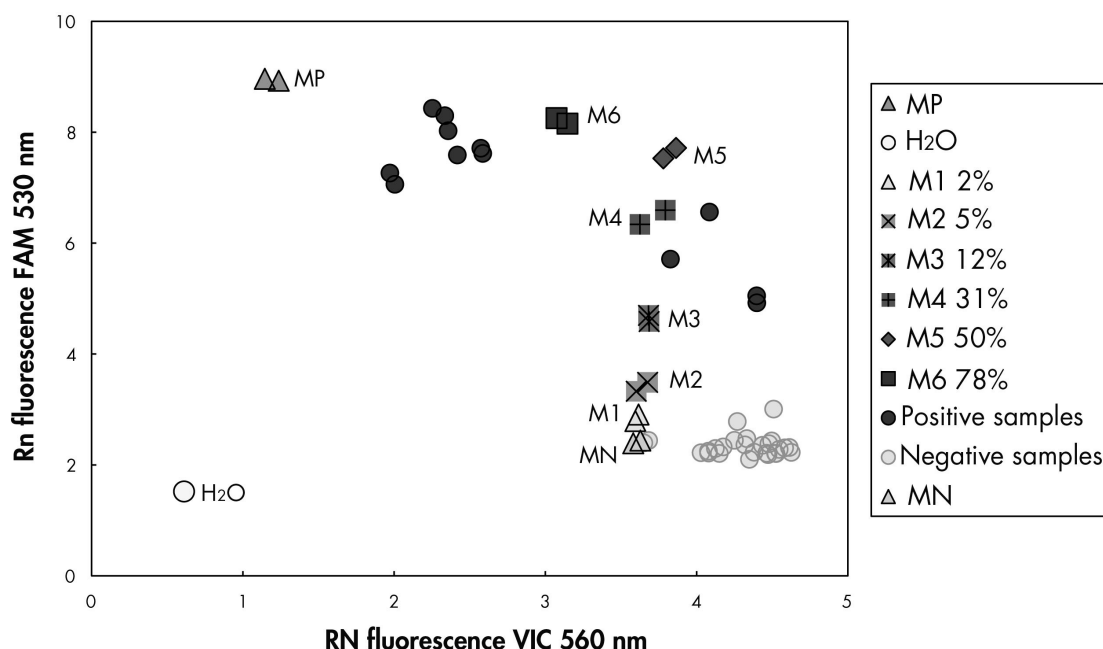
## Interpretation of Results

Obtain a file suitable to extract exported data for all instruments: Rotor-Gene Q MDx 5plex HRM or other Rotor-Gene instrument, LightCycler 2.0, or 480; Applied Biosystems 7300 or 7500 Real-Time PCR System, ABI PRISM 7000 SDS, 7700 SDS, or 7900HT SDS, 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 32.



**Figure 32. Scatter plot of a representative allelic discrimination experiment.**

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

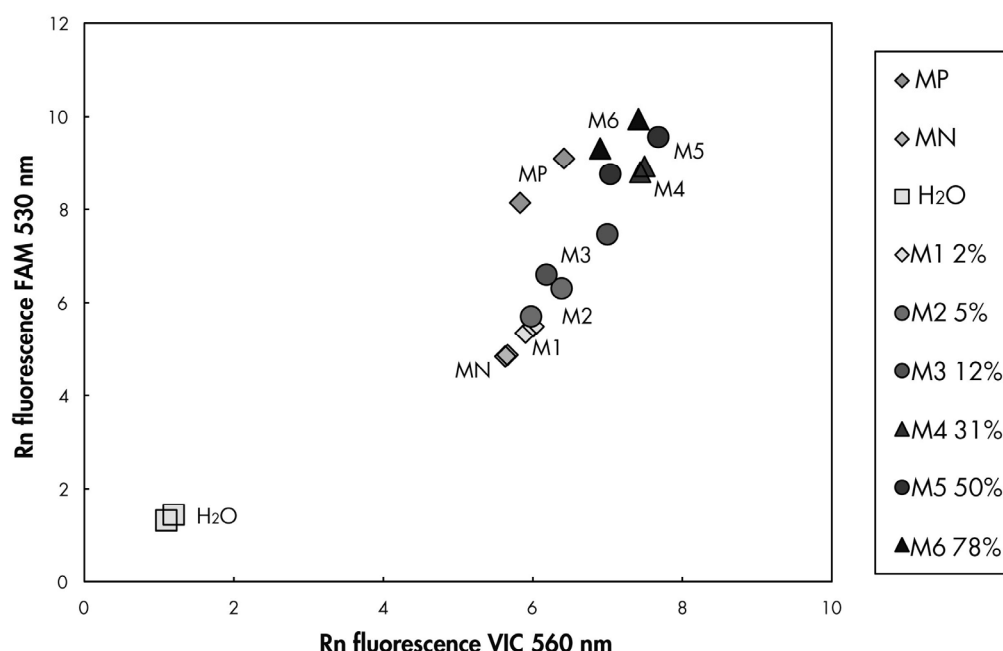
Samples should be located on the arc connecting the negative controls (MN) to the positive controls (MP).

