

therascreen[®] BRAF RGQ PCR Kit Handbook



Version 2

IVD

For in vitro diagnostic use

For use with the Rotor-Gene[®] Q MDx instruments



REF

870211



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Intended Use

The *therascreen* BRAF RGQ PCR Kit is an in vitro diagnostic test for the detection of five somatic mutations found in the BRAF gene and provides qualitative assessment of mutation status. DNA will be extracted from formalin fixed paraffin-embedded (FFPE) tumor tissue and tested using real-time polymerase chain reaction (PCR) on Rotor-Gene Q MDx instruments. The *therascreen* BRAF RGQ PCR Kit is intended to aid the clinician in identifying cancer patients who may benefit from BRAF targeted therapy, such as vemurafenib.

Table 1. List of mutations and COSMIC IDs*

Mutation	Base change	COSMIC ID
V600E	GTG>GAG	476
V600E complex	GTG>GAA	475
V600D	GTG>GAT	473
V600K	GTG>AAG	474
V600R	GTG>AGG	477

*COSMIC IDs are taken from the Catalog of Somatic Mutations in Cancer: (www.sanger.ac.uk/genetics/CGP/cosmic).

Summary and Explanation

The *therascreen* BRAF RGQ PCR Kit is a ready-to-use kit for the detection of five somatic mutations in the BRAF gene using real-time polymerase chain reaction (RT PCR) on the Rotor-Gene Q MDx instrument.

Using ARMS® (Amplification Refractory Mutation System) and Scorpions® technologies, the *therascreen* BRAF RGQ PCR Kit enables detection of the following mutations at codon 600 of the BRAF oncogene against a background of wild-type genomic DNA.

- V600E
- V600E complex (V600Ec)
- V600D
- V600K
- V600R

The methods used are highly selective and, depending on the total amount of DNA present, enable detection of a low percentage of mutant in a background of wild-type genomic DNA. These selectivity and detection limits are superior to technologies such as dye-terminator sequencing.

Principle of the Procedure

The *therascreen* BRAF RGQ PCR Kit utilizes two technologies — ARMS and Scorpions — for detection of mutations in real-time PCR.

ARMS

Allele- or mutation-specific amplification is achieved using ARMS. *Taq* DNA polymerase (*Taq*) is effective at distinguishing between a match and a mismatch at the 3' end of a PCR primer. Specific mutated sequences are selectively amplified even in samples where the majority of the sequences do not carry the mutation. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low level background amplification occurs.

Scorpions

Detection of amplification is performed using Scorpions. Scorpions are bifunctional molecules containing a PCR primer covalently linked to a fluorescently labeled probe. The fluorophore in this probe is associated with a quencher also incorporated into the probe, which reduces fluorescence. During PCR when the probe binds to the amplicon, the fluorophore and quencher become separated. This leads to a measureable increase in fluorescence from the reaction tube.

Kit format

Five assays are supplied in the *therascreen* BRAF RGQ PCR Kit.

- One control assay (Control Reaction Mix; CTRL)
- Four mutation assays (mutant reaction mixes; V600E/Ec, V600D, V600K, V600R)

The V600E/Ec assay detects both V600E and V600Ec mutations but does not distinguish between them.

All reaction mixes are duplex and contain reagents to detect targets that are labeled with FAM™ and an internal control that is labeled with HEX™. The internal control assay controls for the presence of inhibitors that may lead to false negative results.

Assays

The *therascreen* BRAF RGQ PCR Kit comprises a two-step procedure. In the first step, the control assay is performed to assess the total amplifiable BRAF DNA in a sample. In the second step, both the mutation and control assays are performed to determine the presence or absence of mutant DNA.

Control assay

The control assay, labeled with FAM, is used to assess the total amplifiable BRAF DNA in a sample. The control assay amplifies a region of exon 3 of the BRAF gene. The primers and Scorpion probe have been designed to amplify independently of any known BRAF polymorphisms.

Mutation assays

Each mutation assay contains a FAM labeled Scorpion probe and an ARMS primer for discrimination between the wild-type DNA and a specific mutant DNA.

Controls

Note: All experimental runs must contain positive and negative controls.

Positive control

Each run must contain a positive control in tubes 1–5. The *therascreen* BRAF RGQ PCR Kit contains BRAF Positive Control (PC) to be used as the template in the positive control reaction. The positive control results will be assessed to ensure that the kit performs within the stated acceptance criteria.

Negative control

Each run must contain a negative control (“no template control”) in tubes 9–13. The *therascreen* BRAF RGQ PCR Kit contains Water for NTC (NTC) to be used as the “template” for the no template control. The no template control is used to assess any potential contaminations during the run setup and to assess the performance of the internal control reaction.

Internal control reaction assessment

Each reaction mix contains an internal control in addition to the target reaction. A failure indicates that either there may be inhibitors present that could lead to an inaccurate result or an operator setup error has occurred for that tube. If the internal control failure is due to PCR inhibition, diluting the sample may reduce

the effect of inhibitors but it should be noted that this would also dilute the target DNA. A tube of water for sample dilution (Dil.) is provided with the kit. Dilution of samples must be performed with the water for sample dilution (Dil.).

Sample assessment

It is strongly recommended to use the Control Reaction Mix (CTRL) supplied with the *therascreen* BRAF RGQ PCR Kit to assess the total amplifiable BRAF DNA in a sample. The control assay amplifies a region of exon 3 of the BRAF gene. It is recommended to set up samples with only the control assay using the BRAF Positive Control (PC) as a positive control and Water for NTC (NTC) as the no template control.

Note: DNA assessment should be based on PCR and may differ from quantification based on absorbance readings. Additional Control Reaction Mix (CTRL) is supplied to enable assessment of quality and quantity of the DNA in samples before analysis with the *therascreen* BRAF RGQ PCR Kit.

Materials Provided

Kit contents

<i>therascreen</i> BRAF RGQ PCR Kit			(24)
Catalog no.			870211
Number of reactions			24
Control Reaction Mix	Red	1 CTRL	2 x 720 μ l
V600E/Ec Reaction Mix	Purple	2 V600E/Ec	720 μ l
V600D Reaction Mix	Orange	3 V600D	720 μ l
V600K Reaction Mix	Pink	4 V600K	720 μ l
V600R Reaction Mix	Green	5 V600R	720 μ l
BRAF Positive Control	Beige	PC	250 μ l
<i>Taq</i> DNA Polymerase	Mint	<i>Taq</i>	2 x 80 μ l
Water for NTC	White	NTC	1.9 ml
Water for Sample Dilution	White	Dil.	1.9 ml
<i>therascreen</i> BRAF RGQ PCR Kit Handbook (English)			1

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

- DNA extraction kit (see "DNA extraction and preparation", page 13)
- Xylene
- Ethanol (96–100%)*

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Consumables

- 1.5 ml or 2 ml microcentrifuge tubes (for lysis steps)
- 1.5 ml microcentrifuge tubes (for elution steps) (available from Brinkmann [Safe-Lock, cat. no. 022363204], Eppendorf [Safe-Lock, cat. no. 0030 120.086], or Sarstedt [Safety Cap, cat. no. 72.690]) *
- Dedicated pipets[†] (adjustable) for sample preparation
- Dedicated pipets[†] (adjustable) for PCR master mix preparation
- Dedicated pipets[†] (adjustable) for dispensing of template DNA
- Sterile pipet tips with filters (to avoid cross-contamination, we recommend pipet tips with aerosol barriers)

Equipment

- Thermomixer, heated orbital incubator, heating block, or water bath capable of incubation at 90°C[†]
- Benchtop centrifuge[†] with rotor for 2 ml reaction tubes
- Vortex[†]
- Rotor-Gene Q MDx 5plex HRM^{†‡} with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and yellow, respectively)
- Rotor-Gene Q software version 2.3 with the BRAF Assay Package (version 3.1.1) installed for automated mutation detected (see “Appendix II: Installation of the *therascreen* BRAF Assay Package”, page 83)
Note: The Rotor-Gene Q software can be used without the BRAF Assay Package for manual mutation detection. Refer to “Appendix I: *therascreen* BRAF RGQ PCR Kit Manual Protocol”, page 54
- 0.1 ml Strip Tubes and Caps, for use with 72-well rotor (QIAGEN, cat. no. 981103 or 981106)

* This is not a complete list of suppliers.

[†] Ensure that instruments have been checked and calibrated according to the manufacturer’s recommendations.

[‡] In some countries, if applicable the Rotor-Gene Q 5plex HRM instrument with a production date of May 2011, or later can be used. The production date can be obtained from the serial number on the back of the instrument. The serial number is in the format “mmyyynn” where “mm” indicates the production month in digits, “yy” indicates the last two digits of the production year, and “ynn” indicates the unique instrument identifier.

- Sterile microcentrifuge tubes for preparing master mixes
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction setup with a single-channel pipet (QIAGEN, cat. no. 9018901)

Warnings and Precautions

For in vitro diagnostic use

Safety information

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

General precautions

The user should always pay attention to the following.

- Store and extract positive materials (specimens and positive controls) separately from all other reagents and add them to the reaction mix in a spatially separated facility.
- Use extreme caution to prevent contamination of PCRs with synthetic control material. We recommend using separate, dedicated pipets for setting up reaction mixes and adding DNA template. Preparation and dispensing of reaction mixes must be carried out in a separate area to the addition of template. Rotor-Gene Q tubes must not be opened after the PCR run has finished. This is to prevent laboratory contamination with post-PCR products.
- Reagents for the *therascreen* BRAF RGQ PCR Kit have been optimally diluted. We do not recommend further dilution of reagents as this may result in a loss of performance. We do not recommend using reaction volumes of less than 25 μ l since this will increase the risk of false negative results.
- All reagents in the *therascreen* BRAF RGQ PCR Kit are formulated specifically for optimal performance. All reagents supplied in the *therascreen* BRAF RGQ PCR Kit are intended to be used solely with the other reagents in the same *therascreen* BRAF RGQ PCR kit. Substitutions to the reagents in the kit must not be made if optimal performance is to be maintained.

- Only use the *Taq* DNA polymerase (*Taq*) provided in the kit. Do not substitute with *Taq* DNA polymerase from other kits of the same or any other type, or with *Taq* DNA polymerase from another supplier.

Reagent Storage and Handling

The *therascreen* BRAF RGQ PCR Kit is shipped on dry ice and must still be frozen on arrival. If the *therascreen* BRAF RGQ PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, handbook, or reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

The *therascreen* BRAF RGQ PCR Kit should be stored immediately upon receipt at -15 to -30°C in a constant-temperature freezer and protected from light — Scorpions (as with all fluorescently labeled molecules) must be protected from light to avoid photo bleaching and loss of performance.

When stored under the recommended storage conditions in the original packaging, the kit is stable until the expiration date stated on the label. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 6 freeze-thaw cycles.

Specimen Storage and Handling

Note: All samples must be treated as potentially infectious material.

Sample material must be human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue. Specimens must be transported according to standard pathology methodology to ensure specimen quality.

Tumor samples are non-homogeneous and data from a sample of tumor may not be concordant with other sections from the same tumor. Tumor samples may also contain non-tumor tissue. DNA from non-tumor tissue would not be expected to contain mutations detected by the *therascreen* BRAF RGQ PCR Kit.

Procedure

DNA extraction and preparation

The performance characteristics for *therascreen* BRAF RGQ PCR Kit have been generated using DNA extracted with the QIAamp FFPE Tissue Kit (QIAGEN, cat. no. 56404). If using the QIAamp FFPE Tissue kit, carry out the DNA extraction according to the instructions in the handbook noting the following:

- Collect FFPE sections on glass slides.
- Scrape excess paraffin away from around the tissue sections using a fresh, sterile scalpel.
- Scrape tissue sections into microcentrifuge tubes using a fresh scalpel for each sample to be extracted.
- Purified genomic DNA must be eluted in 120–200 µl of Buffer ATE (provided in the QIAamp DNA FFPE Tissue Kit). Store purified genomic DNA at –15 to –30°C.

DNA assessment should be based on the Control Reaction Mix (CTRL) supplied with the *therascreen* BRAF RGQ PCR Kit and may differ from quantification based on absorbance readings. Additional Control Reaction Mix (CTRL) is supplied to enable assessment of quality and quantity of the DNA in samples before analysis with the *therascreen* BRAF RGQ PCR Kit.

Note: To ensure sufficient DNA for analysis it is recommended that a minimum of two FFPE slides be co-extracted in the first instance and assessed with the control assay. If insufficient DNA is obtained for PCR, further slides may be extracted and the DNA pooled.

Note: To ensure sufficient DNA for analysis, FFPE sections must be a minimum of 5 µm thick.

All assays in the *therascreen* BRAF RGQ PCR Kit generate short PCR products. However, the *therascreen* BRAF RGQ PCR Kit will not work with heavily fragmented DNA.

Protocol: Sample assessment

This protocol is used to assess the total amplifiable DNA in samples using the BRAF CE Sample Assessment Locked Template (Assay Package) for automated sample assessment.

Note: For manual sample assessment refer to “Appendix I: *therascreen* BRAF RGQ PCR Kit Manual Protocol”, page 54.

Important points before starting

- Before beginning the procedure read “General precautions”, page 11.
- Take time to familiarize yourself with the Rotor-Gene Q MDx instrument before starting the protocol. See the instrument user manual.
- Do not vortex *Taq* DNA polymerase (*Taq*) or any mix containing *Taq* DNA polymerase, as this may inactivate the enzyme.
- Pipet *Taq* DNA polymerase (*Taq*) by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.
- Up to 24 samples can be assessed using the Control Reaction Mix (CTRL) available.

Things to do before starting

- Ensure that the *therascreen* BRAF Assay Package software is installed before first use of the Rotor-Gene Q instrument (see “Appendix II: Installation of the *therascreen* BRAF Assay Package”, page 83).
- Before each use, all reagents need to be thawed for a minimum of 1 hour at room temperature (15–25°C), mixed by inverting 10 times, and centrifuged briefly to collect the contents at the bottom of the tube.
- Ensure that *Taq* DNA polymerase (*Taq*) is at room temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

Procedure

- 1. Thaw the Control Reaction Mix (CTRL), Water for No Template Control (NTC), and the Positive Control (PC) at room temperature (15–25°C) for a minimum of 1 hour. When the reagents have thawed, mix them by inverting each tube 10 times to avoid localized concentrations of salts, and then centrifuge briefly to collect the contents at the bottom of the tube.**

2. Prepare sufficient master mixes (Control Reaction Mix [CTRL] plus *Taq* DNA polymerase [*Taq*]) for the DNA samples, one positive control reaction, and one no template control reaction according to the volumes given in Table 2. Include reagents for 1 extra sample to allow sufficient overage for the PCR setup.

The master mix contains all of the components needed for PCR except the sample.

Table 2. Preparation of control assay master mix*

Component	Volume
Control Reaction Mix (CTRL)	19.5 μl x (n+1)*
<i>Taq</i> DNA polymerase (<i>Taq</i>)	0.5 μl x (n+1)*
Total volume	20.0 μl/reaction

* n = number of reactions (samples plus controls). When preparing the master mix, prepare enough for 1 extra sample (n+1) to allow for sufficient overage for the PCR setup. The value n should not exceed 26 (24 samples, plus 2 controls).

3. Thoroughly mix the master mix by gently pipetting up and down 10 times. Place the appropriate number of strip tubes in the loading block according to the layout in Figure 1. Immediately add 20 μl master mix to each PCR strip tube.

Caps will remain in the plastic container until required. For sample assessment, control assay master mix should be added to one positive control well, one negative control well, and one well for each sample.

Assay									
Control	1 (PC)	9	17	25	-	-	-	-	-
Control	2 (NTC)	10	18	26	-	-	-	-	-
Control	3	11	19	-	-	-	-	-	-
Control	4	12	20	-	-	-	-	-	-
Control	5	13	21	-	-	-	-	-	-
Control	6	14	22	-	-	-	-	-	-
Control	7	15	23	-	-	-	-	-	-
Control	8	16	24	-	-	-	-	-	-

Figure 1. Layout of sample assessment assays in the loading block. Numbers denote positions in the loading block and indicate final rotor position.

4. Immediately add 5 µl of Water for No Template Control (NTC) to the no template control tube (PCR tube number 2) and cap the tube. Add 5 µl of each sample to the sample tubes (PCR tube numbers 3–26) and cap the tubes. Add 5 µl of BRAF Positive Control (PC) to the positive control tube (PCR tube number 1) and cap the tube.

Mark the lids of the tubes to show the direction to load the tubes into the Rotor-Gene Q MDx instrument.

5. After all PCR tubes are capped, perform a visual check on sample tube fill levels to ensure sample has been added to all tubes.
6. Invert all PCR tubes (4 times) to mix samples and reaction mixes.
7. Place the PCR strip tubes into the appropriate positions in the 72-well rotor (Figure 1). If the rotor is not fully occupied, all empty positions on the rotor must be filled with a capped, empty tube.
8. Immediately place the 72-well rotor into the Rotor-Gene Q MDx instrument. Make sure that the locking ring (accessory of the Rotor-Gene Q instrument) is placed on top of the rotor to secure the tubes during the run.

- Start the Rotor-Gene Q series software by double clicking the “*therascreen* BRAF CE Sample Assessment Locked Template” icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument (see Figure 2).

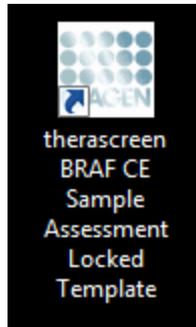


Figure 2. The “*therascreen* BRAF CE Sample Assessment Locked Template” icon.

- The “Setup” tab appears as the default (Figure 3). Ensure that the locking ring is properly attached and check the “Locking Ring Attached” box. Close the lid of the Rotor-Gene Q instrument.

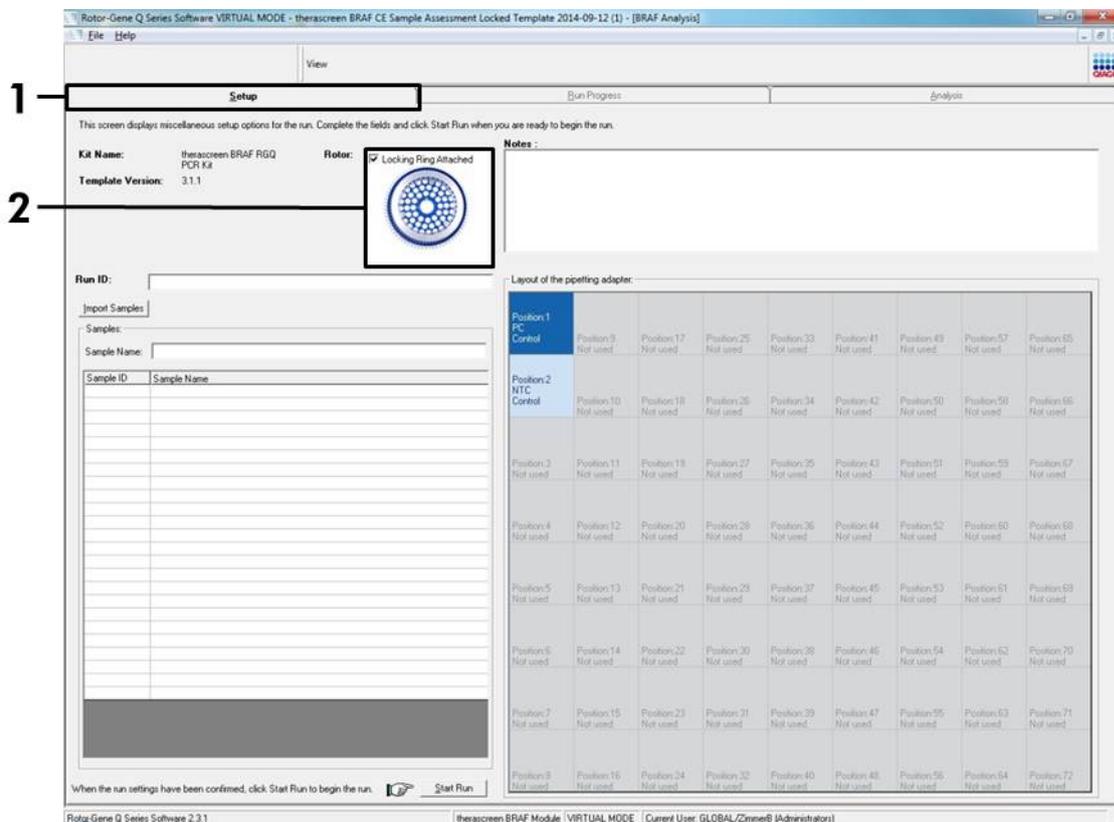


Figure 3. The “Setup” tab (1) and “Locking Ring Attached” box (2).

11. Enter the run ID in the “Run ID” dialog field according to your local naming convention. Enter the sample name in the “Sample Name” dialog field according to your local naming convention and press the return key. This will add the sample name to the sample list below and assign the sample a “Sample ID” (1, 2, 3, etc.). In addition, the “Layout of the pipetting adapter” panel on the right side will update to include the sample name (Figure 4).

Note: Alternatively, sample names stored in the *.smp (Rotor-Gene Q sample file) or *.csv (comma separated values) format can be imported using the “Import Samples” button. Sample names will be populated automatically using this method.

Note: In the “Layout of the pipetting adapter” panel, check that addition of the sample name has been highlighted by a change in color and the sample name is in the sample position (Figure 4).

Note: Sample names with more than 8 characters may not be completely displayed in the “Layout of the pipetting adapter” panel.

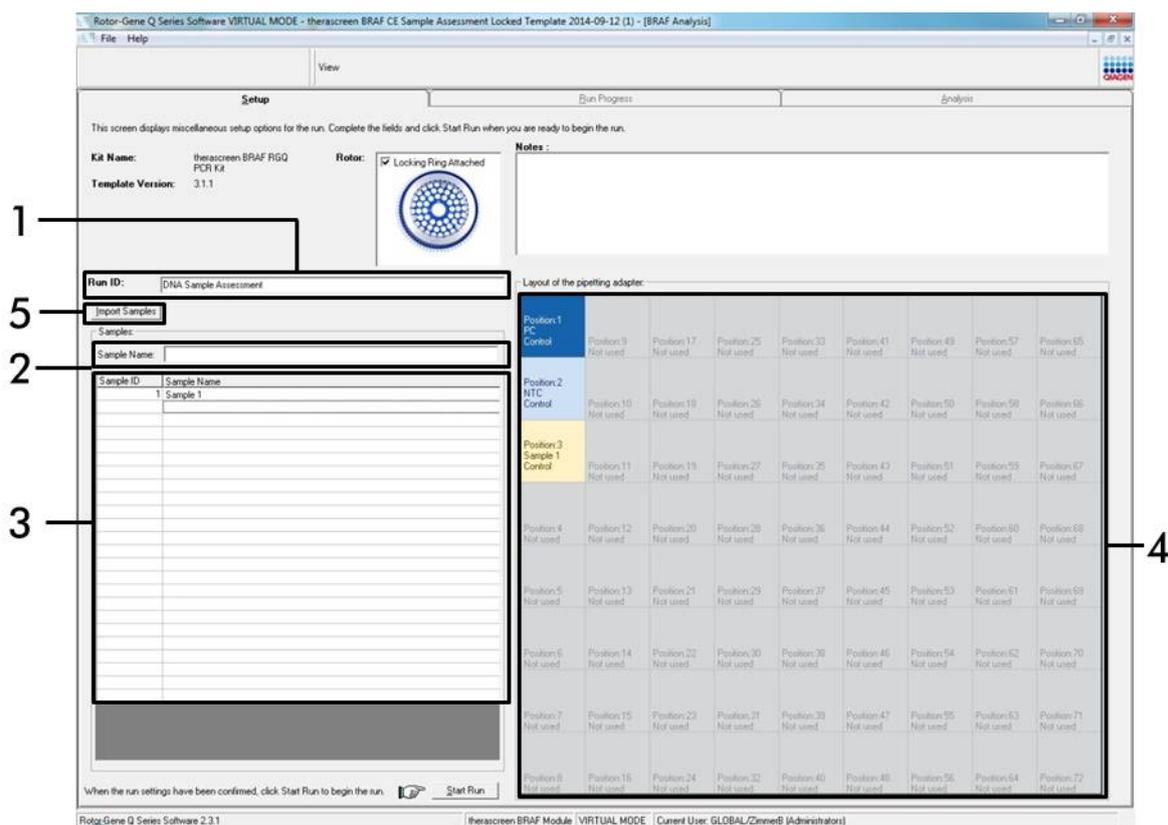


Figure 4. Entering the “Run ID” and “Sample Name”. (1 = “Run ID” dialog field, 2 = “Sample Name” dialog field, 3 = Sample List, 4 = “Layout of the pipetting adapter” panel, 5 = “Sample Import” button).

12. Repeat step 11 to enter the names of all additional samples (Figure 5).

Note: To edit a sample name, click on the “Sample Name” in the sample list and the selected sample will appear in the “Sample Name” dialog field above. Edit the sample name according to your local naming convention and press the return key to update the name.

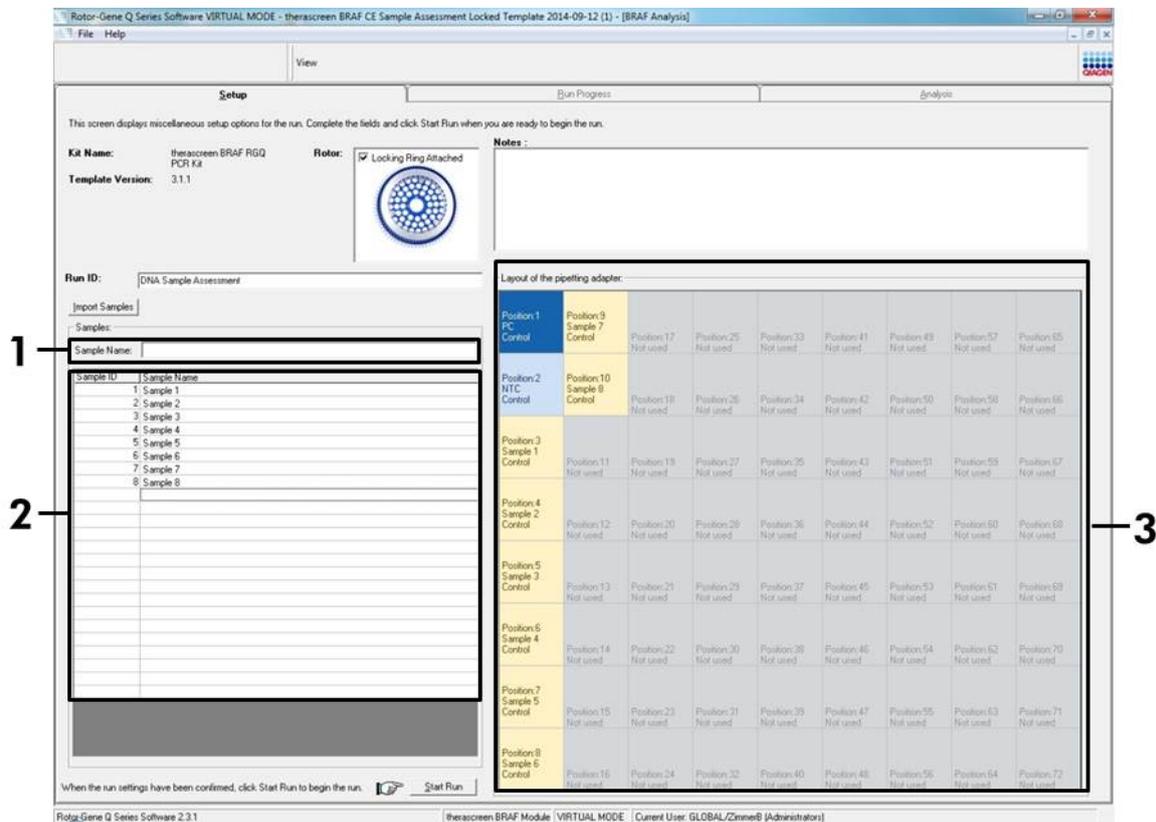


Figure 5. Entering additional sample names in the “Sample Name” dialog field. (1 = “Sample Name” dialog field, 2 = Sample List, 3 = “Layout of the pipetting adapter” panel).

13. When all sample names have been entered, verify they are correct. Add any additional information in the “Notes” dialog field if necessary and then click the “Start Run” button (Figure 6).

Note: If any rotor position is unused, a “Warning” will appear (Figure 6) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube. Check that all unused rotor positions are filled with a capped, empty tube and click “OK” to proceed.

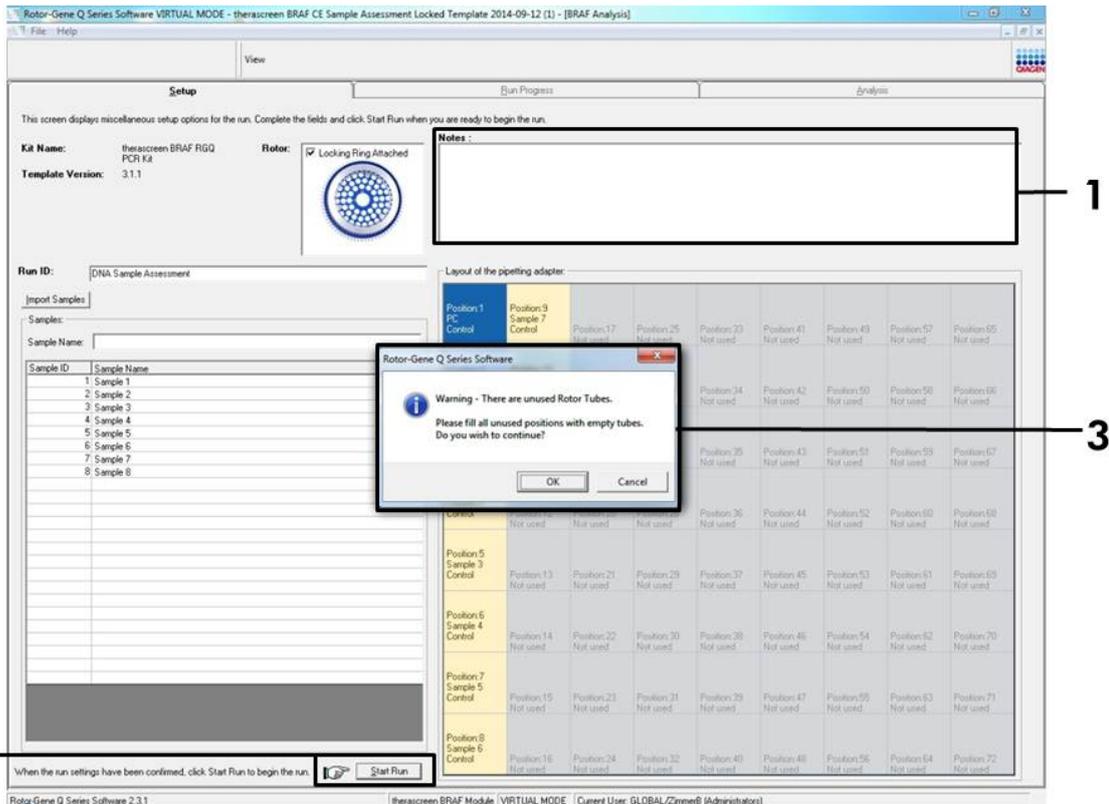


Figure 6. “Notes” dialog field (1), the “Start Run” button (2) and “Warning” of unused rotor positions (3).

14. A "Save As" window appears. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location and click the "Save" button (Figure 7).

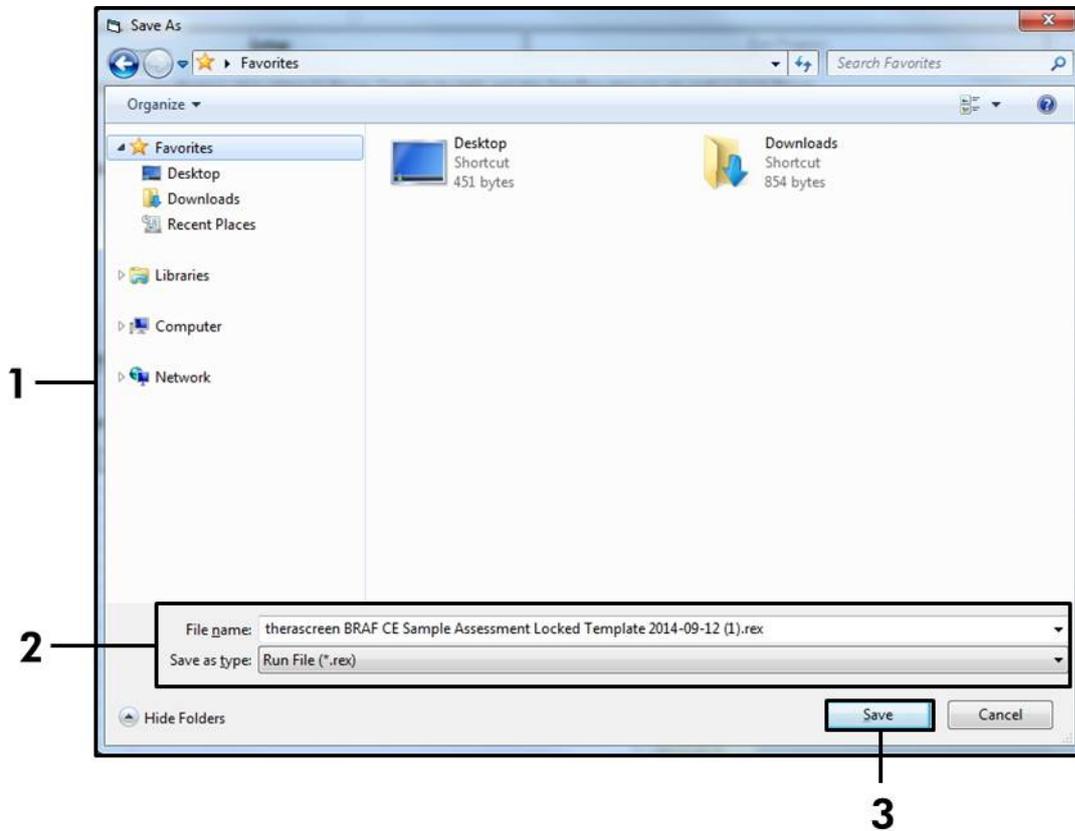


Figure 7. Saving the run file. (1 = "Save As" window, 2 = "File Name" and "Save as type" fields, 3 = "Save" button).

15. The PCR run starts.

Note: When the run starts, the “Run Progress” tab will open automatically to show the temperature trace and remaining run time (Figure 8).

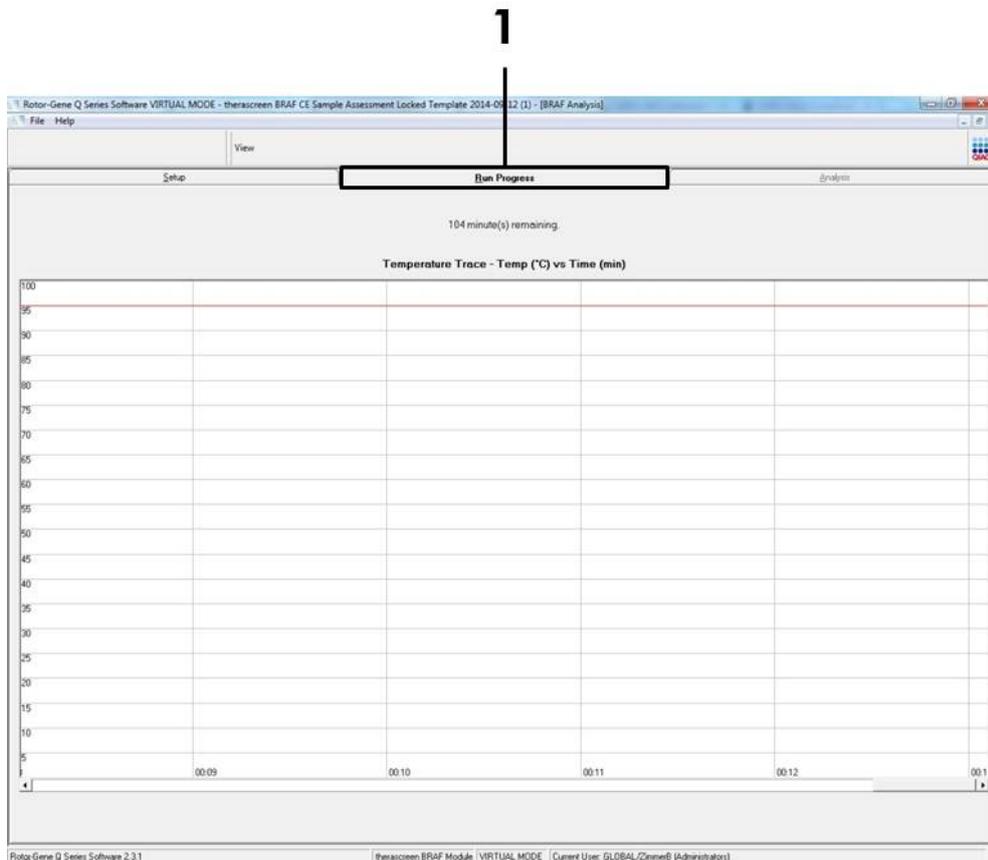


Figure 8. The “Run Progress” tab.

16. After the run is finished, the “Analysis” tab will open automatically.

Note: If the “Analysis” tab fails to open, click the “Analysis” tab (Figure 9).

Note: An explanation of the calculation method is presented in the “Interpretation of Results” section, page 36.

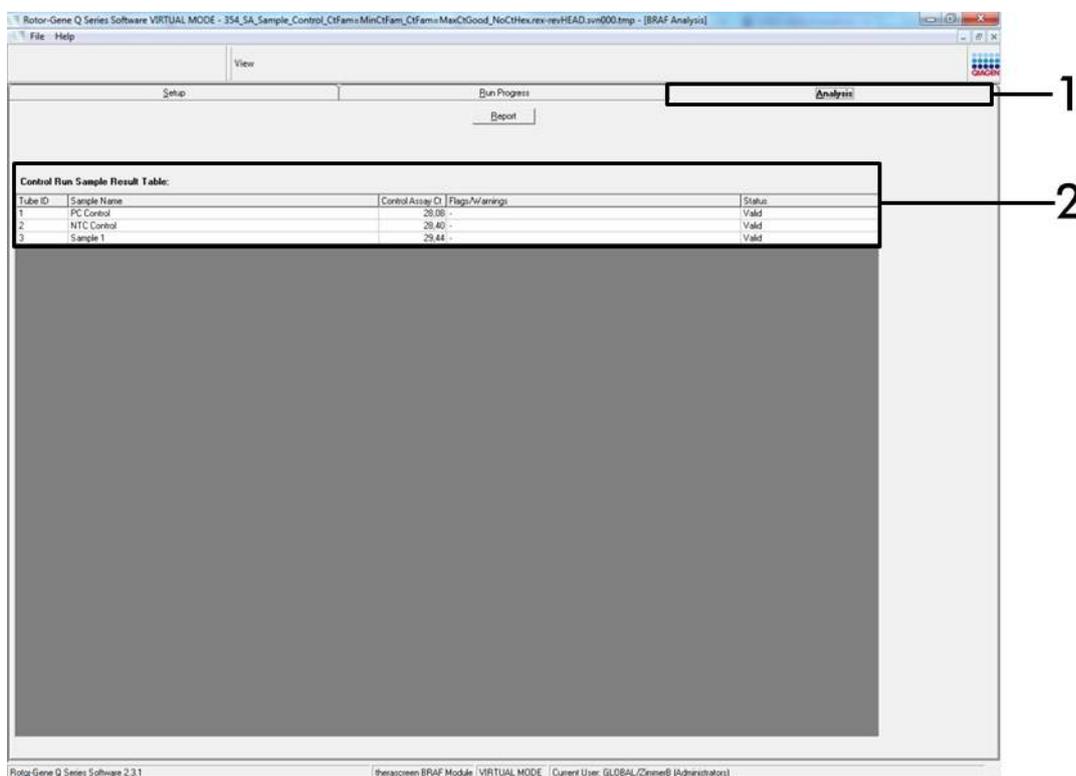


Figure 9. The “Analysis” tab and reporting of results. (1 = “Analysis” tab, 2 = “Sample Result Table”).

17. Control results will be reported as follows in the “Sample QC Result Table” (Figure 9).

- **Run controls (PC and NTC, tube positions 1 and 2 respectively).** If the results are within acceptable ranges, each will display “Valid”, otherwise an “Invalid” result will appear.
- **Sample control reaction $C_T > 32.00$, will display “Invalid”.** Quantity of DNA is not sufficient for mutation analysis. Retest the sample. If the quantity of DNA is still insufficient, extract more tumor tissue if available (see “Troubleshooting guide”, page 37).
- **Sample control reaction $C_T < 21.95$, will display “Invalid”.** DNA concentration is too high for mutation analysis. Dilute with Nuclease-Free Water for Dilution (Dil.) and retest. Dilute to a C_T of 21.95–32.00. A 1:1 dilution increases the C_T value by approximately 1.0.

- **Sample control reaction C_T of 21.95–32.00, ($21.95 \leq \text{Control } C_T \leq 32.00$) will display “Valid”.** DNA concentration is suitable for mutation analysis.

Note: If re-extraction or dilution is required, repeat the control reaction to confirm that the DNA concentration is suitable for use.

18. Report files can be produced by clicking the “Report” button. The “Report Browser” window will appear. Select “BRAf CE Analysis Report” under “Templates” and then click the “Show” button (Figure 10).

Note: Reports can be saved to an alternative location in Web Archives format by clicking the “Save As” button on the top left corner of each report.