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 (M1 of the reference scale) should appear above the negative controls.
- Water controls should be located at the bottom left.

- Poor positioning of a water control (higher than MN for FAM measurement or higher than MP for VIC) may indicate contamination.

**Note:** Positioning of the controls may be different on analysis of LightCycler 2.0 instrument data (see Figure 33). The water controls should still be located at the bottom left.



**Figure 33. Scatter plot of a representative allelic discrimination experiment.** Instrument: LightCycler 2.0.

## 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 (MP), the cut-off sample (M1), the negative control (MN), and the reference scale (M2 to M6). The ratios must be consistent between duplicates. Calculate the average ratio of all duplicates.

Calculate the normalized ratio (NRatio) for the cut-off sample (M1) and for all the samples:

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

**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 cut-off sample (M1) (NRatio<sub>M1</sub>):

$$\text{GZ: } [(N\text{Ratio}_{M1} \times 0.94); (N\text{Ratio}_{M1} \times 1.06)]$$

Compare the Normalized Ratio of each sample to the NRatio<sub>M1</sub> GZ.

Interpretation of results is outlined in Table 14.

**Table 14. Interpretation of genotyping results using normalized ratios**

| Results  | Interpretation             |
|--|----------------------------|
| $N\text{Ratio}_{\text{Sample}} > N\text{Ratio}_{M1} \times 1.06$ | JAK2 V617F is detected     |
| $N\text{Ratio}_{\text{Sample}} < N\text{Ratio}_{M1} \times 0.94$ | JAK2 V617F is not detected |
| $N\text{Ratio}_{\text{Sample}}$ within $N\text{Ratio}_{M1}$ GZ   | Result inconclusive        |

A semiquantitative result for the mutation burden can be obtained by comparing the value of each unknown sample ratio (Ratio<sub>Sample</sub>) with the mean ratio values of the reference scale (Ratio<sub>M1-6</sub>) (see Table 15).

**Table 15. Semiquantitative values for the JAK2 V617F mutation burden using the reference scale**

| Results  | Mutation burden     |
|--|---------------------|
| $\text{Ratio}_{M1} < \text{Ratio}_{\text{Sample}} < \text{Ratio}_{M2}$ | 2–5% JAK2 V617F     |
| $\text{Ratio}_{M2} < \text{Ratio}_{\text{Sample}} < \text{Ratio}_{M3}$ | 5–12.5% JAK2 V617F  |
| $\text{Ratio}_{M3} < \text{Ratio}_{\text{Sample}} < \text{Ratio}_{M4}$ | 12.5–31% JAK2 V617F |
| $\text{Ratio}_{M4} < \text{Ratio}_{\text{Sample}} < \text{Ratio}_{M5}$ | 31–50% JAK2 V617F   |
| $\text{Ratio}_{M5} < \text{Ratio}_{\text{Sample}} < \text{Ratio}_{M6}$ | 50–78% JAK2 V617F   |
| $\text{Ratio}_{M6} < \text{Ratio}_{\text{Sample}}$                     | 78–100% JAK2 V617F  |

An example of data calculation and interpretation is given in Table 16.