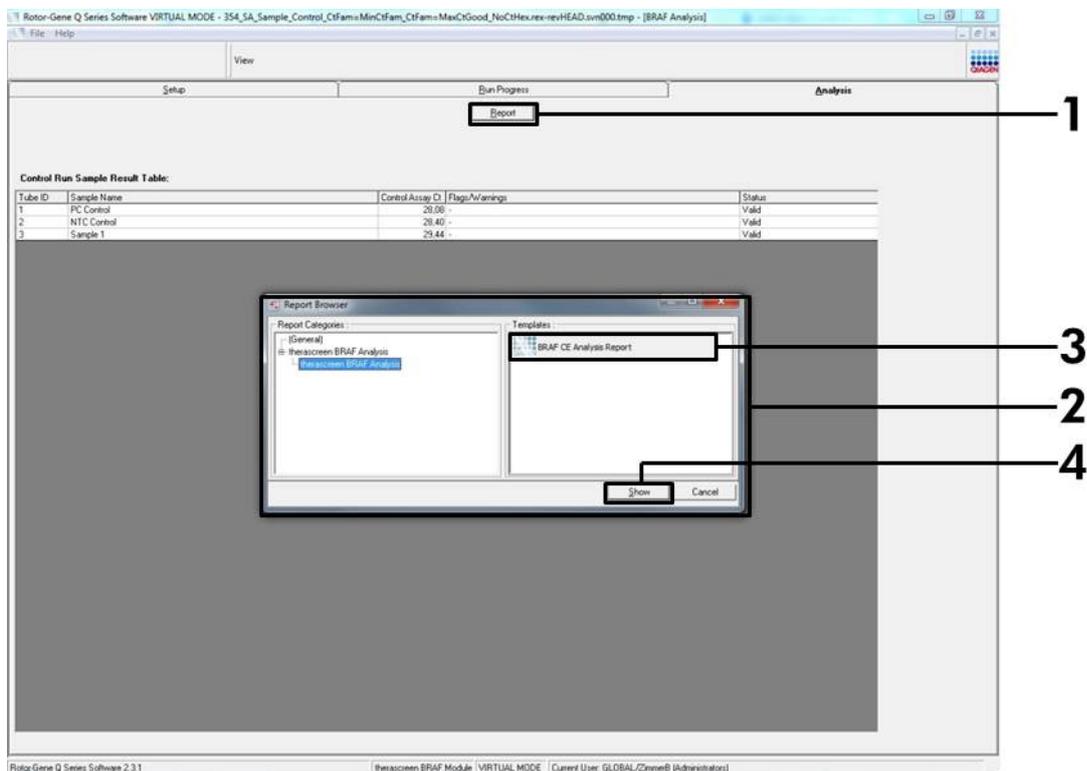


Figure 10. Selecting the “BRAf CE Analysis Report”. (1 = “Report” button, 2 = “Report Browser”, 3 = “BRAf CE Analysis Report”, 4 = “Show” button).

Protocol: BRAF mutation detection

This protocol is for the detection of BRAF mutations. Once a sample has passed the sample assessment, it can be tested using the BRAF mutation assays using automated software.

Note: For manual mutation detection refer to “Appendix I: *therascreen* BRAF RGQ PCR Kit Manual Protocol”, page 54.

Important points before starting

- Before beginning the procedure read “General precautions”, page 11.
- Take time to familiarize yourself with the Rotor-Gene Q MDx before starting the protocol. See the instrument user manual.
- Do not vortex *Taq* DNA polymerase (*Taq*), or any mix containing *Taq* DNA polymerase, as this may inactivate the enzyme.
- For efficient use of the *therascreen* BRAF RGQ PCR Kit, samples must be grouped into batches of no less than 6. Smaller batch sizes will mean that fewer samples can be tested with the *therascreen* BRAF RGQ PCR Kit.
- Pipet *Taq* DNA polymerase (*Taq*) by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.

Things to do before starting

- Ensure that the *therascreen* BRAF Assay Package software is installed before first use of the Rotor-Gene Q instrument (see “Appendix I: *therascreen* BRAF RGQ PCR Kit Manual Protocol”, page 54)
- Before each use, all reagents need to be thawed for a minimum of 1 hour at room temperature (15–25°C), mixed by inverting 10 times, and centrifuged briefly to collect the contents at the bottom of the tube.
- Ensure that *Taq* DNA polymerase (*Taq*) is at room temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

Procedure

- 1. Thaw the reaction mixes, Water for No Template Control (NTC), and BRAF Positive Control (PC) at room temperature (15–25°C) for a minimum of 1 hour. When the reagents have thawed, mix them by inverting each tube 10 times to avoid localized concentrations of salts and then centrifuge briefly to collect the contents at the bottom of the tube.**

2. Prepare sufficient master mixes (reaction mix plus *Taq* DNA polymerase [*Taq*]) for the DNA samples, one positive control reaction, and one no template control reaction according to the volumes given in Table 3. Include reagents for 1 extra sample to allow sufficient coverage for the PCR setup.

The master mixes contain all of the components needed for PCR except the sample.

Table 3. Preparation of assay master mixes*

Assay	Volume of reaction mix	Volume of <i>Taq</i> DNA polymerase (<i>Taq</i>)
Control	19.5 μ l x (n+1)	0.5 μ l x (n+1)
V600E/Ec	19.5 μ l x (n+1)	0.5 μ l x (n+1)
V600D	19.5 μ l x (n+1)	0.5 μ l x (n+1)
V600K	19.5 μ l x (n+1)	0.5 μ l x (n+1)
V600R	19.5 μ l x (n+1)	0.5 μ l x (n+1)

* n = number of reactions (samples plus controls). When preparing the master mix, prepare enough for 1 extra sample (n+1) to allow for sufficient coverage for the PCR setup.

3. Thoroughly mix the master mix by gently pipetting up and down 10 times. Place the appropriate number of strip tubes in the loading block according to the layout in Figure 11. Immediately add 20 μ l master mix to each PCR strip tube (not provided).

Caps will remain in the plastic container until required.

Assay	Controls		Sample number						
	PC	NTC	1	2	3	4	5	6	7
Control	1	9	17	25	33	41	49	57	65
V600E/Ec	2	10	18	26	34	42	50	58	66
V600D	3	11	19	27	35	43	51	59	67
V600K	4	12	20	28	36	44	52	60	68
V600R	5	13	21	29	37	45	53	61	69
–	6	14	22	30	38	46	54	62	70
–	7	15	23	31	39	47	55	63	71
–	8	16	24	32	40	48	56	64	72

Figure 11. Layout of control and mutation assays in the loading block. Numbers denote position in the loading block and indicate final rotor position.

4. Immediately add 5 µl of Water for No Template Control (NTC) to the no template control PCR strip tubes (PCR tube numbers 9–13) and cap the tubes. Add 5 µl of each sample to the sample tubes (PCR tube numbers 17–21, 25–29, 33–37, 41–45, 49–53, 57–61, and 65–69) and cap the tubes. Add 5 µl of BRAF Positive Control (PC) to the positive control tubes (PCR tube numbers 1–5) and cap the tubes. Each DNA sample must be tested with both the control and all of the mutation assays.

Mark the lids of the tubes to show the direction to load the tubes into the Rotor-Gene Q MDx instrument.

5. After all PCR tubes are capped, perform a visual check on sample tube fill levels to ensure sample has been added to all tubes.
6. Invert all PCR tubes (4 times) to mix samples and reaction mixes
7. Place the PCR strip tubes into the appropriate positions in the 72-well rotor (Figure 11).

A maximum of 7 samples can be included in each PCR run. If the rotor is not fully occupied, all empty positions on the rotor must be filled with a capped, empty tube.

8. Immediately place the 72-well rotor into the Rotor-Gene Q MDx instrument. Make sure that the locking ring (accessory of the Rotor-Gene Q instrument) is placed on top of the rotor to secure the tubes during the run.

- Start the Rotor-Gene Q software and open the template at the same time by double-clicking on the “*therascreen* BRAF CE Mutation Analysis Locked Template” icon on the desktop of the laptop connected to the Rotor-Gene Q instrument (Figure 12).



Figure 12. The “*therascreen* BRAF CE Mutation Analysis Locked Template” icon.

- The “Setup” tab appears as the default (Figure 13). Ensure that the locking ring is properly attached and check the “Locking Ring Attached” box. Close the lid of the Rotor-Gene Q instrument.

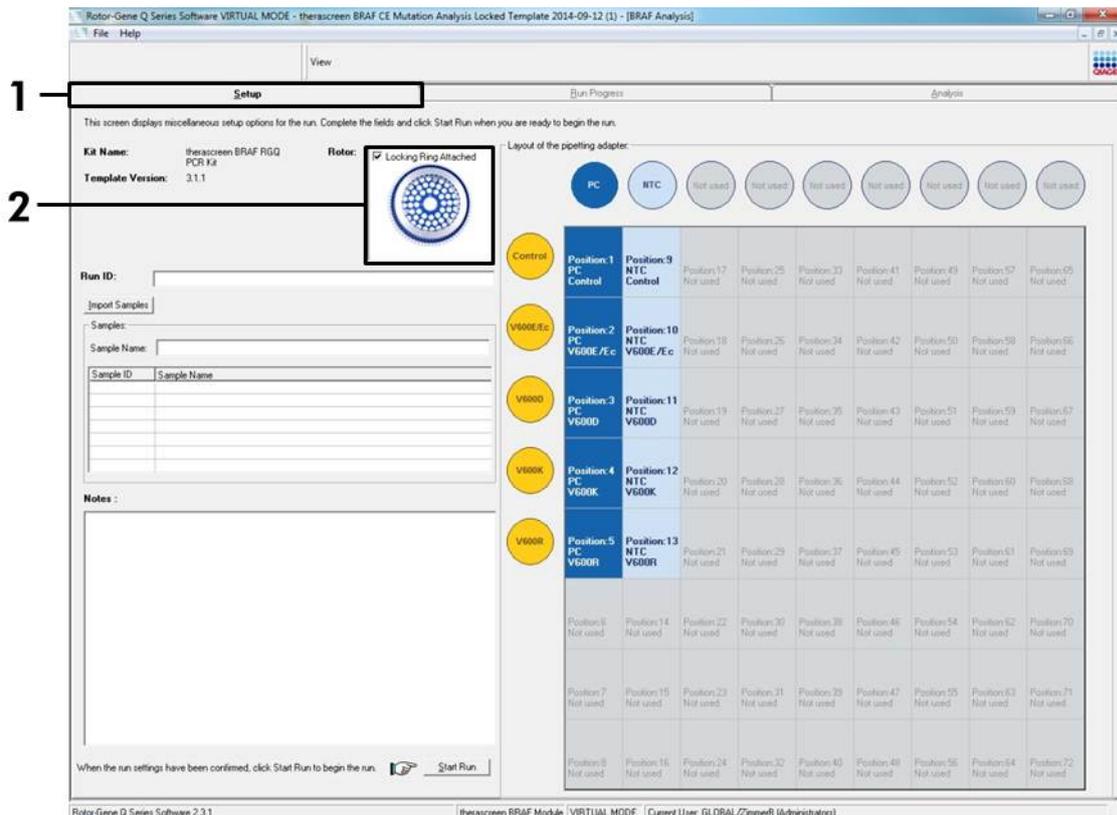


Figure 13. The “Setup” tab (1) and “Locking Ring Attached” box (2).

11. Enter the run ID in the “Run ID” dialog field according to your local naming convention. Enter the sample name in the “Sample Name” dialog field according to your local naming convention and press the return key. This will add the sample name to the sample list below and assign the sample a “Sample ID” (1, 2, 3, etc.). In addition, the “Layout of the pipetting adapter” panel on the right side will update to include the sample name (Figure 14).

Note: Alternatively, sample names stored in the *.smp (Rotor-Gene Q sample file) or *.csv (comma separated values) format can be imported using the “Import Samples” button. Sample names will be populated automatically using this method.

Note: In the “Layout of the pipetting adapter” panel, check the sample name has been highlighted by a change in color and that all assays in the column under the sample circle are highlighted (Figure 14).

Note: A maximum of 7 samples can be added. The sample IDs (in the sample circles) will automatically be assigned from 1 to 7.

Note: Sample names with more than 8 characters may not be completely displayed in the “Layout of the pipetting adapter” panel.

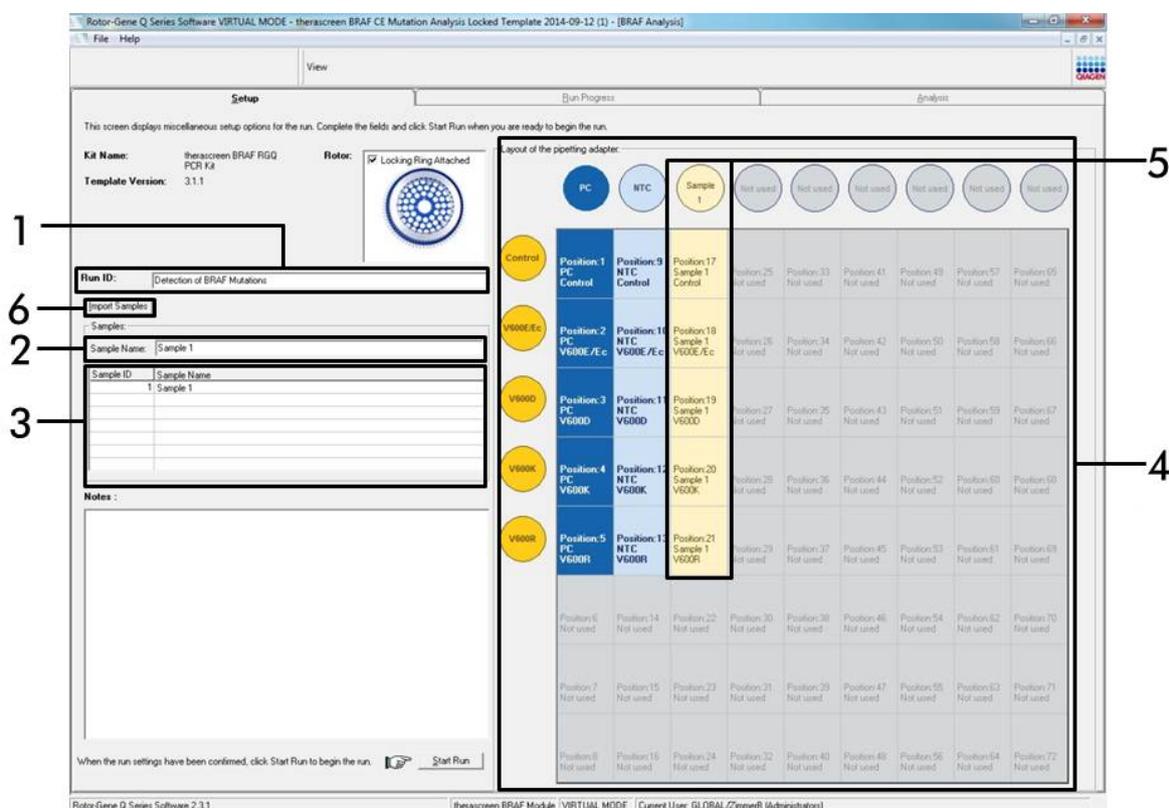


Figure 14. Entering the “Run ID” and “Sample Name”. (1 = “Run ID” dialog field, 2 = “Sample Name” dialog field, 3 = Sample List, 4 = “Layout of the pipetting adapter” panel, 5 = Highlighted sample circle and column of 5 assays underneath panel, 6 = “Sample Import” button).

12. Repeat step 11 to enter the names of all additional samples (Figure 15).

Note: To edit a sample name, click on the “Sample Name” in the sample list and the selected sample will appear in the “Sample Name” dialog field above. Edit the sample name according to your local naming convention and press the return key to update the name.

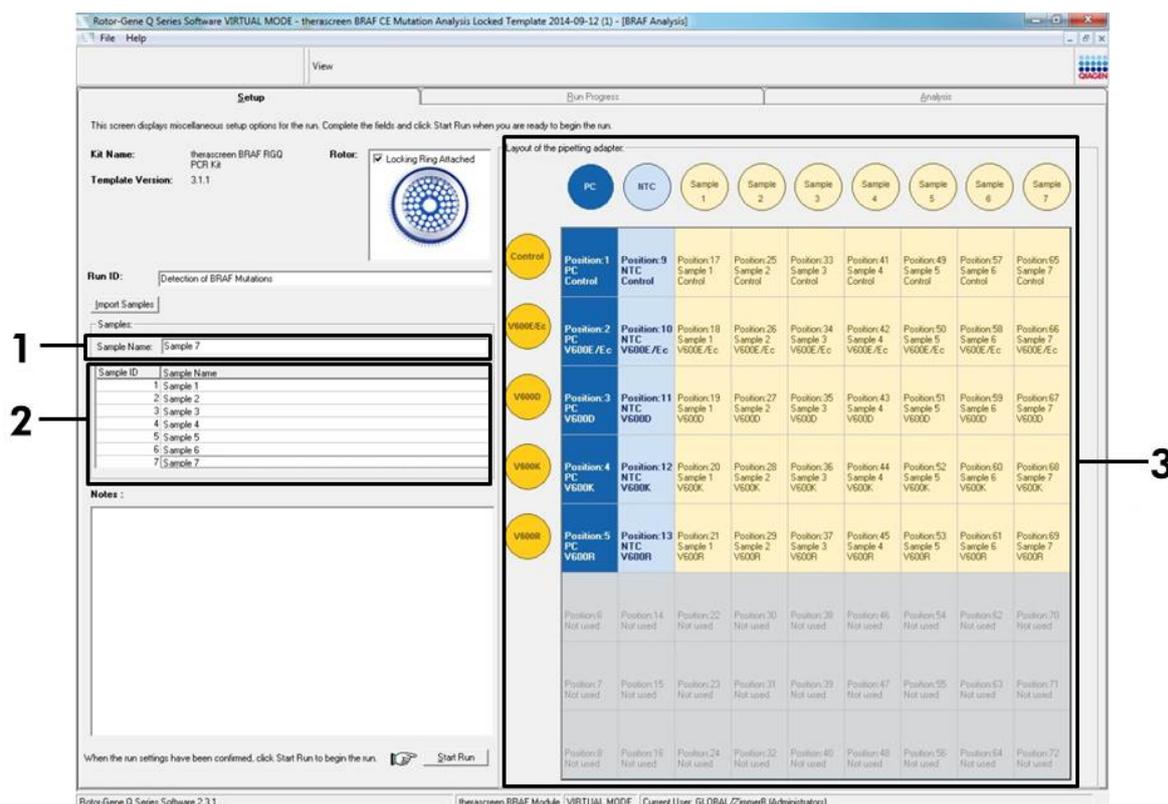


Figure 15. Entering additional sample names in the “Sample Name” dialog field. (1 = “Sample Name” dialog field, 2 = Sample list, 3 = “Layout of the pipetting adapter”).

13. When all sample names have been entered, verify they are correct. Add any additional information in the “Notes” dialog field if necessary and then click the “Start Run” button (Figure 16).

Note: If any rotor position is unused, a “Warning” will appear (Figure 16) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube. Check that all unused rotor positions are filled with a capped, empty tube and click “OK” to proceed.

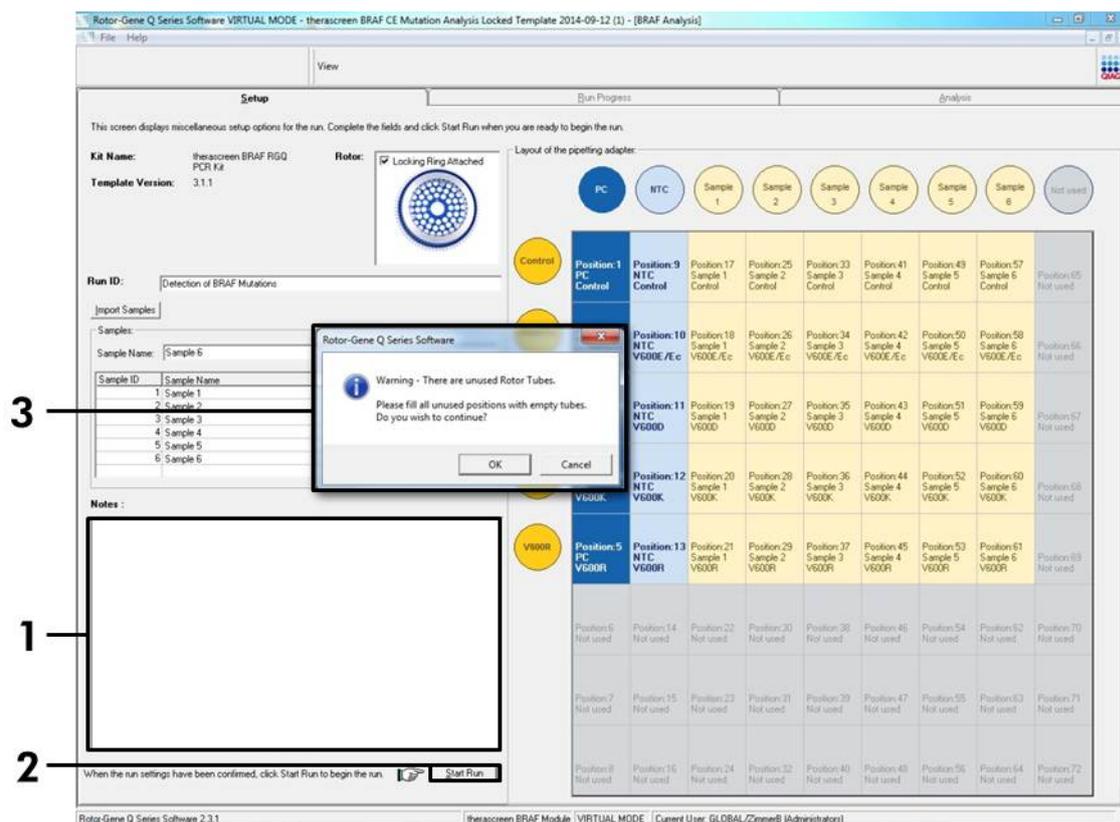


Figure 16. “Notes” dialog field (1), the “Start Run” button (2) and “Warning” of unused rotor positions (3).

14. A "Save As" window appears. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location (Figure 17).

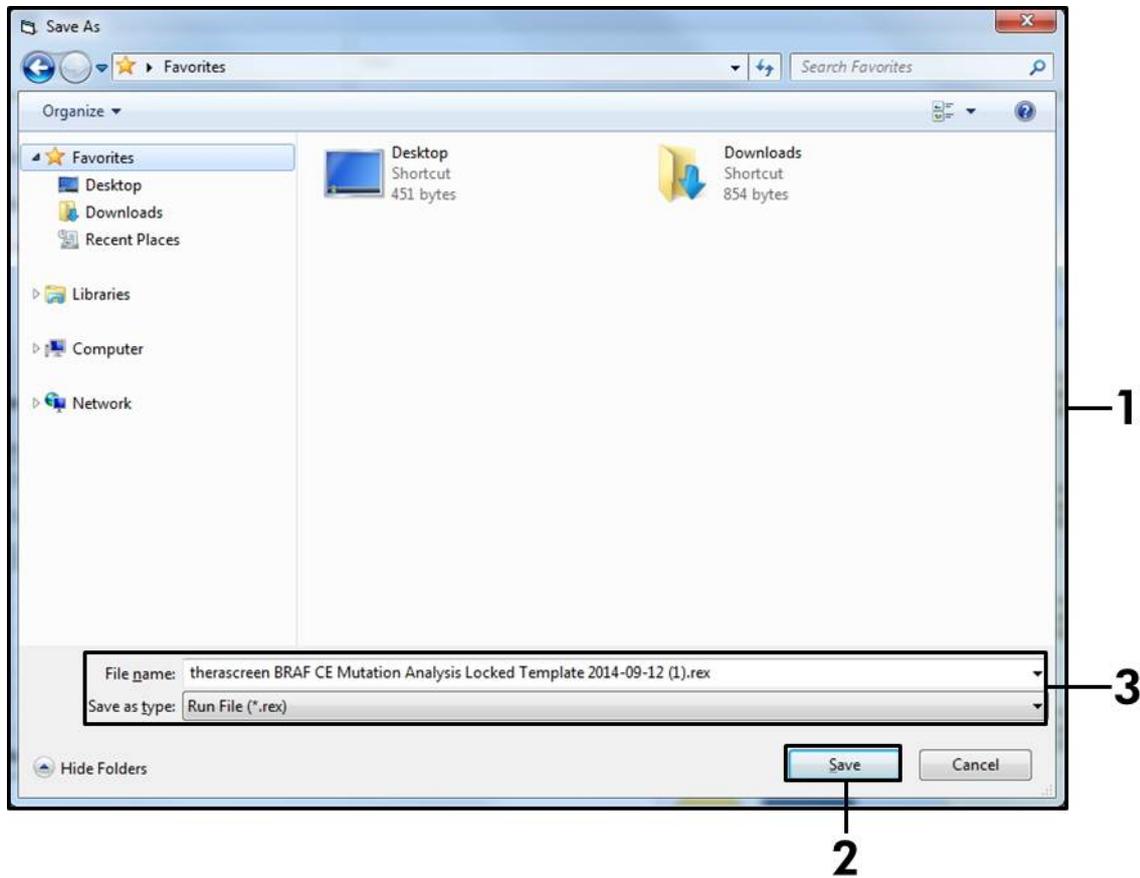


Figure 17. Saving the run file. (1 = "Save As" window, 2 = "File Name" and "Save as type" fields, 3 = "Save" button).

15. The PCR run starts.

Note: When the run starts, the “Run Progress” tab will open automatically to show the temperature trace and remaining run time (Figure 18).

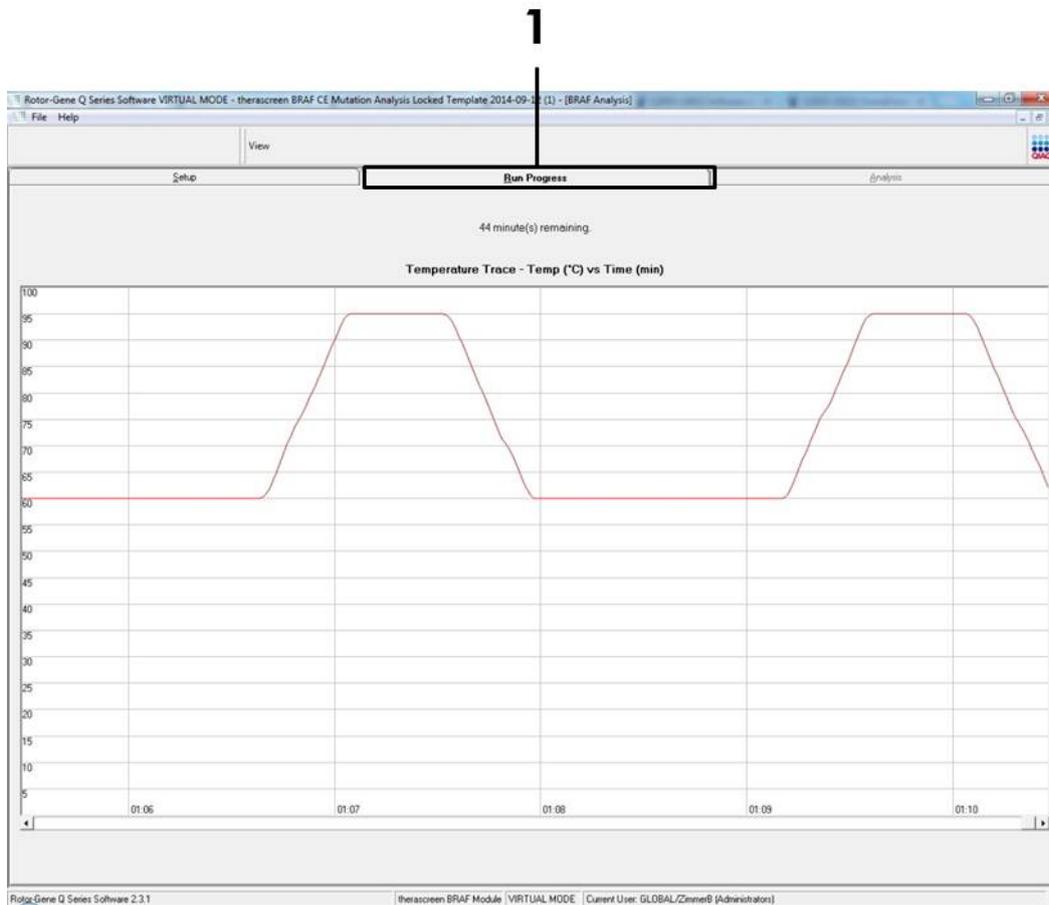


Figure 18. The “Run Progress” tab (1).

16. After the run is finished, the “Analysis” tab will open automatically.

Note: If the “Analysis” tab fails to open, click the “Analysis” tab (Figure 19).

Note: An explanation of the calculation method is presented in the “Interpretation of Results” section, page 36.

The screenshot shows the Rotor-Gene Q Series Software interface in VIRTUAL MODE. The 'Analysis' tab is selected. The interface is divided into three main sections:

- Run Controls, Positive Control:** A table with 5 rows. All 'Positive Control Status' values are 'Valid'.
- Run Controls, Negative Control:** A table with 5 rows. All 'Negative Control Status' values are 'Valid'.
- Sample Result Table:** A table with 1 row for 'Sample 1'. The 'BRAF Status' is 'Mutation Detected'. The 'BRAF Mutation Status' column also shows 'Mutation Detected'.

Numbered callouts in the image point to: 1 = Analysis Tab, 2 = Run Controls, Positive Control panel, 3 = Run Controls, Negative Control panel, 4 = Sample Result Table, 5 = BRAF Mutation Status panel.

Figure 19. The “Analysis” tab and reporting of results. (1 = “Analysis Tab” 2 = “Run Controls, Positive Controls” panel, 3 = “Run Controls, Negative Control” panel, 4 = “Sample Result Table”, 5 = “BRAF Mutation Status” panel).

17. Assay results will be reported as follows (Figure 19):

- **The “Run Controls, Positive Control” panel.** If the results are within acceptable range, the “Positive Control Status” will display “Valid”, otherwise an “Invalid” result will appear.
- **The “Run Controls, Negative Control” panel.** If both the “NTC” and “Internal Control” results are within acceptable ranges, the “Negative Control Status” will display “Valid”, otherwise an “Invalid” result will appear.
- **The “Sample Result Table” panel.** Specific mutations will be reported for the Mutation Positive samples under the “BRAF Mutation Status” column.

18. Report files can be produced by clicking the “Report” button. The “Report Browser” window will appear. Select “BRAF CE Analysis Report” under “Templates”, and then click the “Show” button (Figure 20).

Note: Reports can be saved to an alternative location in Web Archives format by clicking the “Save As” button on the top left corner of each report.

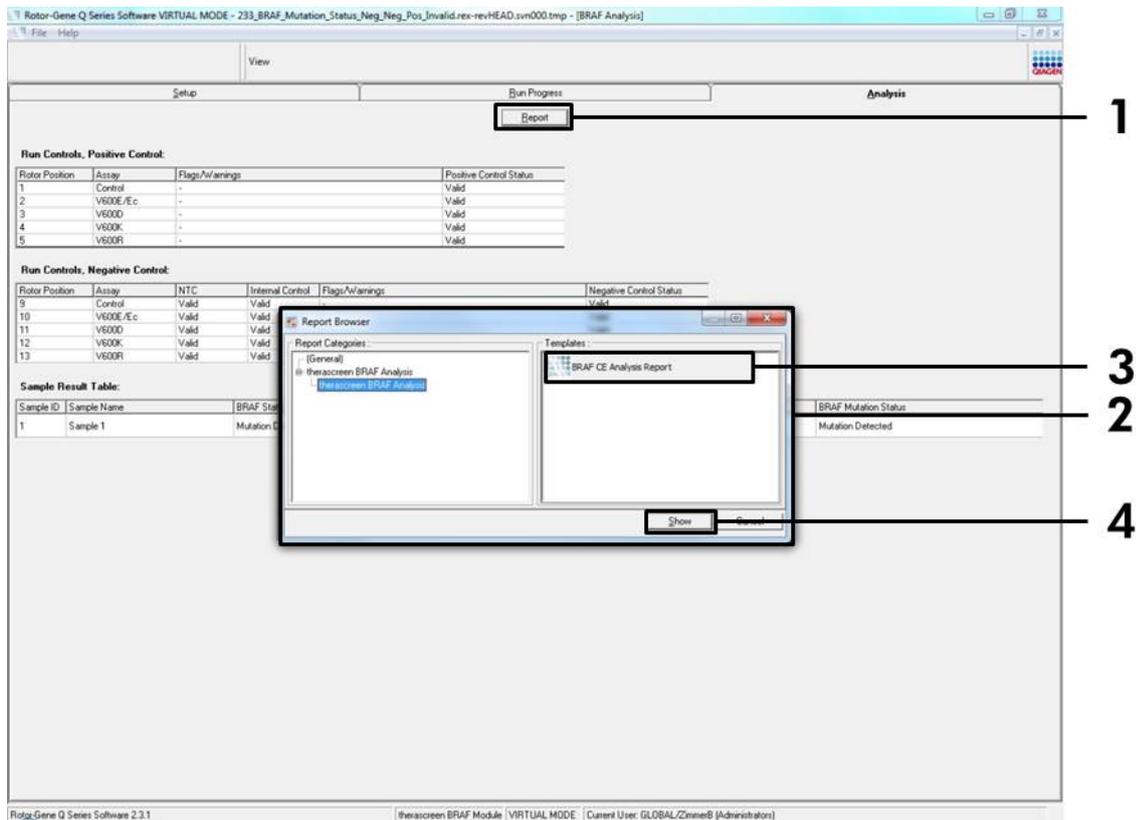


Figure 20. Selecting the “BRAF CE Analysis Report”. (1 = “Report” button, 2 = “Report Browser” panel, 3 = “BRAF CE Analysis Report” button, 4 = “Show” button).

Interpretation of Results (Automated)

The analysis and mutation calls are performed automatically by the *therascreen* BRAF Assay Package once a run is completed. The following information explains how the *therascreen* BRAF Assay Package makes the analysis and mutation calls.

Note: For manual analysis refer to “Appendix I: *therascreen* BRAF RGQ PCR Kit Manual Protocol”, page 54.

The PCR cycle at which the fluorescence from a particular reaction crosses a threshold value is defined as the C_T value. C_T values indicate the quantity of specific input DNA. Low C_T values indicate higher input DNA levels and high C_T values indicate lower input DNA levels. Reactions with a C_T value are classed as positive amplification.

The Rotor-Gene Q software interpolates fluorescence signals between any two recorded values. The C_T values can therefore be any real number (not limited to integers) within the range of 0 to 40.

For the *therascreen* BRAF RGQ PCR Kit, the threshold values of the Green and Yellow channels are set at 0.15 and 0.05 relative fluorescence units respectively. These values are automatically configured in the *therascreen* BRAF Assay Package.

The run controls (positive control, NTC, and internal controls) are assessed to ensure that acceptable C_T values are met and the reactions are performing correctly.

Sample ΔC_T values are calculated, for each mutation assay using the equation:

$$\Delta C_T = [\text{mutation assay } C_T \text{ value}] - [\text{control assay } C_T \text{ value}]$$

Samples are classed as mutation positive if they give a ΔC_T less than or equal to the cutoff ΔC_T value for that assay. Above this value, the sample may either contain less than the percentage of mutation able to be detected by the *therascreen* BRAF RGQ PCR Kit (beyond the limit of the assays), or the sample is mutation negative which would be reported as “No Mutation Detected”.

No amplification in mutation reactions will be scored as “No Mutation Detected”. ΔC_T values calculated from background amplification are expected to be greater than the cutoff ΔC_T values and the sample will be classed as “No Mutation Detected”.

The assay results will be displayed as “Mutation Detected”, “No Mutation Detected”, “Invalid” or, if a run control fails, “Run Control Failed”. For the mutation-positive samples, specific mutations will be reported according to the cross reactivity logic in “Table 8. Calling sample mutation status” on page 48.

Other possible results that may be displayed are discussed in the “Protocol: Sample assessment” on page 14, “Protocol: BRAF mutation detection” on page 25, and “

therascreen BRAF Assay Package flags” on page 37 in this handbook.

Rarely, a tumor may contain more than one mutation. In such instances, the report will show the BRAF Status as “Mutation Detected” however, all positive mutations will be listed along with the warning flag “SAMPLE_POSITIVE_AND_UNCLASSIFIABLE”.

Troubleshooting guide

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

Comments and suggestions

Invalid results

- | | |
|--|---|
| a) The storage conditions for one or more components did not comply with the instructions given in “Reagent Storage and Handling”, page 12 | Check the storage conditions and expiration date (see label) of the box and use a new kit, if necessary. |
| b) The <i>therascreen</i> BRAF CE RGQ PCR Kit has expired | Check the storage conditions and expiration date (see kit label) of the box and, if necessary, use a new <i>therascreen</i> BRAF RGQ PCR Kit. |

***therascreen* BRAF Assay Package flags**

Table 4 lists the possible flags that may be generated by the *therascreen* BRAF Assay Package, their meaning, and actions to be taken.

Table 4. *therascreen* BRAF Assay Package flags

Flag	Meaning	Action to be taken
PC_CTRL_ASSAY_FAIL	PCR run invalid — FAM C _T out of range for positive control in control reaction.	Repeat the entire PCR run.
PC_CTRL_INVALID_DATA	PCR run invalid — Fluorescence data in positive control (control reaction) can't be interpreted.	Repeat the entire PCR run.
PC_MUTATION_ASSAY_FAIL	PCR run invalid — FAM C _T out of range for one or more mutation reactions.	Repeat the entire PCR run.
PC_MUTATION_INVALID_DATA	PCR run invalid — Fluorescence data in positive control (mutation reaction) can't be interpreted.	Repeat the entire PCR run.
NTC_INVALID_DATA	PCR run invalid — Fluorescence data in negative control can't be interpreted.	Repeat the entire PCR run.
NTC_ASSAY_CT_INVALID	PCR run invalid — FAM invalid (smaller than limit) for negative control.	Repeat the entire PCR run.
NTC_INT_CTRL_FAIL	PCR run invalid — Internal control above range for negative control.	Repeat the entire PCR run.
NTC_INT_CTRL_EARLY_CT	PCR run invalid — Internal control is below range for negative control.	Repeat the entire PCR run.

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_CTRL_INVALID_DATA	Sample invalid — Fluorescence data in sample control can't be interpreted.	Setup new PCR run to repeat the relevant sample(s).
SAMPLE_CTRL_HIGH_CONC	Sample invalid — FAM C _T too low in sample control.	Dilute sample to increase control C _T value. This dilution should be calculated on the assumption that diluting 1:1 with the water supplied in the kit will increase the C _T by 1.0; once sample is diluted, setup new PCR run to repeat sample.
SAMPLE_CTRL_LOW_CONC	Sample valid — Low concentration in sample control (warning, not error).	No action.
SAMPLE_CTRL_FAIL	Sample invalid — FAM C _T too high in sample control reaction.	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_CTRL_ INT_CTRL_EARLY_ CT	Sample invalid — HEX C _T too low for sample (internal control).	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.
SAMPLE_CTRL_ INT_CTRL_FAIL	C _T too high (or no C _T) for internal control (HEX) and C _T too high (or no C _T) for the control assay (FAM).	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_INT_CTRL_FAIL	C _T too high (or no C _T) for internal control (HEX) and no C _T for the mutation assay (FAM).	<p>If sample given “mutation detected” status — No action.</p> <p>If sample given “invalid” status, setup new PCR run to repeat sample.</p> <p>Note: If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of inhibitors, but it should be noted that this would also dilute the target DNA. A tube of Water for Sample Dilution (Dil.) is included with the kit.</p> <p>If “invalid” on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.</p>

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_INT_ CTRL_EARLY_CT	Mutation tube invalid — C _T HEX too low for sample (internal control).	If sample given valid “mutation detected” status — No action. If sample given “invalid” status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_INVALID_DATA	Mutation tube invalid – Fluorescence data in internal control can't be interpreted.	<p>If sample given valid "mutation detected" status – No action.</p> <p>If sample given "invalid" status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.</p>

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_ MUTATION_ EARLY_DELTA_CT	Mutation tube invalid —C _T FAM too low for sample.	<p>If sample given valid “mutation detected” status — No action.</p> <p>If sample given “invalid” status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.</p>

Table continued on next page

Table 4 cont.

Flag	Meaning	Action to be taken
SAMPLE_POSITIVE_ AND_INVALID	<p>Result valid — One or more mutation tubes for a sample are valid and positive, at the same time one or more mutation tubes for the same sample are invalid (warning, not an error).</p> <p>The sample is called “mutation detected” since a mutation is present. However, the specific mutation shown on the report may not represent the actual mutation present due to cross reactivity of the assays. Therefore, the sample must be called “mutation detected”.</p>	No action.
SAMPLE_POSITIVE_ AND_UNCLASSIFIABLE	<p>Result valid — More than one mutation tube is valid for the same sample. The combination is not compatible with the expected cross reactivity patterns. Refer to Table 8. Although rare the sample may contain more than one mutation.</p>	No action.

Quality Control

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

Limitations

Results from the product must be interpreted within the context of all relevant clinical and laboratory findings and are not to be used alone for diagnosis.

Verification studies were performed using human DNA extracted from formalin-fixed paraffin-embedded tumor samples and synthetic standards as appropriate for the individual studies.

The product has been verified using the QIAamp DNA FFPE Tissue Kit from QIAGEN.

The product is intended only for use on Rotor-Gene Q MDx instruments

Strict compliance with the *therascreen BRAF RGQ PCR Kit Handbook* is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended, and will result in a loss of performance.

It is important that the amount and quality of DNA in the sample is assessed prior to performing sample analysis using the *therascreen BRAF RGQ PCR Kit*. Additional control mix is provided to determine that the C_T value is acceptable for the assay. Absorbance readings must not be used as they do not correlate with the C_T values in fragmented DNA samples.

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

Performance Characteristics

Limit of blank (LOB), working range, and cutoff values

A total of 143 FFPE samples were tested in a study following guidance in NCCLS EP17-A (2004) to determine the LOB and cutoff values for each mutation assay. In addition, the working range for the control assay was determined. The cutoff values were established and are shown in Table 5.

Table 5. Established cutoff values for each mutation assay

	Mutant analysis (ΔC_T)			
	V600E/Ec	V600D	V600K	V600R
Cutoff (ΔC_T)	≤ 7.0	≤ 6.9	≤ 6.0	≤ 7.0

The control reaction C_T range was established as 21.95 to 32.00 C_T .

The assay cutoffs and working range were verified using standards and a further (unique) 102 FFPE samples. During verification the cutoffs were assessed for ability to distinguish the correct mutation in background of wild-type DNA by assessing each assay with high input genomic DNA and high input mutation (see “Cross-reactivity”, page 48). The effect of input DNA on mutation call was also assessed (see “Effect of input DNA on Δ CT values”, page 47).