**Table 16. An example of fluorescence data calculation and interpretation using the reference scale**

| Sample    | VIC          | FAM          | Ratio | Mean ratio | NRatio | Interpretation        |
|-----------|--------------|--------------|-------|------------|--------|-----------------------|
| MN        | 49.613       | 3.8          | 0.077 | 0.078      | 1.000  | Mutation not detected |
| MN        | 49.797       | 3.976        | 0.08  |            |        |                       |
| MP        | 12.516       | 37.037       | 2.959 | 2.951      | 37.722 | Mutation detected     |
| MP        | 12.958       | 38.121       | 2.942 |            |        |                       |
| M1        | 54.394       | 6.39         | 0.117 | 0.119      | 1.516  | Cut-off sample        |
| M1        | 58.266       | 6.973        | 0.12  |            |        |                       |
| M2        | 61.798       | 10.882       | 0.176 | 0.172      | 2.202  | Mutation detected     |
| M2        | 54.814       | 9.231        | 0.168 |            |        |                       |
| M3        | 57.364       | 16.604       | 0.289 | 0.297      | 3.797  | Mutation detected     |
| M3        | 59.742       | 18.192       | 0.305 |            |        |                       |
| M4        | 56.965       | 28.99        | 0.509 | 0.505      | 6.462  | Mutation detected     |
| M4        | 58.077       | 29.158       | 0.502 |            |        |                       |
| M5        | 54.251       | 37.221       | 0.686 | 0.672      | 8.586  | Mutation detected     |
| M5        | 54.979       | 36.125       | 0.657 |            |        |                       |
| M6        | 46.185       | 44.498       | 0.963 | 0.954      | 12.2   | Mutation detected     |
| M6        | 45.077       | 42.598       | 0.945 |            |        |                       |
| S 1       | 13.47        | 37.409       | 2.777 | 2.852      | 36.464 | Mutation (78–100%)    |
| S 1       | 14.559       | 42.616       | 2.927 |            |        |                       |
| S 2       | 50.432       | 24.958       | 0.495 | 0.505      | 6.46   | Mutation (12.5–31%)   |
| S 2       | 53.797       | 27.746       | 0.516 |            |        |                       |
| S 3       | 52.038       | 5.995        | 0.115 | 0.117      | 1.49   | Inconclusive result   |
| S 3       | 54.01        | 6.364        | 0.118 |            |        |                       |
| S 4       | 50.811       | 4.842        | 0.095 | 0.048      | 0.609  | Inconclusive result   |
| S 4       | 0.01         | –            | 0     |            |        |                       |
| <b>GZ</b> | <b>1.425</b> | <b>1.607</b> |       |            |        |                       |

## 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 54).

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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 RS Kit at –30 to –15°C and keep primers and probes mix (PPM) protected from light. See “Reagent Storage and Handling”, page 11.<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.  |

| <b>Comments and suggestions</b>                    |  |
|--|--|
| 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 RS Kit at –30 to –15°C and keep primers and probes mix (PPM) protected from light. See “Reagent Storage and Handling”, page 11.<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.  |
| b) Insufficient centrifugation of the capillaries | The prepared PCR mix may still be in the upper vessel of the capillary, or an air bubble could be trapped in the capillary tip.<br><br>Always centrifuge capillaries loaded with the reaction mix as described in the specific operating manual of the apparatus. |
| c) Outer surface of the capillary tip dirty       | Always wear gloves when handling the capillaries.   |

## Quality Control

Quality control of the complete kit has been performed on a LightCycler 480. This kit is manufactured according to ISO 13485:2003 standard. Certificates of Analysis are available upon request at [www.qiagen.com/support/](http://www.qiagen.com/support/).

## Limitations

The users must be trained and familiar with this technology prior to the use of this device. This kit should be used following the instructions given in this manual, in combination with a validated instrument mentioned in "Materials Required but Not Provided", page 9.

Any diagnostic results that are 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 used in their laboratory which are not covered by the QIAGEN performance studies.

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

## Performance Characteristics

### Nonclinical studies

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

#### Precision

Three dilution levels of genomic DNA from cell lines harboring the JAK2 V617F mutation in wild type DNA were tested. The dilutions corresponded to mutation loads of 1%, 2%, and 3%. Independent dilution batches were obtained for each level, and replicates of these dilutions were tested in 3 independent experiments. Ratios obtained for each DNA sample ( $\text{Ratio}_{\text{Sample}}$ ) were compared with the negative control ratio (JAK2 100% wild type DNA,  $\text{Ratio}_{\text{NC}}$ ). Results are summarized in Table 17.