Accuracy: Comparison to the analytical reference method

A study demonstrated the concordance in mutation status of the *therascreen* BRAF RGQ PCR Kit relative to bidirectional Sanger sequencing. In this study, 126 FFPE samples were tested using statistical measures of agreement/disagreement from the CLSI EP12-A2 Guidance (2008). Valid results were returned for 102 of the FFPE samples for both the *therascreen* BRAF RGQ PCR Kit and bidirectional Sanger sequencing. Pyrosequencing® was used to confirm mutation status where sample mutation status call was not concordant between bidirectional Sanger sequencing and the *therascreen* BRAF RGQ PCR Kit.

Table 6 shows the analysis of agreement between the *therascreen* BRAF RGQ PCR Kit and sequencing.

Table 6. Analysis of agreement

	Measure of agreement	Frequency (%)
Score	Overall agreement	96.08
	Positive agreement	100.00
	Negative agreement	95.29

The negative agreement frequency is due to mutation detection for 4 samples that were called wild-type by sequencing and V600E/Ec mutation positive by the *therascreen* BRAF RGQ PCR Kit. This is due to the increased sensitivity of the Scorpions and ARMS technologies.

Effect of input DNA on Δ C_T values

The effect of total input DNA levels on the determination of mutation status using the *therascreen* BRAF RGQ PCR Kit was evaluated as part of the Verification of Assay Cut Offs and Working Range study. This was to verify that mutation calls

generated by the *therascreen* BRAF RGQ PCR Kit are consistent at different DNA input levels across the working range.

Mutation standards containing high, medium, and low percentage mutation (100%, 50%, and 3 x LOD%, respectively) in a background of wild-type DNA were prepared at high, medium, and low DNA input levels. Therefore, a total of 9 mutation standards were tested for each mutation assay. The results for all assays are shown in Table 7.

The estimated differences in mean ΔC_T between each pair of DNA input levels, as estimated from the linear regression analysis, are all within $\pm 1 C_T$. All 4 mutation assays were therefore considered equivalent at high, medium, and low DNA input levels.

Table 7. Estimated differences between DNA input levels

Assay	Parameter (DNA input level)	Estimated difference (ΔC_T)	95% confidence interval (lower, higher)
V600E (E)	High — Medium	0.56	0.22, 0.90
V600E (E)	Low — Medium	0.01	-0.33, 0.35
V600E (Ec)	High — Medium	0.48	0.12, 0.84
V600E (Ec)	Low — Medium	0.26	-0.10, 0.62
V600D	High — Medium	-0.32	-0.58, -0.06
V600D	Low — Medium	-0.43	-0.69, -0.17
V600K	High — Medium	0.10	-0.10, 0.30
V600K	Low — Medium	-0.33	-0.53, -0.13
V600R	High — Medium	-0.12	-0.28, 0.04
V600R	Low — Medium	-0.62	-0.78, -0.46

Cross-reactivity

Standards at high input DNA with high mutation content (100%) were tested to assess potential cross-reactivity of each assay. Cross-reactivity results enabled compilation of a mutation status logic table as shown in Table 8. The BRAF CE Assay Package uses cross-reactivity logic to determine mutation status.

Table 8. Calling sample mutation status

V600E/Ec	V600D	V600K	V600R	Mutation status
Positive	Negative	Negative	Negative	V600E or V600Ec positive
Positive	Negative	Positive	Negative	V600Ec or V600K positive
Positive	Positive	Negative	Negative	V600D positive
Negative	Positive	Negative	Negative	V600D positive
Negative	Negative	Positive	Negative	V600K positive
Negative	Negative	Negative	Positive	V600R positive

Limit of detection (LOD) values

A study was conducted to determine the LOD of each of the 4 mutation-specific reactions incorporated in the *therascreen* BRAF RGQ PCR Kit. In this study, LOD was defined as the lowest amount of mutant DNA in a background of wild-type DNA at which a mutant sample will provide mutation-positive results in 95% of the test results (C_{95}).

To determine the LOD for each assay, different percentage mutation standards were prepared at medium input DNA concentration and tested with the *therascreen* BRAF RGQ PCR Kit. The LOD for each assay was calculated by logistic regression. To verify the LOD for each assay, mutation standards at the determined LOD were prepared. Sixty replicates were tested and the positive test rate verified.

The verified LOD at medium input DNA concentration is given in Table 9. At higher input DNA concentrations, the LOD values are expected to be lower than the values stated in Table 9.

Table 9. LOD values for each mutation assay (medium input)

Assay (mutation)*	LOD C₉₅ at medium input DNA (percentage of mutant DNA in wild-type DNA)
V600E (E)	1.82%
V600E (Ec)	4.31%
V600D	3.19%
V600K	4.34%
V600R	4.85%

* Limit of detections for the V600E assay were calculated for both V600E and V600Ec mutations.

Effect of melanin on kit performance

The objective of this study was to evaluate the impact of melanin, a known PCR inhibitor found in melanoma samples, on the performance of the *therascreen* BRAF RGQ PCR Kit. This was performed by spiking melanin directly into DNA samples prior to testing with the *therascreen* BRAF RGQ PCR Kit across a range of concentrations (0–250 ng/reaction) and assessing the effect on the ΔC_T values and mutation status of test samples.

The results showed low melanin concentration had no effect on ΔC_T and minimal effect on ΔC_T at medium levels of melanin concentration. Therefore, at low and medium concentration levels melanin did not affect the ability of the assays to detect mutation. At 180 ng/reaction the internal control failed, indicating the presence of inhibitor and thus enabling detection of inhibitors prior to mutation calling being affected.

Melanin concentrations expected to be encountered in normal use do not impact the ability of the *therascreen* BRAF RGQ PCR Kit to distinguish between mutation-positive and mutation-negative samples.

A summary of results is shown in Table 10.

Table 10. Amount of melanin tested in each assay

Melanin concentration (ng/reaction)	Change in ΔC_T	Internal control status (pass/fail)
0	0	Pass
60	-0.20	Pass
100	-0.61	Pass
150	-1.21	Pass
180	-2.15	Fail

Repeatability

A matrix study design was implemented to vary operator, day, plate layout, and instrument to determine assay precision both within run and between runs. Repeatability was demonstrated at low level DNA input at 3 x LOD for mutation assays. In addition, the percentage of mutation-positive calls was assessed for each assay when tested with its specific mutation standard. Each mutation assay gave 100% positive mutation calls.

Precision values are given in Table 11.

Reproducibility

A matrix study design was implemented to assess assay reproducibility by testing standards at 3 laboratories (sites), with 3 lots of *therascreen* BRAF RGQ PCR Kits (2 at each site), using 2 operators per site, on 2 instruments per site, over 4 alternate days. Reproducibility was demonstrated at low level mutation (3 x LOD) for mutation assays and low input wild-type for the control assay. The precision for each assay was calculated across the 3 sites, along with 95% precision estimates (Table 12).

Table 11. Repeatability precision estimates

Assay	Precision (between runs)	95% confidence interval (lower, higher)	Precision (within run)	95% confidence interval (lower, higher)
Control	0.30	0.25, 0.39	0.16	0.13, 0.20
V600E (E)	0.74	0.61, 0.94	0.57	0.46, 0.74
V600E (Ec)	0.79	0.64, 1.01	0.76	0.62, 0.99
V600D	0.47	0.38, 0.60	0.46	0.38, 0.60
V600K	0.37	0.31, 0.48	0.37	0.30, 0.49
V600R	0.44	0.36, 0.56	0.44	0.36, 0.58

Table 12. Reproducibility precision estimates

Assay	Precision	95% confidence interval (lower, higher)
Control	0.54	0.42, 0.76
V600E (E)	0.87	0.67, 1.22
V600E (Ec)	0.86	0.66, 1.21
V600D	0.80	0.62, 1.14
V600K	0.61	0.47, 0.86
V600R	0.63	0.49, 0.89

Symbols



Contains reagents sufficient for <24> reactions



Use by



In vitro diagnostic medical device



Catalog number



Lot number



Material number



Components



Contains



Number



Global Trade Item Number



Temperature limitation



Manufacturer



Consult instructions for use



Keep away from sunlight



Caution

Appendix I: *therascreen* BRAF RGQ PCR Kit Manual Protocol

This section contains instructions for using the *therascreen* BRAF RGQ PCR Kit with RGQ software version 2.3 in the open mode (i.e. without using the BRAF Assay Package).

General Information

- For a list of materials required, refer to page 9.
- For full instructions on sample preparation and sample layout refer to sections “Protocol: Sample assessment”, page 14, and “Protocol: BRAF mutation detection”, page 25.

Protocol: Creating a temperature profile

Before you start, create a temperature profile for the BRAF analysis. The cycling parameters are the same for both Sample Assessment and Mutation Assessment.

Procedure

To summarize, the cycling parameters are as follows:

Table 13. Cycling parameters

Cycles	Temperature	Time	Data acquisition
1	95°C	15 minutes	None
40	95°C	30 seconds	None
	60°C	60 seconds	Green and yellow

1. Double-click the Rotor-Gene Q Series Software 2.3 software icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument.
2. To create a new template, select "Empty Run" and then click "New" to enter the "New Run Wizard".

3. Select 72-Well Rotor as the rotor type. Confirm that the locking ring is attached and check the “Locking Ring Attached” box. Click “Next” (Figure 21).

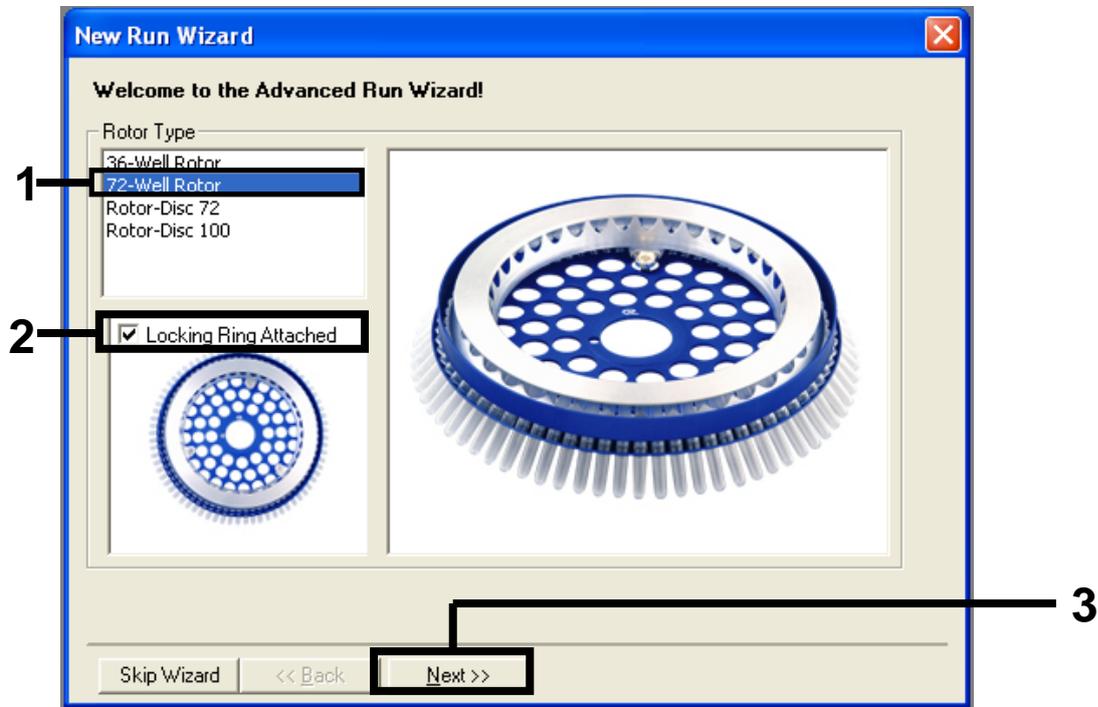


Figure 21. The “New Run Wizard” dialog box. (1 = “Rotor type”, 2 = “Locking Ring Attached box”, 3 = “Next” button).

4. Enter the name of the operator. Add any notes and enter the reaction volume as 25. Ensure that "Sample Layout" reads "1, 2, 3...". Click "Next" (Figure 22).

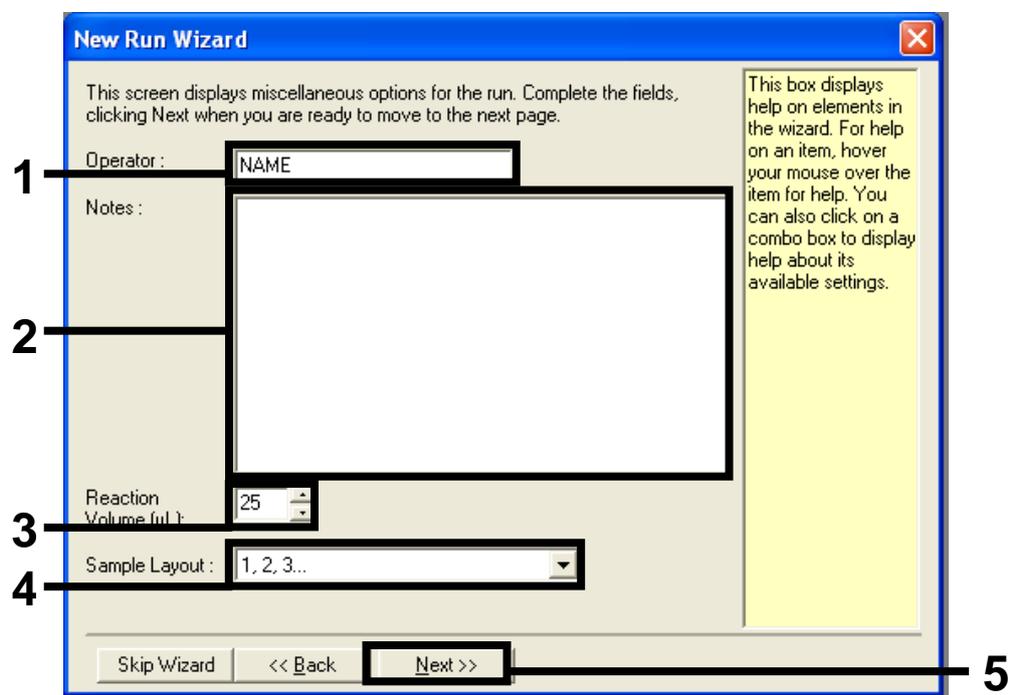


Figure 22. Entering operator name and reaction volumes. (1 = "Operator" dialog field, 2 = "Notes" dialogue field, 3 = "Reaction Volume" field, 4 = "Sample Layout" field, 5 = "Next" button).

5. Click the "Edit Profile" button in the "New Run Wizard" dialog box (Figure 23) and check the run parameters according to the following steps.

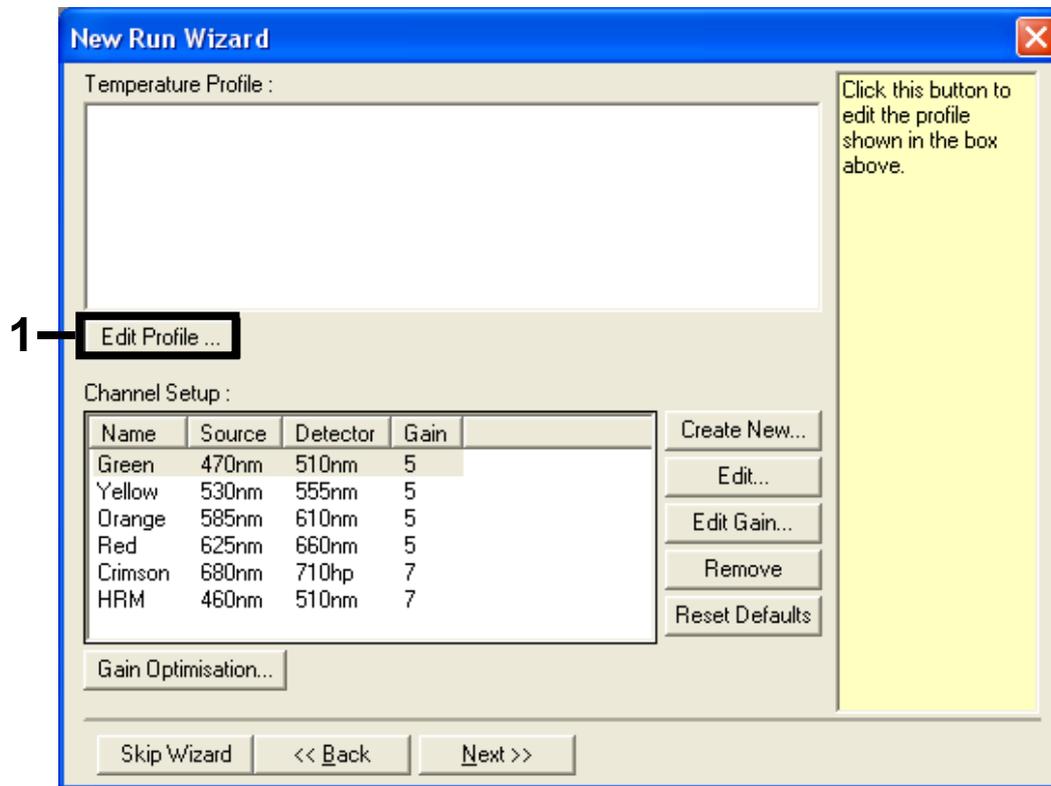


Figure 23. Editing the profile (1).

6. Click the "Insert after" button and select New Hold at Temperature (Figure 24).

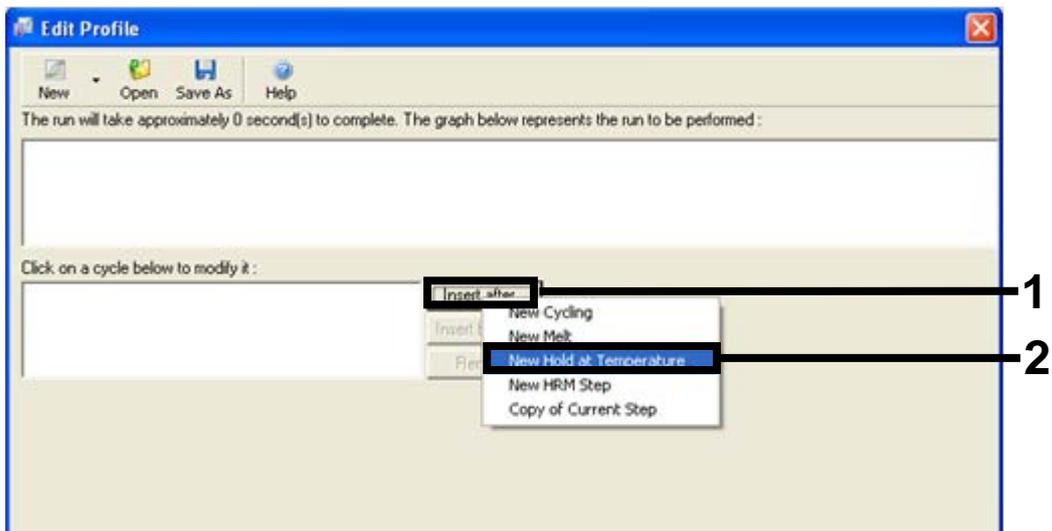


Figure 24. Inserting an initial incubation step. (1 = "Insert after" button, 2 = "New Hold at Temperature").

7. Change "Hold Temperature" to 95°C and "Hold Time" to 15 mins 0 secs. Click the "Insert After" button and then select New Cycling (Figure 25).

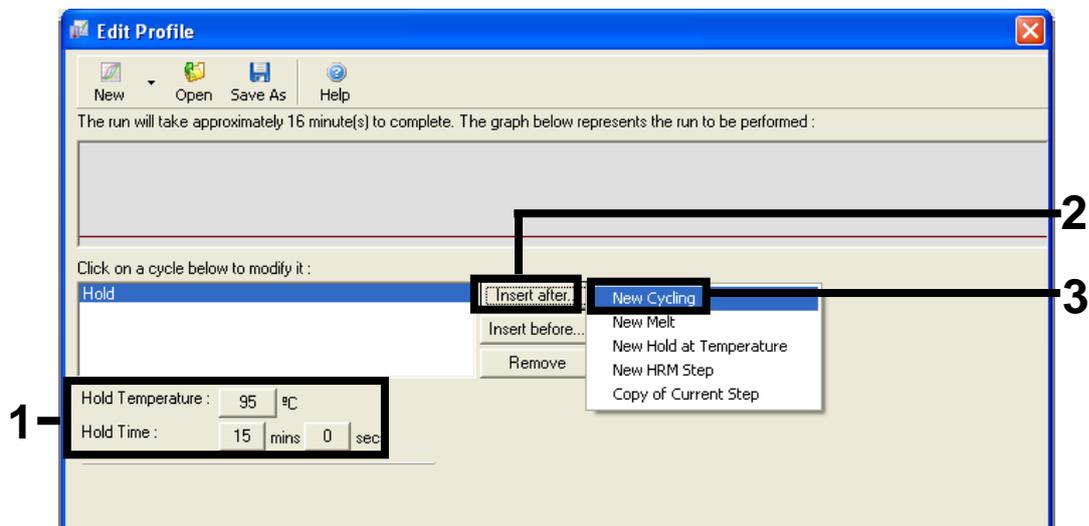


Figure 25. Initial incubation step at 95°C. (1 = "Hold Temperature and Hold Time" buttons, 2 = "Insert after" button, 3 = "New Cycling").

8. Change the number of cycle repeats to 40. Select the first step and set to 95°C for 30 secs (Figure 26).

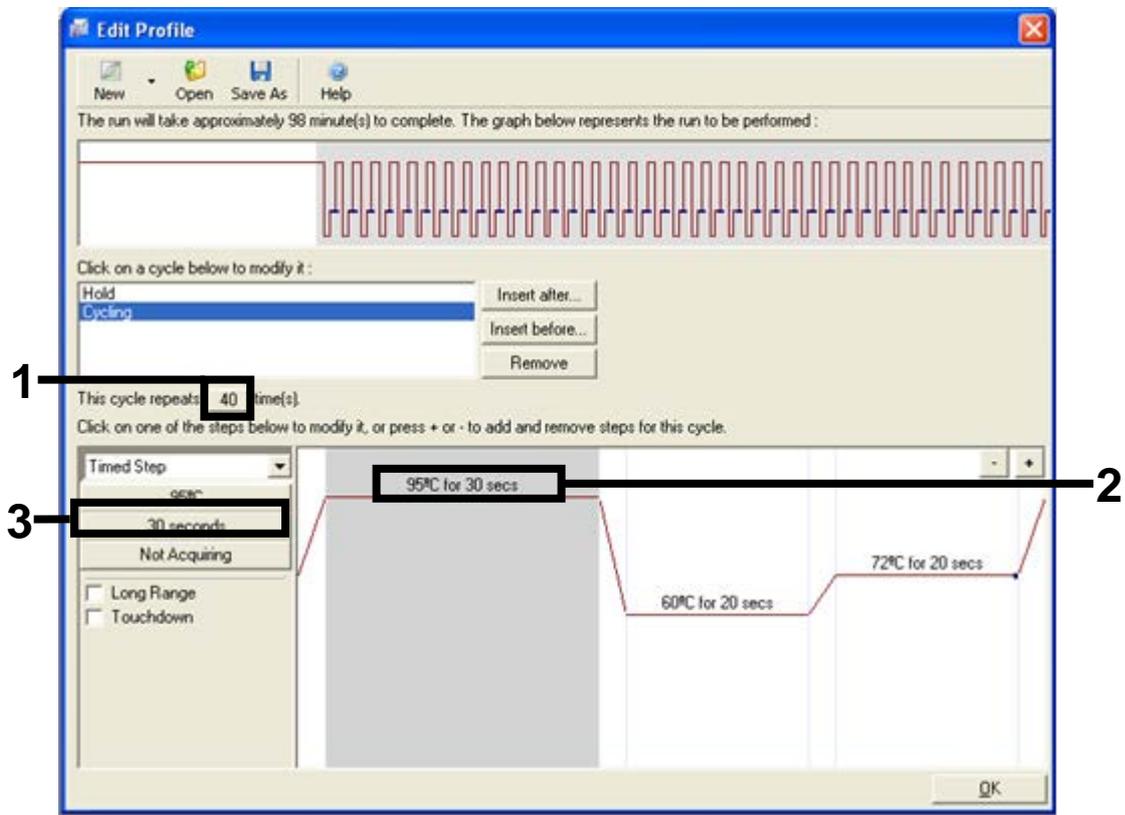


Figure 26. Cycling step at 95°C. (1 = "Cycle repeats" box, 2 = Step one temperature setting, 3 = Step one time setting).

9. Highlight the second step and set to 60°C for 60 secs. Enable data acquisition during this step by selecting the “Not Acquiring” button (Figure 27).

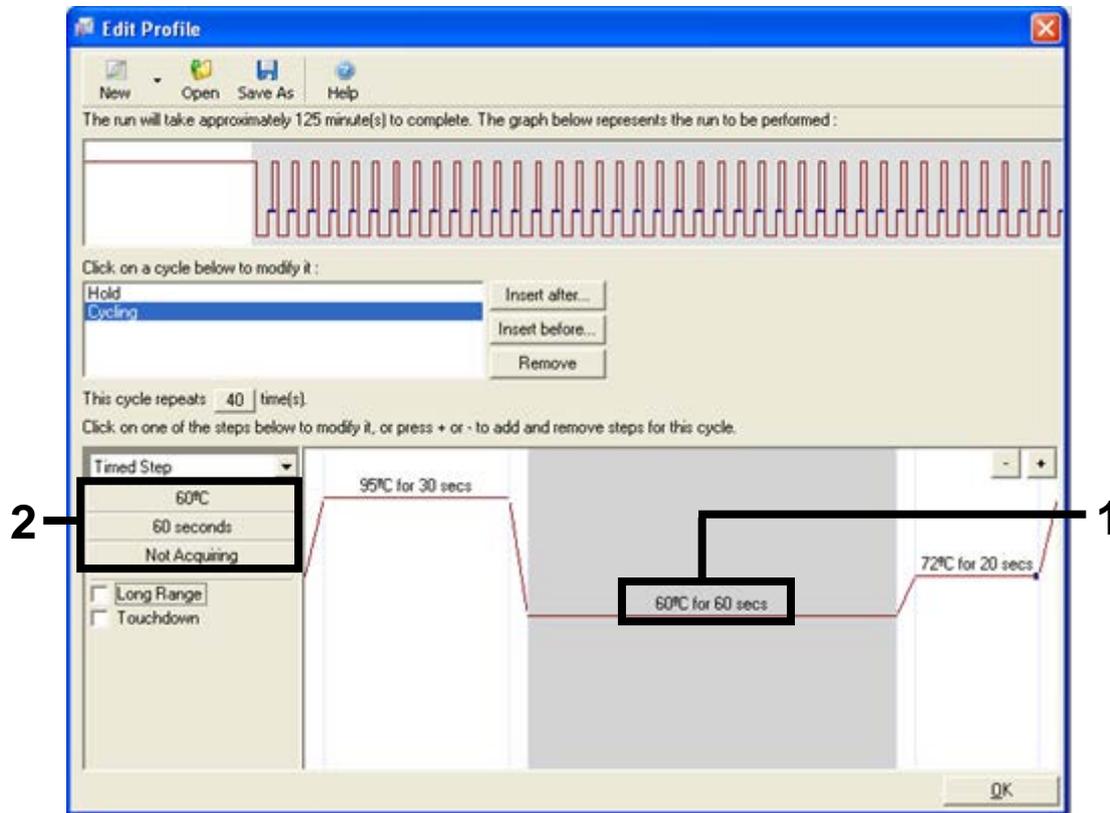


Figure 27. Cycling step at 60°C. (1 = Second step temperature and time setting, 2 = “Not Acquiring” button).

10. Set Green and Yellow as acquiring channels by selecting the ">" button to transfer these from the "Available Channels" list. Click "OK" (Figure 28).

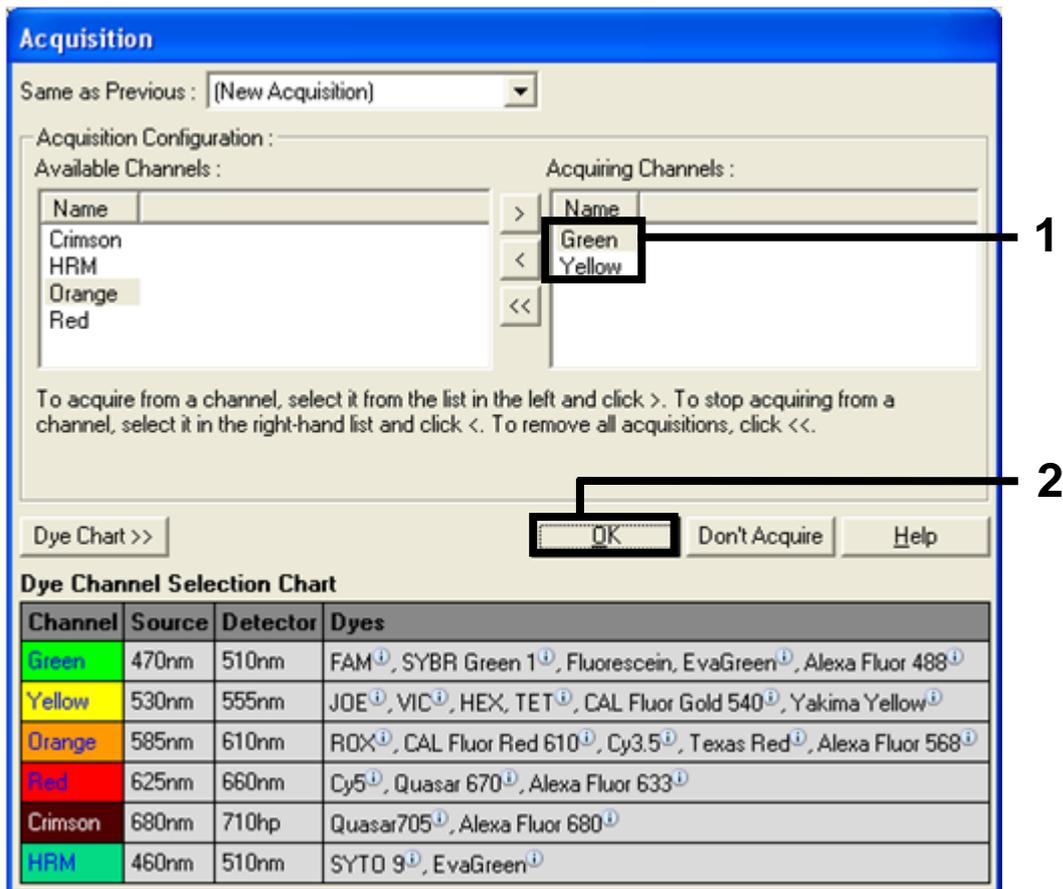


Figure 28. Acquiring at cycling step of 60°C. (1 = Selected channels, 2 = "OK" button).

11. Highlight the third step and delete by clicking the “-” button. Click “OK” (Figure 29).

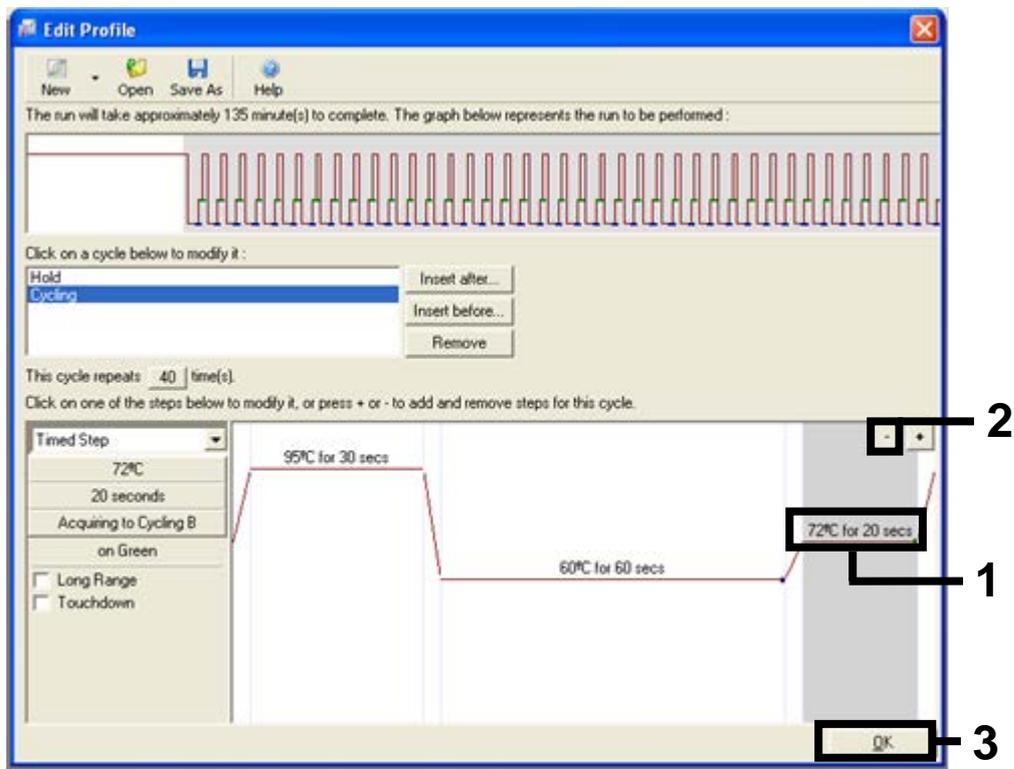


Figure 29. Removal of the extension step. (1 = Third step, 2 = Delete button, 3 = “OK” button).

12. In the next dialog box, click the "Gain Optimisation" button (Figure 30)

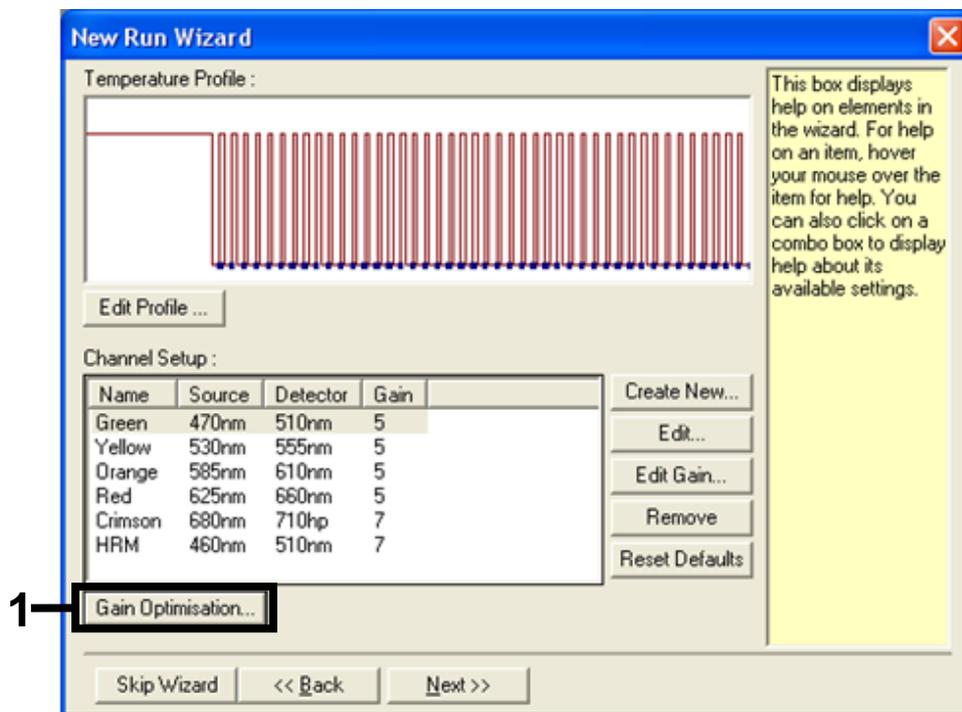


Figure 30. Gain optimization (1).

13. Click the "Optimise Acquiring" button. Channel settings are displayed for each channel. Accept these default values by clicking "OK" for both channels (Figure 31).

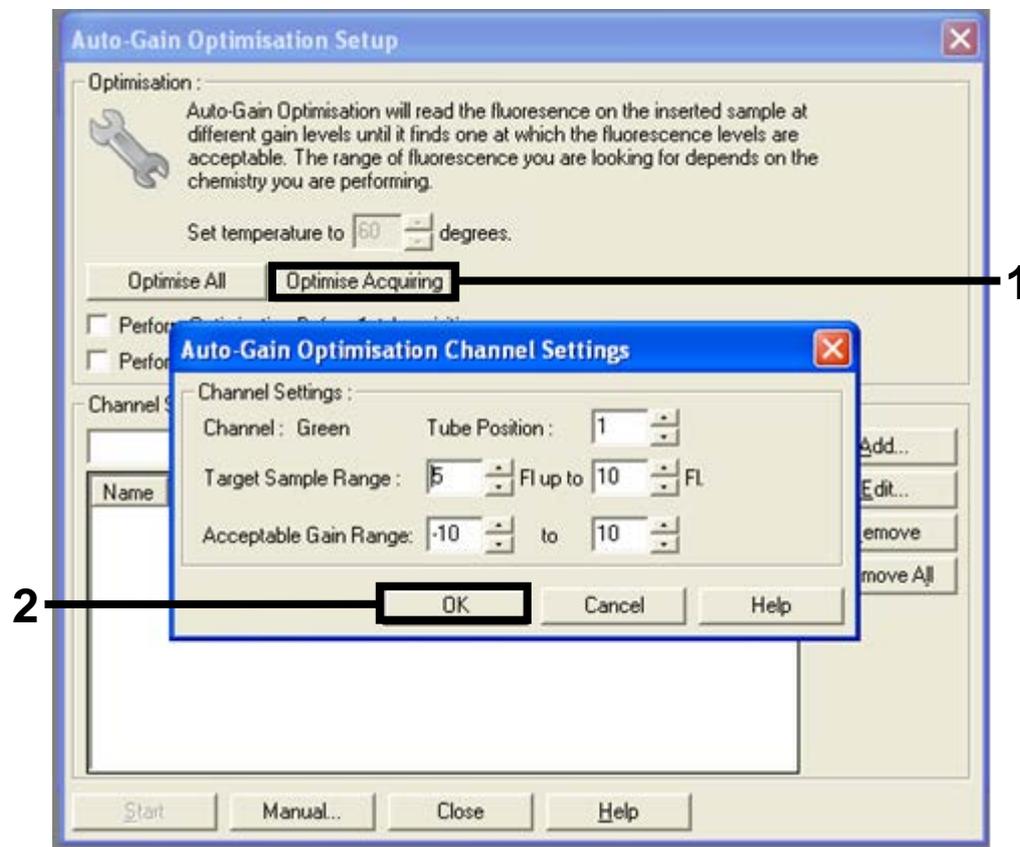


Figure 31. Auto-gain optimization for the Green channel. (1 = "Optimise Acquiring" button, 2 = "OK" button).

14. Check the “Perform Optimisation before 1st Acquisition” box, and then click the “Close” button to return to the wizard (Figure 32).

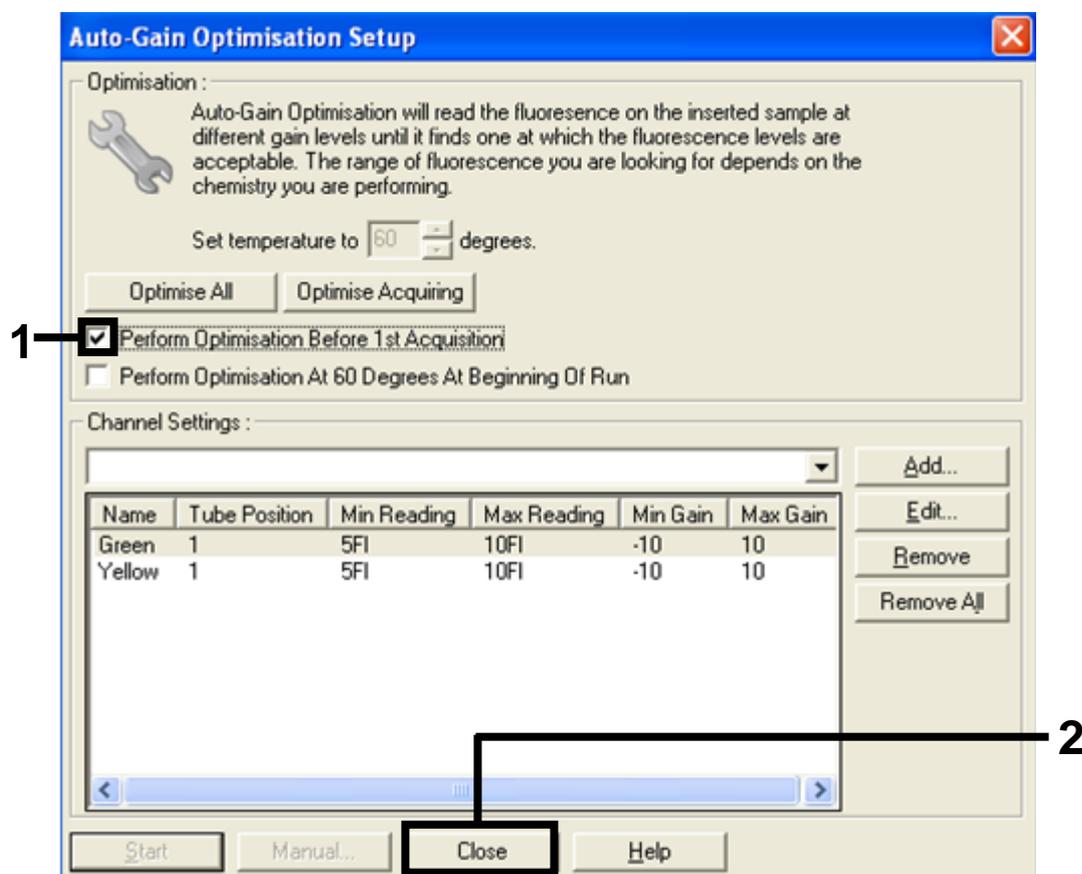


Figure 32. Selection of Green and yellow channels. (1 = “Perform Optimisation Before 1st Acquisition” tick box, 2 = “Close” button).