**Table 17. Precision data for nonclinical studies.**

| Mutation level | $\text{Ratio}_{\text{Sample}} > \text{Ratio}_{\text{NC}}$ | %CV (ratio) |
|----------------|---|-------------|
| 1% V617F DNA   | 100% (n = 183)  | 6.8         |
| 2% V617F DNA   | 100% (n = 72)   | 4.5         |
| 3% V617F DNA   | 100% (n = 135)  | 5.1         |

## Interlaboratory analytical data

A multi-center study was performed involving 13 laboratories. Analytical data were collected on dilutions of genomic DNA harboring JAK2 V617F mutation in wild type DNA. Three experiments were performed in each laboratory. For each experiment, the following DNA samples were tested from cell lines:

- 1 negative control (NC) 0% V617F
- 1 positive control (PC) 100% V617F
- 1 cut-off sample (COS) 2% V617F
- 3 samples harboring intermediate mutation loads (20%, 50%, and 80%)

The experiments were performed on 7 different instrument models: ABI PRISM 7000 SDS (11), Applied Biosystems 7300 Real-Time PCR System (1), Applied Biosystems 7500 Real-Time PCR System (3), ABI PRISM 7700 SDS (11), ABI PRISM 7900 SDS (1), LightCycler 2.0 (1), and iCycler® (1). Results are summarized in Table 18.

**Table 18. Interlaboratory analytical data obtained from dilutions of genomic DNA from cell lines harboring the JAK2 V617F mutation in wild type DNA**

| Sample detection | Positive samples | Negative samples |
|------------------|------------------|------------------|
| JAK2 V617F       | 177*             | 0                |
| JAK2 wild type   | 0                | 36               |

\* Positive samples included 36 positive controls (PC), 36 cut-off samples (COS; 2% V617F), 34 samples harboring 20% JAK2 V617F, 35 samples harboring 50% JAK2 V617F, and 36 samples harboring 80% JAK2 V617F.

## Clinical studies

### Comparison between *ipsogen* JAK2 MutaScreen Kit and ARMS® method

DNA samples from 141 patients with suspected MPN were tested in parallel with the *ipsogen* JAK2 MutaScreen Kit and a qPCR assay based on the amplification refractory mutation system (ARMS) principle (5). Results of the comparison are shown in Table 19 (2 x 3 contingency table) and Table 20 (percentage agreement).



**Table 19. Comparison between methods: *ipsogen* JAK2 MutaScreen Kit and ARMS**

|   |                                    | Results of ARMS testing method |                   |         |
|---|------------------------------------|--------------------------------|-------------------|---------|
|   |                                    | JAK2 V617F<br>>2%              | JAK2 V617F<br><2% | Total   |
| Results of<br><i>ipsogen</i><br>JAK2<br>MutaScreen<br>testing<br>method | JAK2 V617F<br>Mutation<br>detected | 91                             | 0                 | 91      |
|   | Inconclusive<br>result             | 1                              | 2                 | 3       |
|   | JAK2 WT<br>No mutation<br>detected | 1                              | 46                | 47      |
| Total   |                                    | 93                             | 48                | n = 141 |

**Table 20. Comparison between methods: *ipsogen* JAK2 MutaScreen Kit and ARMS**

|  | Agreement<br>(%) | 95% CI* (%)      |
|--|------------------|------------------|
| Positive data  |                  |                  |
| Agreement between <i>ipsogen</i> JAK2<br>MutaScreen Kit and ARMS | 98.9             | 94.1–99.8        |
| Negative data  |                  |                  |
| Agreement between <i>ipsogen</i> JAK2<br>MutaScreen Kit and ARMS | 100              | 92.3–100         |
| <b>Total agreement</b>   | <b>99.3</b>      | <b>96.0–99.9</b> |

\* Confidence intervals were calculated according to CLSI EP12-A “User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline”.

### Comparison between *ipsogen* JAK2 MutaScreen Kit and sequencing

DNA samples from 51 patients with suspected MPN were tested in parallel with the *ipsogen* JAK2 MutaScreen Kit and the reference technique (“gold standard”), direct sequencing. One sample could not be interpreted due to sequencing failure. Comparisons of results obtained from the 50 interpretable samples are summarized in Table 21 (2 x 3 contingency table) and Table 22 (percentage agreement).