15. Click “Next” to save the template in an appropriate location by selecting “Save Template”.

Procedure (Manual)

Protocol: Sample assessment (manual)

This protocol is used to assess the total amplifiable DNA in samples and should be performed prior to BRAF mutation analysis.

- Prepare samples as described in section "Protocol: Sample assessment" on page 14.
- Set up the PCR run on the Rotor-Gene Q MDx instrument as described in section "Protocol: *therascreen* BRAF PCR RGQ Setup" on page 69.
- After the run is complete, analyze the data according to the instructions in section "Sample assessment data analysis" on page 75.

Protocol: BRAF mutation detection (manual)

Once a sample has passed the sample assessment, it can be tested to detect BRAF mutations.

- Prepare samples as described in section “Protocol: BRAF mutation detection” on page 25.
- Set up the PCR run on the Rotor-Gene Q MDx instrument as described in section “Protocol: *therascreen* BRAF PCR RGQ Setup” on page 69”.
- After the run is complete, analyze the data according to the instructions in section “BRAF mutation detection data analysis” on page 76.

Protocol: *therascreen* BRAF PCR RGQ Setup

1. Open the Rotor-Gene Q series software (2.3) and open the appropriate temperature profile (.ret file).

For instructions on how to create the temperature profile and check the run parameters, see “Protocol: Creating a temperature profile” on page 55.

2. Ensure that the correct rotor is selected, and check the box to confirm that the locking ring is attached. Click “Next” (
3. Figure 33).

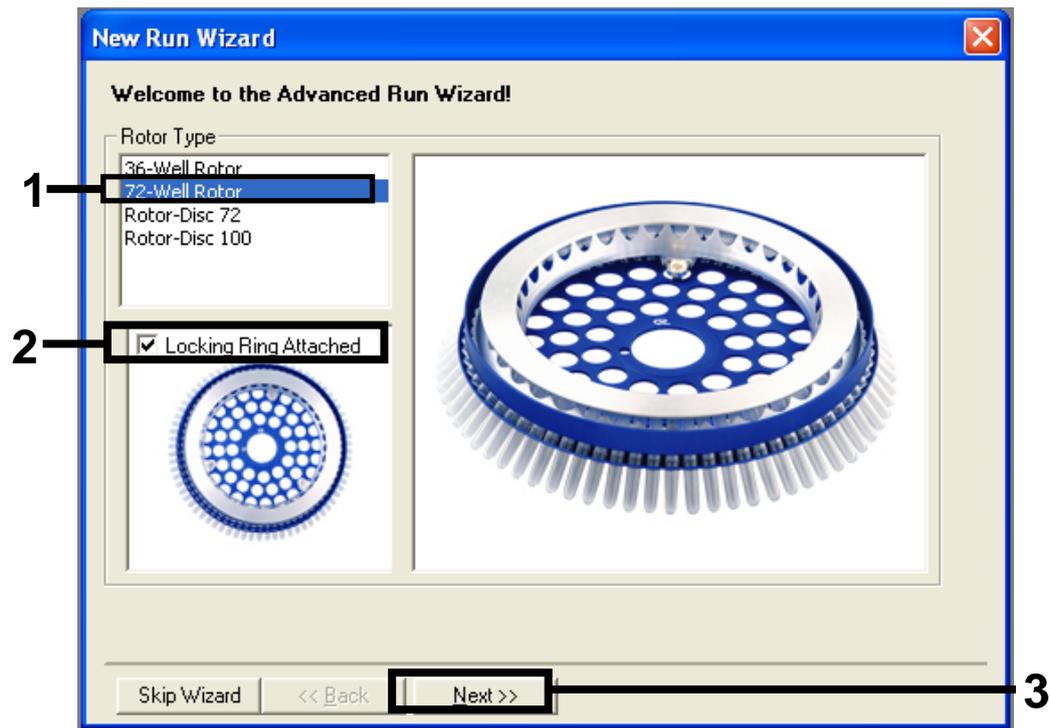


Figure 33. The “New Run Wizard” dialog box and welcome screen. (1 = “Rotor type”, 2 = “Locking Ring Attached box”, 3 = “Next” button).

4. Enter the name of the operator. Add any notes, check that the reaction volume is set to 25 and "Sample Layout" reads "1, 2, 3...". Click "Next" (Figure 34).

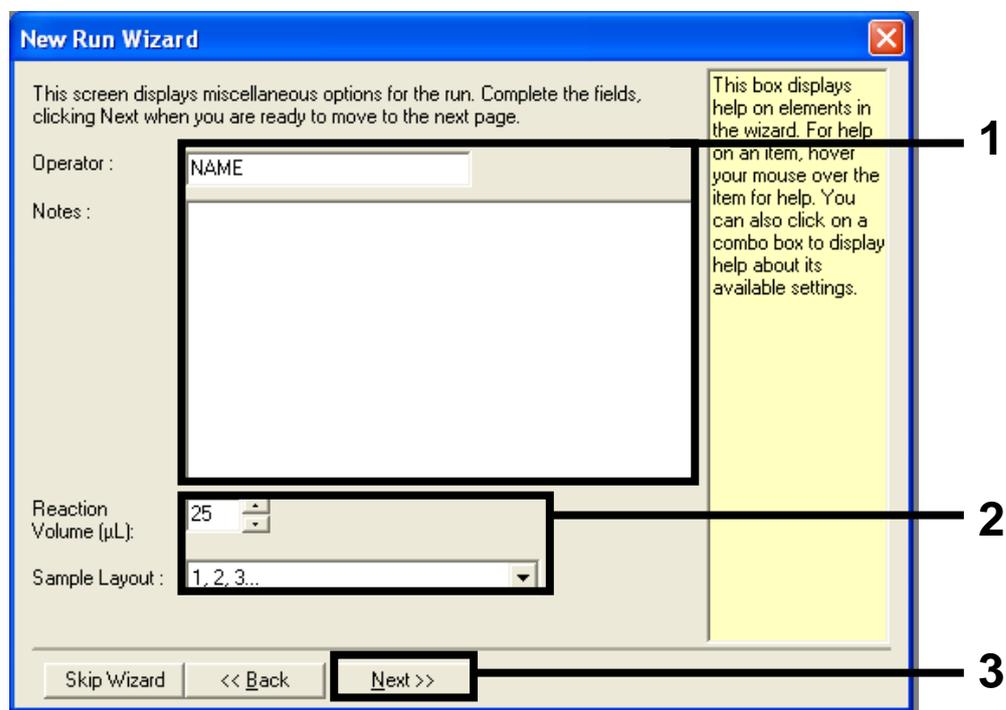


Figure 34. The "New Run Wizard" option screen. (1 = "Operator" and "Notes" fields, 2 = "Reaction Volume" and "Sample Layout" fields, 3 = "Next" button).

5. The next window allows editing of the temperature profile. No editing is required as the temperature profile has already been created according to the instructions in "Protocol: Creating a temperature profile", page 55. Click "Next" (Figure 35).

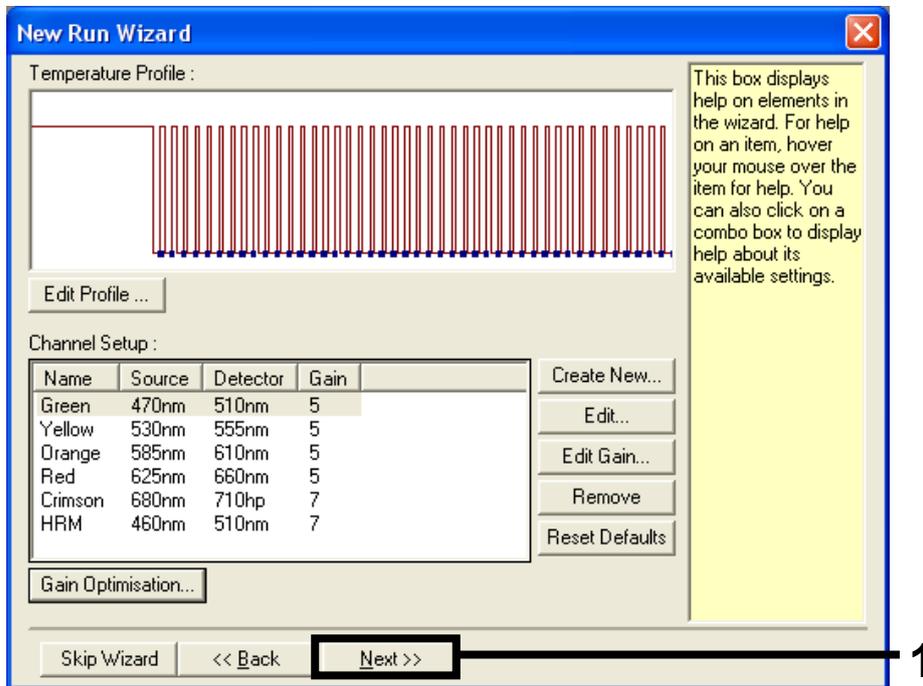


Figure 35. The "New Run Wizard" dialog box and temperature editing screen. (1 = "Next" button).

6. Check the summary and click "Start Run" to save the run file and start the run (Figure 36).

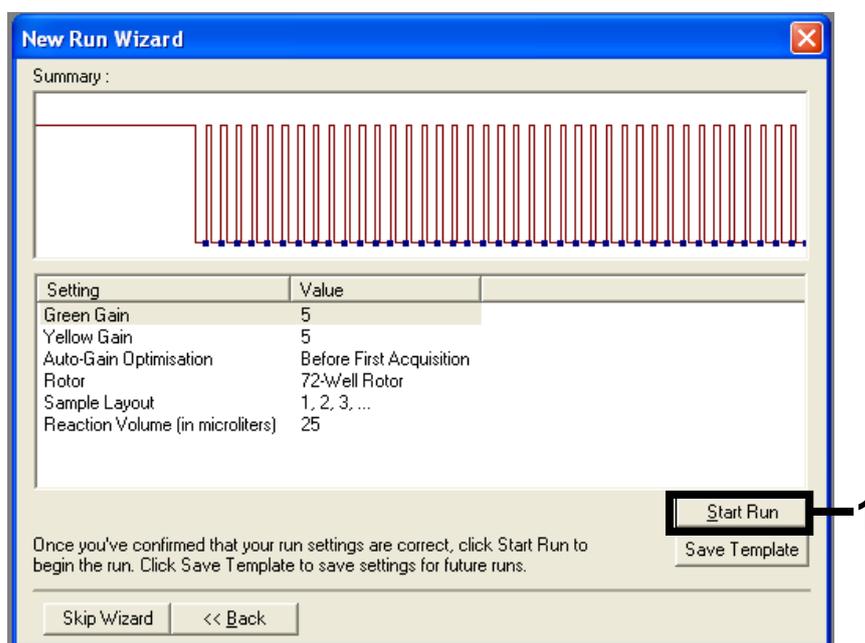


Figure 36. The "New Run Wizard" dialog box and summary screen. (1 = "Start Run" button).

7. After the run starts, a new window appears in which you can either enter sample names now or click "Finish" and enter them later by selecting the "Sample" button during the run, or once the run is complete.

Clicking "Finish and Lock Samples" will prevent you from editing the sample names. The user should take particular care when entering sample names to ensure correct sample testing and analysis.

Note: When naming samples, empty wells should be left blank in the "Name" column.

8. After the run is complete, analyze the data according to sections "Sample assessment data analysis", page 75 or "BRAF mutation detection data analysis", page 76 as appropriate.
9. If quantitation reports are required, click the "Reports" icon of the toolbar in the Rotor-Gene Q run file.

10. In the report browser, click “Cycling A Green (page 1)” under “Report Categories” (Figure 37).

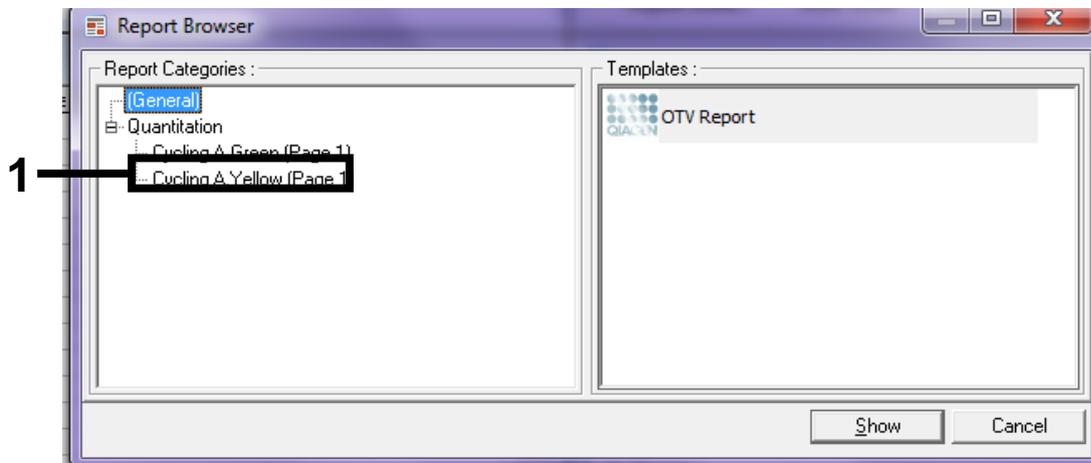


Figure 37. Report browser. (1 = “Cycling A. Green (Page 1)” button).

11. Select “Quantitation (Full Report)” under “Templates” (Figure 38).

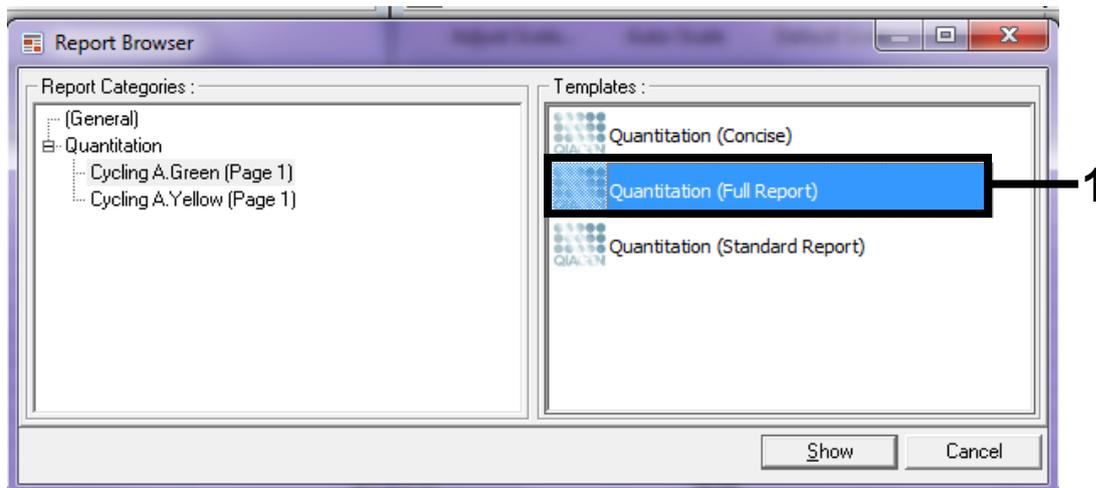


Figure 38. “Quantitation report (Full Report)” (1).

12. Click “Show” to generate the report.

13. Click “Save As” to save an electronic version.

14. Repeat for “Cycling A Yellow (Page1)”.

Interpretation of Results (Manual)

After the sample assessment run or the mutation analysis run is complete, analyze the data according to the following procedure.

Software analysis settings

1. Open the appropriate file using the Rotor-Gene Q series software 2.3.
2. If you haven't already named your samples prior to performing the run, click "Edit Samples".
3. Insert the names of your samples in the "Name" column.
Note: Leave the names of any empty wells blank.
4. Click "Analysis". On the analysis page, click "Cycling A. Yellow" to view the Yellow channel.
5. Click "Named On".
Note: This ensures that empty wells do not feature in the analysis.
6. Select "Dynamic tube".
7. Select "Slope correct".
8. Select "Linear scale".
9. Select "Take off Adj" and enter the values 15.01 in the top box ("If take off point was calculated before cycle") and 20.01 in the bottom box ("then use the following cycle and take off point")
10. Set the threshold to 0.05.
11. Set "Eliminate Cycles before" to 15.
12. Check the Yellow C_T values.
13. On the analysis page, click "Cycling A. Green" to view the Green channel.
14. Select "Named On".
15. Select "Dynamic tube".
16. Select "Slope correct".
17. Select "Linear scale".
18. Select "TOA" and enter the values 15.01 in the top box ("If take off point was calculated before cycle") and 20.01 in the bottom box ("then use the following cycle and take off point")
19. Set the threshold to 0.15.
20. Set "Eliminate Cycles before" to 15.
21. Check the Green C_T values.

Sample assessment data analysis

Run control analysis

After the run has finished, analyze the data as follows.

- **Negative control:** To ensure that there is no template contamination, the no template control must not generate a C_T value in the green (FAM) channel below 40. To ensure that the run is set up correctly, the no template control must show amplification in the range of 32.53–38.16 in the yellow (HEX) channel. The values specified are within and including these values.
- **Positive control:** The BRAF Positive Control (PC) must give a control assay C_T value in the green (FAM) channel of 30.37–36.38. The values specified are within and including those values. A value outside this range indicates an assay setup problem and is therefore a run failure.

Sample data must not be used if either of these two run controls has failed.

Provided that both run controls are valid, each sample C_T value must be within the range of 21.95–32.00 in the green channel. If the sample is out of this range, the following guidance is provided.

Sample analysis — control assay

- **Sample control assay $C_T < 21.95$:** Samples with a control $C_T < 21.95$ must be diluted since this represents the lower end of the validated assay range. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range on the basis that diluting by half will increase the C_T by 1. If the sample is close to 21.95, dilution is recommended to ensure a result is obtained from the sample test (BRAF mutation detection) run. Samples must be diluted using the water provided in the kit (Water for Dilution [Dil.]).
- **Sample control assay $C_T > 32.00$:** Re-extraction of the sample is recommended since insufficient starting DNA template will be present to detect all mutations at the stated cutoff values for the assay.

BRAF mutation detection data analysis

Run control analysis

Refer to the run control analysis flowchart in Figure 39.

- **Negative control:** To ensure that there is no template contamination, the no template control must not generate a C_T value in the green (FAM) channel below 40. To ensure that the run is set up correctly, the no template control must show amplification in the range of 32.53–38.16 in the yellow (HEX) channel. The values specified are within and including these values.
- **Positive control:** The BRAF Positive Control (PC) must give a C_T value for each BRAF assay as shown in Table 14 in the green channel. The values specified are within and including those values. A value outside this range indicates an assay setup problem and is therefore a run failure.

Note: Sample data must not be used if either of these two run controls has failed.

Table 14. Acceptable C_T range for reaction controls.

Reaction mix	Sample	Channel	C_T range
Control	PC	Green	30.37–36.38
V600E/Ec	PC	Green	29.62–35.73
V600D	PC	Green	29.75–35.79
V600K	PC	Green	29.32–35.32
V600R	PC	Green	29.41–35.41

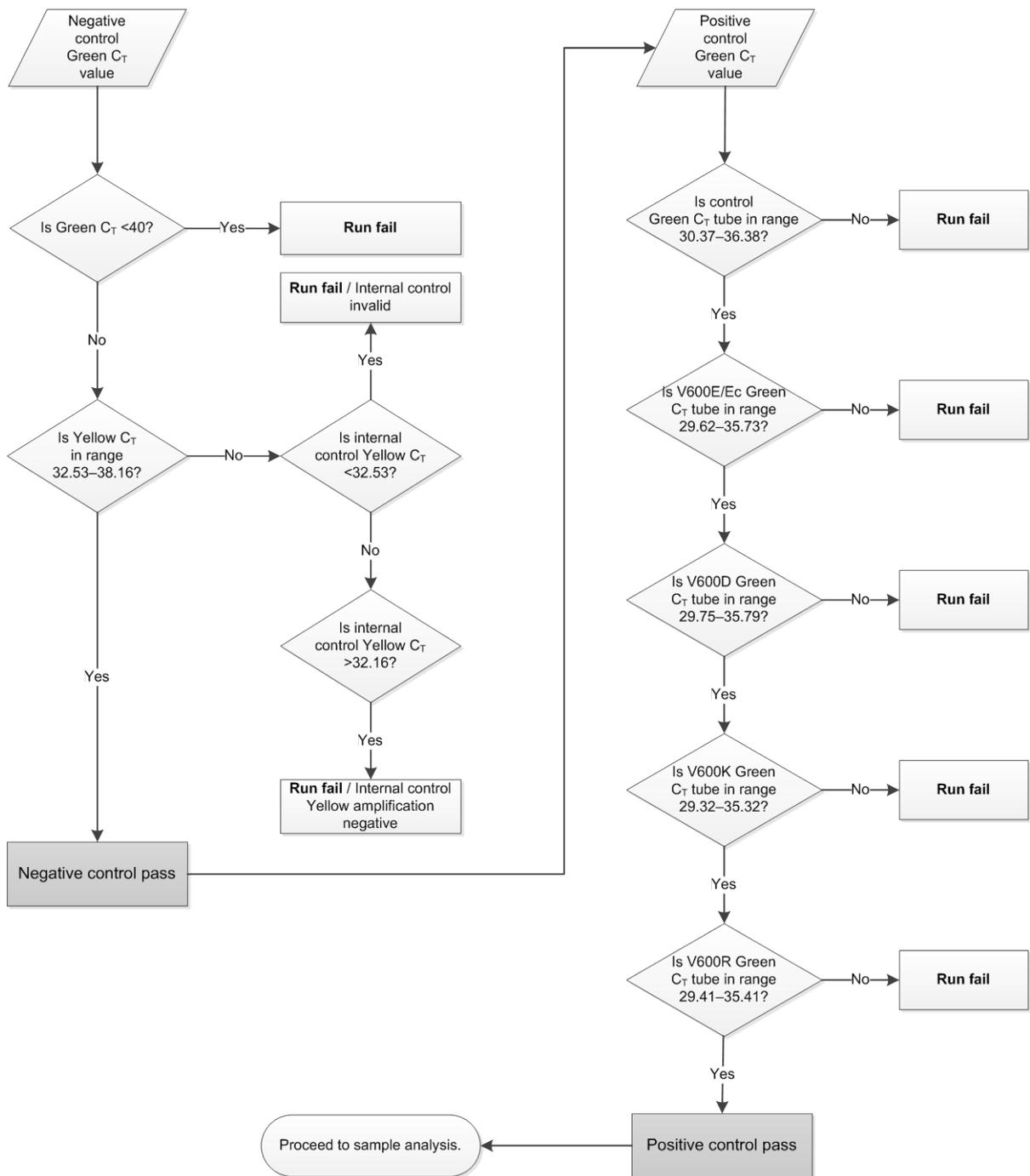


Figure 39. Run control analysis flowchart.