**Table 21. Comparison between methods: *ipsogen* JAK2 MutaScreen Kit and sequencing**

|   |                                    | Results of direct sequencing |                   |        |
|---|------------------------------------|------------------------------|-------------------|--------|
|   |                                    | JAK2 V617F<br>>2%            | JAK2 V617F<br><2% | Total  |
| Results of<br><i>ipsogen</i><br>JAK2<br>MutaScreen<br>testing<br>method | JAK2 V617F<br>Mutation<br>detected | 26                           | 1                 | 27     |
|   | Inconclusive<br>result             | 0                            | 1                 | 1      |
|   | JAK2 WT<br>No mutation<br>detected | 2                            | 20                | 22     |
| Total   |                                    | 28                           | 22                | n = 50 |

**Table 22. Comparison between methods: *ipsogen* JAK2 MutaScreen Kit and sequencing**

|  | Agreement<br>(%) | 95% CI* (%)      |
|--|------------------|------------------|
| Positive data  |                  |                  |
| Agreement between <i>ipsogen</i> JAK2<br>MutaScreen Kit and sequencing | 92.9             | 77.4–98.0        |
| Negative data  |                  |                  |
| Agreement between <i>ipsogen</i> JAK2<br>MutaScreen Kit and sequencing | 95.2             | 77.3–99.2        |
| <b>Total agreement</b>   | <b>93.9</b>      | <b>83.5–97.9</b> |

\* Confidence intervals were calculated according to CLSI EP12-A “User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline”.

### Multicenter study on 228 patient samples

DNA samples from patients were analyzed with home brew techniques in 13 laboratories contributing to an interlaboratory study. In each laboratory, 3 experiments were performed, using DNA from cell lines as described for the nonclinical precision data (see above), and with DNA from 10 patients available in the laboratory.

The 228 samples with a known JAK2 genotype were tested in parallel with the *ipsogen* JAK2 MutaScreen Kit and by home brew methods, including qualitative PCR, allele specific PCR, fluorescence energy resonance transfer (FRET), sequencing, allele specific oligonucleotide PCR, RFLP, and allelic discrimination. Results of the comparisons are shown in Table 23 (2 x 3 contingency table) and Table 24 (percentage agreement).

**Table 23. Comparison between methods: *ipsogen* JAK2 MutaScreen Kit and home brew methods**

|   |                                    | Results of home brew testing |                   |         |
|---|------------------------------------|------------------------------|-------------------|---------|
|   |                                    | JAK2 V617F<br>>2%            | JAK2 V617F<br><2% | Total   |
| Results of<br><i>ipsogen</i><br>JAK2<br>MutaScreen<br>testing<br>method | JAK2 V617F<br>Mutation<br>detected | 139                          | 3                 | 142     |
|   | Inconclusive<br>result             | 5                            | 17                | 22      |
|   | JAK2 WT<br>No mutation<br>detected | 3                            | 61                | 64      |
| Total   |                                    | 147                          | 81                | n = 228 |

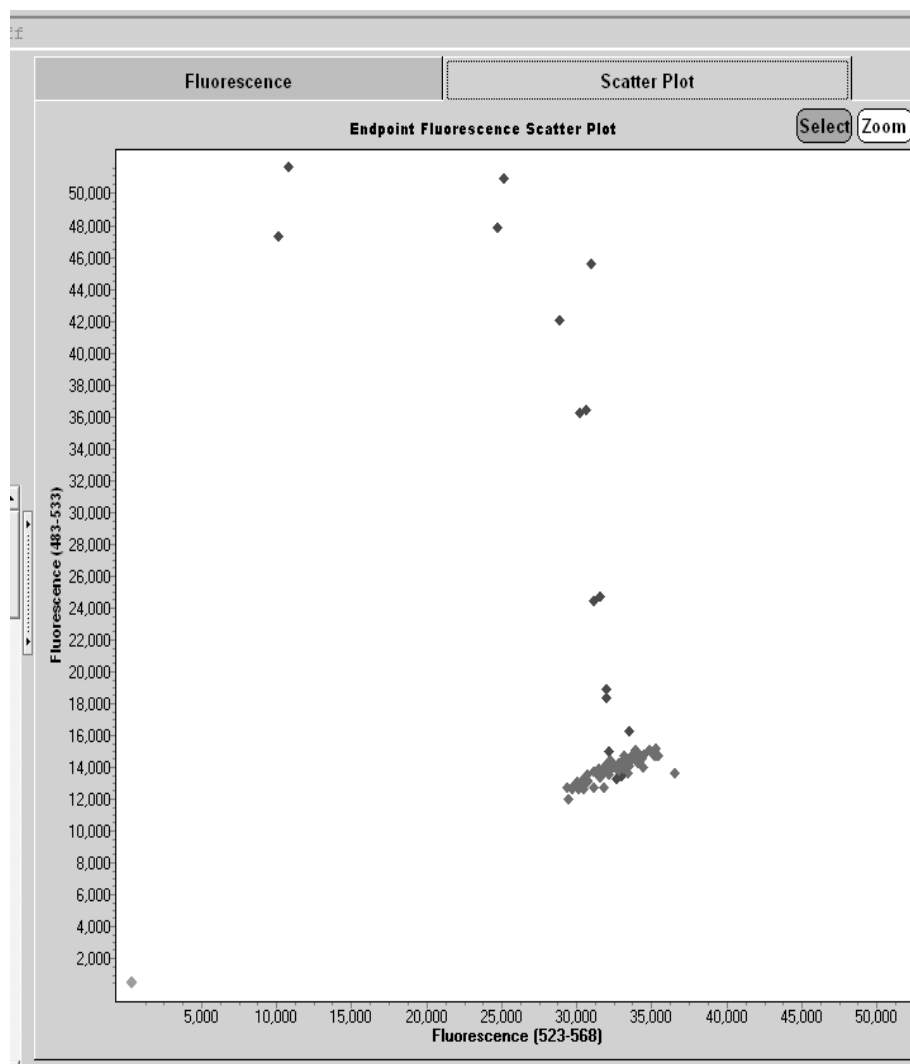
**Table 24. Comparison between methods: JAK2 MutaScreen Kit and home brew methods**

|   | Agreement<br>(%) | 95% CI* (%)      |
|---|------------------|------------------|
| Positive data   |                  |                  |
| Agreement between <i>ipsogen</i> JAK2<br>MutaScreen Kit and home brew | <b>97.9</b>      | 94.0–99.3        |
| Negative data   |                  |                  |
| Agreement between <i>ipsogen</i> JAK2<br>MutaScreen Kit and home brew | <b>95.3</b>      | 87.1–98.4        |
| <b>Total agreement</b>  | <b>97.1</b>      | <b>93.8–98.7</b> |

\* Confidence intervals were calculated according to CLSI EP12-A "User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline".

## Robustness: testing of samples from healthy donors

DNA samples from 103 healthy blood-donors were analyzed with the *ipsogen* JAK2 MutaScreen RS Kit. All of the samples were detected as JAK2 wild-type. Analysis of 38 samples with the LightCycler 480 instrument is shown in Figure 34.



**Figure 34. Analysis of healthy donors.** LightCycler 480 analysis of 38 healthy donors (◆) with the *ipsogen* JAK2 MutaScreen RS Kit (cat. no. 673123). Positive results in duplicate (◆) correspond to a reference scale supplied with the kit. VIC fluorescence values are plotted on the x axis and FAM values are plotted on the y axis.

## References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

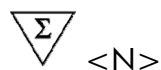
For a complete list of references, visit the QIAGEN Reference Database online at [www.qiagen.com/RefDB/search.asp](http://www.qiagen.com/RefDB/search.asp) or contact QIAGEN Technical Services or your local distributor.

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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



Temperature limitation



Manufacturer



Consult instructions for use

## Contact Information

For technical assistance and more information, please see our Technical Support Center at [www.qiagen.com/Support](http://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](http://www.qiagen.com)).

## Ordering Information

| Product   | Contents   | Cat. no. |
|---|--|----------|
| <i>ipsogen</i> JAK2<br>MutaScreen RS Kit (19)   | For 19 reactions: V617F Positive Control, V617F Negative Control, V617F Reference Scale, Primers and Probes Mix JAK2 wild type and JAK2 V617F  | 673123   |
| <b>Rotor-Gene Q MDx — for IVD-validated real-time PCR analysis in clinical applications</b> |  |          |
| Rotor-Gene Q MDx<br>5plex HRM Platform  | Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included | 9002032  |
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