Sample analysis — Sample control Green C_T value

Refer to the sample analysis flowchart in Figure 40.

Provided that both run controls are valid for the control assay, each sample control C_T value must be within the range of 21.95–32.00 in the green channel.

If the sample is out of this range, the following guidance is provided.

- **Sample control assay C_T <21.95:** Samples with a control C_T <21.95 will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range on the basis that diluting by half will increase the C_T by 1. Samples should be diluted using the water provided in the kit (Water for Dilution [Dil.]).
- **Sample control assay C_T >32.00:** Re-extraction of the sample is recommended since insufficient starting DNA template will be present to detect all mutations at the stated cutoff values for the assay.

Sample analysis — Sample internal control mutation assays Yellow C_T value

Refer to the sample analysis flowchart in Figure 40.

All wells of each sample must be analyzed. Check that each well generates a HEX signal in the Yellow channel from the internal control. There are 3 possible outcomes.

- If the internal control C_T falls within the specified range (32.53–38.16), it is Yellow amplification positive and valid.
- If the internal control C_T is above the specified range (>38.16), the tube is Yellow amplification negative. If there is amplification in the Green channel for that tube, the Yellow amplification is valid. If there is no amplification in the Green channel for that tube, the Yellow amplification is invalid.
- If the internal control C_T is below the specified range (<32.53), the tube is invalid.

If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of inhibitors, but it should be noted that this would also dilute the target DNA. A tube of Water for Sample Dilution (Dil.) is included with the kit.

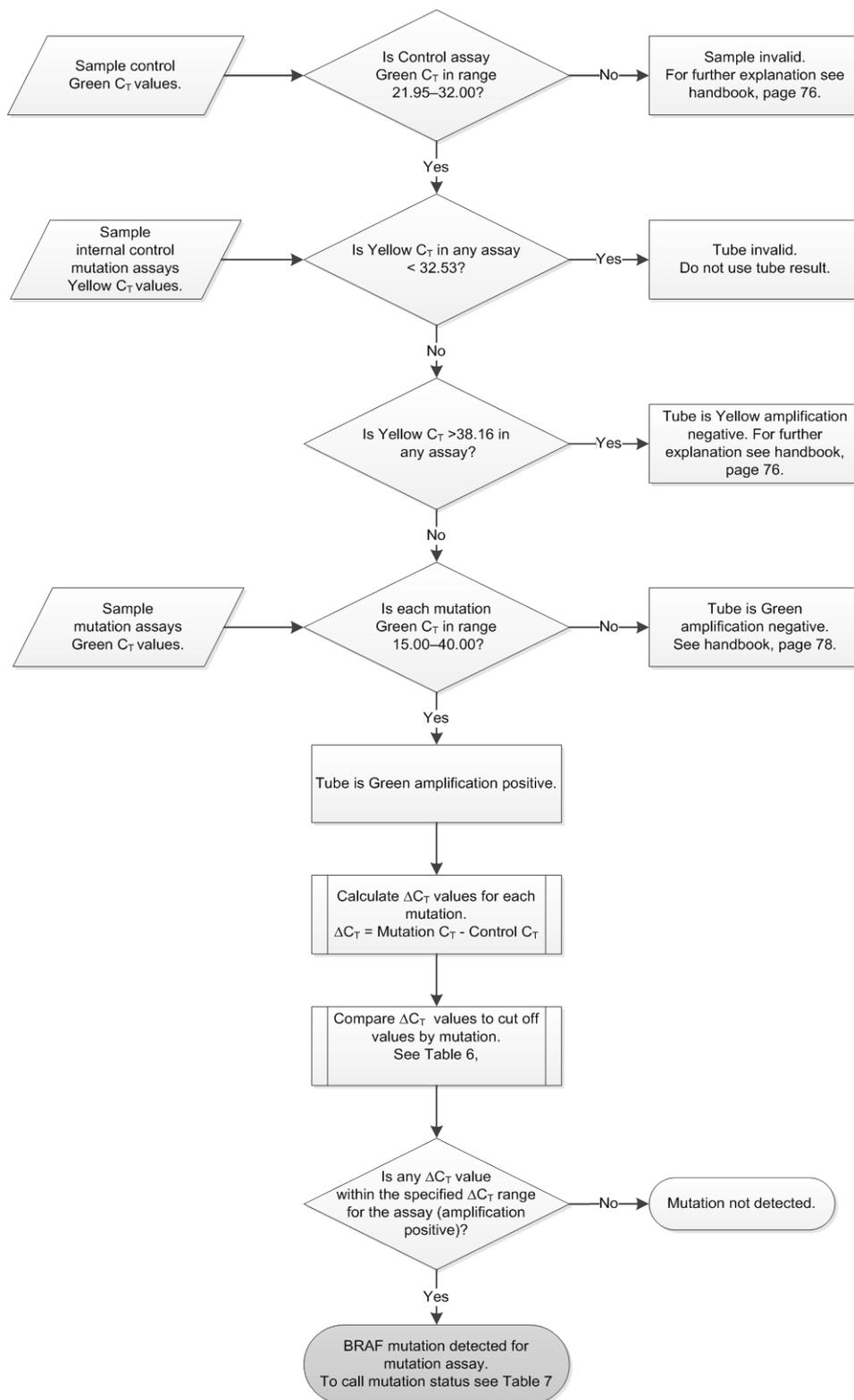


Figure 40. Sample analysis flowchart.

Sample analysis — Sample mutation assays Green C_T value

The Green values for all 4 reaction mixes should be checked against the values listed in Table 15.

Table 15. Acceptable sample mutation reaction values (Green channel)*

Assay	Acceptable C _T range	ΔC _T range
V600E/Ec	15.00–40.00	≤7.0
V600D	15.00–40.00	≤6.9
V600K	15.00–40.00	≤6.0
V600R	15.00–40.00	≤7.0

* Acceptable values are within and including the values shown.

- If the Green C_T falls within the specified range, it is FAM amplification positive.
- If the Green C_T is above the specified range or there is no amplification, it is Green amplification negative.

Calculate the ΔC_T value for each mutation tube that is FAM amplification positive as follows, ensuring that the mutation and control C_T values are from the same sample.

$$\Delta C_T = \text{mutation } C_T - \text{control } C_T$$

Compare the ΔC_T value for the sample with the cutoff point for the assay in question (Table 15), ensuring that the correct cutoff point is applied to each assay.

The cutoff point is the point above which a positive signal could potentially be due to background signal of the ARMS primer on wild-type DNA. If the sample ΔC_T value is higher than the cutoff point, it is classed as negative or beyond the limits of detection of the kit.

For every sample, each mutation reaction will be given a status of mutation detected; mutation not detected, or invalid using the following criteria.

■ **Mutation detected:**

Green amplification positive and the ΔC_T are at or below the cutoff value. If multiple mutations are detected, the mutation status should be assigned according to Table 16.

■ **Mutation not detected:**

Green amplification positive and the ΔC_T are above the cutoff value.

Green amplification negative and Yellow (internal control) amplification are positive.

■ **Invalid:**

Yellow (internal control) is invalid.

Green amplification is negative and Yellow amplification is negative.

For further explanation, see the flowchart (Figure 40). If a sample is Yellow amplification negative in a tube but Green amplification positive in a different tube, then a “mutation detected” result in the different tube can still be considered valid but the particular mutation identified may not be reliably assigned.

If a sample is Yellow amplification negative and Green amplification positive in the same tube, then the “mutation detected” result should be considered valid.

If a tube is Yellow (internal control) invalid, the result of that tube must not be used.

Sample analysis — Assigning sample mutation status

Once all mutation reaction tubes are assessed, the mutation status of the sample is determined as follows.

- **Mutation detected:** One or more of the 4 mutation reactions are positive. If multiple mutations are detected, the mutation reported should be in accordance with Table 16 (see next page).
- **Mutation not detected:** All 4 mutation reactions are negative.
- **Invalid:** No mutation reactions are positive and 1 or more mutation reactions are invalid.

Table 16. Calling sample mutation status

V600E/Ec	V600D	V600K	V600R	Mutation status
Positive	Negative	Negative	Negative	V600E or V600Ec positive
Positive	Negative	Positive	Negative	V600Ec or V600K positive
Positive	Positive	Negative	Negative	V600D positive
Negative	Positive	Negative	Negative	V600D positive
Negative	Negative	Positive	Negative	V600K positive
Negative	Negative	Negative	Positive	V600R positive

Note: The *therascreen* BRAF RGQ PCR Kit is intended to detect mutations in the BRAF gene in a DNA sample. When a sample is called BRAF mutation detected, only one specific mutation should be reported. If multiple mutations are detected, the mutation reported should be in accordance with Table 16.

Some cross-reactivity occurs between mutation reactions. For example, the V600E/Ec assay may give a positive result if a V600D mutation is present, the V600E/Ec assay may give a positive result if a V600K mutation is present, and the V600K assay may give a positive result if a V600E complex mutation is present. However, mutation status can be distinguished using Table 16.

Cross-reactivity is due to the ARMS primer detecting other mutations of similar sequence to one another. If a second mutation assay gives a positive result, this is likely to be cross-reactivity. Double mutants have been observed, although these are rare.

Therefore, in rare cases, combinations of positive results may be detected that are not given in Table 16. The sample can still be called BRAF mutation detected. However, due to cross-reactivity, a particular mutation cannot be distinguished. Therefore, the sample should only be called BRAF mutation detected.

If one or more of the mutation reactions are invalid but one or more are positive, the sample can still be called BRAF mutation detected since a mutation is present. However, the specific mutation reported may not be accurate and may be a result of cross-reactivity. Therefore, the sample should only be called BRAF mutation detected.

Appendix II: Installation of the *therascreen* BRAF Assay Package

The *therascreen* BRAF RGQ PCR Kit is designed for use with the Rotor-Gene Q MDx instrument with a 72-well rotor. The *therascreen* BRAF Assay Package is available for download on the corresponding *therascreen* BRAF RGQ PCR Kit product webpage on www.qiagen.com. The download information can be found in the “Product Resources” section under the tab “Supplementary Protocols”. The Assay Packages can also be ordered on a CD (QIAGEN, cat. no. 9023820).

The assay package includes “*therascreen* BRAF CE Sample Assessment Locked Template” and “*therascreen* BRAF CE Mutation Analysis Locked Template”.

Note: The *therascreen* BRAF Assay Package is only compatible with Rotor-Gene Q software version 2.3. Make sure the correct version of the Rotor-Gene Q software is installed before proceeding with the *therascreen* BRAF Assay Package installation. If your Rotor-Gene Q MDx instrument was delivered with a previous software version you can easily upgrade by downloading the software version 2.3 from the Rotor-Gene Q MDx product page on www.qiagen.com. The new software can be found in the respective “Product Resources” section under the tab “Operating Software”.

Procedure (download)

1. Download the *therascreen* BRAF RGQ Assay Package CE on the corresponding *therascreen* BRAF RGQ PCR Kit product webpage on www.qiagen.com.
2. Open the downloaded zip-file by double-clicking on the file and extracting the file inside the archive.
3. Start the installation by double-clicking the extracted file `therascreen_BRAF_Assay_Package_3.1.1.exe`.

Procedure (CD)

1. Order the *therascreen* BRAF RGQ Assay Package CE CD (QIAGEN, cat. no. 9023820), which is available separately, from QIAGEN
2. Insert the CD into the CD drive of the laptop connected to the Rotor-Gene Q instrument.
3. Start the installation by double-clicking the file `therascreen_BRAF_Assay_Package_3.1.1.exe` if the CD auto-loads or alternatively locate this executable file using the file browser on the connected laptop.

4. The setup wizard will appear. Click "Next" to continue (Figure 41).

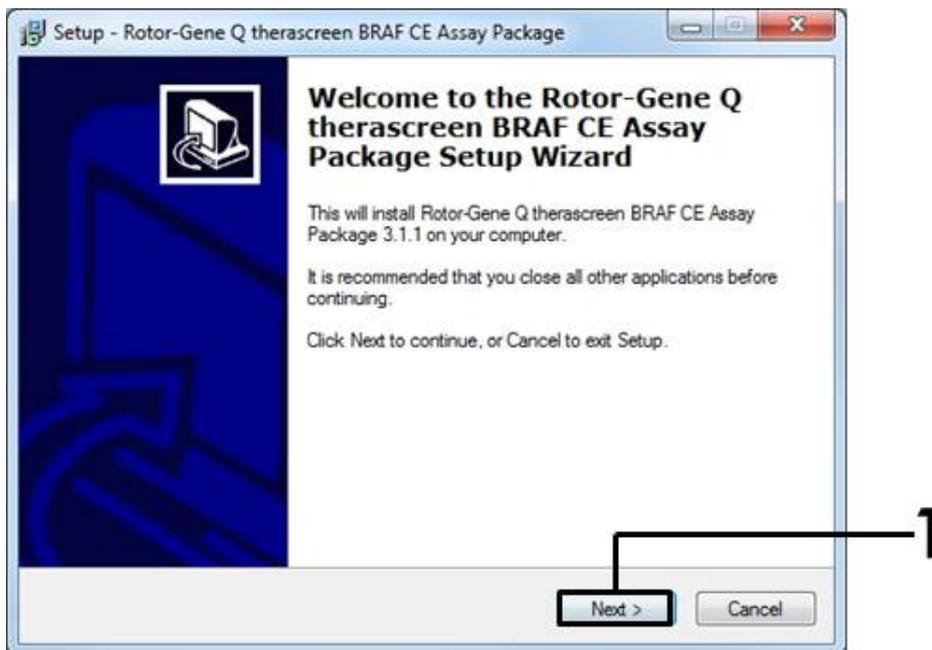


Figure 41. The "Setup" dialog box. (1 = "Next" button).

5. Read the License Agreement in the "License Agreement" dialog box and accept the agreement by checking the statement "I accept the agreement". Click "Next" to continue (Figure 42).

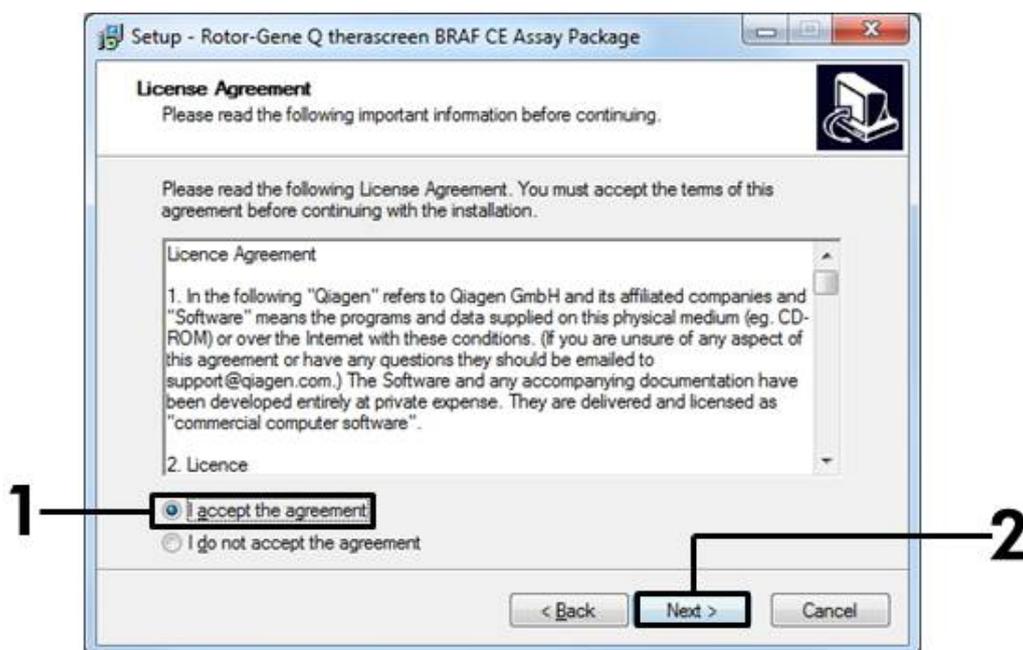


Figure 42. The "License Agreement" dialog box. (1 = "Accept" button, 2 = "Next" button).

6. The template setup will start automatically and when this is complete, a final “Setup” dialog box will appear. Click “Finish” to exit the setup wizard (Figure 43).

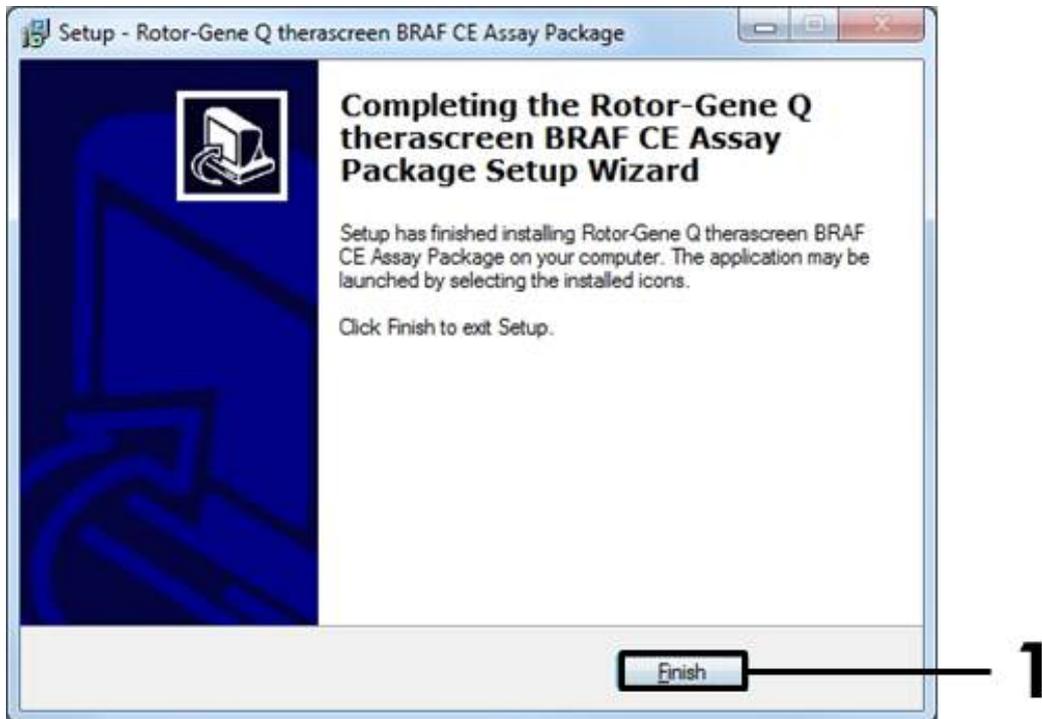


Figure 43. Completing the setup wizard. (1 = “Finish” button).

7. Restart the computer. Shortcuts to both the “therascreen BRAF CE Sample Assessment Locked Template” and “therascreen BRAF CE Mutation Analysis Locked Template” will be generated automatically and appear on the desktop.

Contact Information

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

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> BRAF RGQ PCR Kit (24)	For 24 reactions: Control Assay, 4 Mutation Assays, Positive Control, <i>Taq</i> DNA Polymerase, Water for NTC, and Water for Sample Dilution	870211
Rotor-Gene Q and other accessories		
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
Rotor-Gene Q MDx 5plex HRM System	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	9002033
<i>therascreen</i> BRAF Assay Package CD	CD with the <i>therascreen</i> BRAF CE Sample Assessment Locked Template and the <i>therascreen</i> BRAF CE Mutation Analysis Locked Template.	9023820
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106

Product	Contents	Cat. no.
QIAamp DNA FFPE Tissue Kit – for purification of genomic DNA from paraffin-embedded tissues		
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

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*Not For Use In Determining Risk Of Developing Endometriosis

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the *therascreen* BRAF RGQ PCR Kit to the following terms:

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