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Storage Condition

Store all of reagents at -20°C .

For research use only, not for use in diagnostic procedures.

Limited Product Warranty

It is imperative that users strictly adhere to this manual. Failure to do so will void our guarantee of this product. TrimGen Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose.

Notice to Purchaser

The purchase of eQ-PCR™ products includes a limited, nonexclusive license to use the reagents and systems. This license does not grant rights to use the reagents and systems for the reproduction of the eQ-PCR™ reagents and systems, to modify the eQ-PCR™ reagents and systems for resale, or to use the eQ-PCR™ reagents and systems to manufacture commercial products without written approval of TrimGen Corporation. No other license, expressed, implied, or by estoppel is granted.

Product Use and Limitations

The eQ-PCR™ Kit is designed "For Research Use Only, not for use in diagnostic procedures." For all of other applications, user should follow the instructions provided by the appropriate regulatory authorities.

Product Safety and Liabilities

Some reagents included in this product may cause harmful conditions. The user should read the instructions on the bottle or tube, and in the User Manual carefully before starting to use the reagents. Handle the chemicals with caution. When working with the reagents, always wear appropriate Personal Protective Equipment (PPE), lab coat, gloves, and protective goggles. TrimGen Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the misuse, the results of use, or the inability to use this product.

Storage

Upon receipt of the eQ-PCR™ BRAF V600E Detection kit, store all reagents at -20°C and protect them from light until use. After use, store at $2-8^{\circ}\text{C}$ and protect them from light for up to three months.

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Introduction

The eQ-PCR™ BRAF V600E Detection Kit is designed to detect the BRAF V600E mutation presented in DNA samples. The mutation is detected by TrimGen's proprietary eQ-PCR (enhanced Quantitative-PCR) technology (US patent pending) and the assay is conducted by real-time PCR system.

BRAF is a member of the RAF family, a group of serine/threonine kinases that play important roles in the extracellular signal regulated kinase cascade. RAF kinases are critical for cell development and differentiation. Mutations in these proteins may influence the prognosis of certain types of tumors. BRAF mutation is commonly found in many cancers such as melanomas, colorectal cancers and papillary thyroid carcinoma. BRAF V600E is a somatic mutation with the highest pathogenic cancer-associated prevalence.

TrimGen's eQ-PCR™ BRAF V600E Detection Kit provides an accurate, sensitive and rapid assay to detect the BRAF V600E mutation.

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Materials Provided:

The eQ-PCR™ BRAF V600E Detection Kit contains pre-packaged reagents for 32 reactions.

Tube Label	Cap Color	Quantity
PCR Mix 4	<i>Purple</i>	480 µl
BRAF-TP	<i>White</i>	40 µl
BRAF-WT CTL	<i>Yellow</i>	20 µl
BRAF-Mut CTL	<i>Orange</i>	20 µl
NF Water	<i>Clear</i>	500 µl

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***Note:** The BRAF-TP is **light sensitive**. Keep this reagent protected from direct light.

PCR Mix 4 (*Purple cap*)

Pre-mixed reagents for DNA amplification

BRAF-TP (*White cap*)

Mix of primers and probes to detect BRAF V600E mutation

BRAF-WT CTL (*Yellow cap*)

Control DNA samples for BRAF wild type

BRAF-Mut CTL (*Orange cap*)

Control DNA samples for BRAF V600E mutant identification

NF Water (*Clear cap*)

Nuclease-free water for the blank control

Sample Preparation:

TrimGen provides high efficiency DNA extraction kits for the following samples:

Paraffin-embedded (FFPE) tissues
Fresh or frozen tissues
Fine Needle Aspiration tissues
Cells

Kit Name	Cat No.
WaxFree DNA	WF-50 (50 extractions)
	WF-100 (100 extractions)

Blood samples

Any commercially available DNA extraction kit is acceptable.

Adjustment of DNA concentration:

When using a column or bead DNA extraction method, adjust the DNA concentration to **10-80 ng/μl**.

When using TrimGen's DNA preparation kit, follow the kit protocol to perform the PCR amplification.

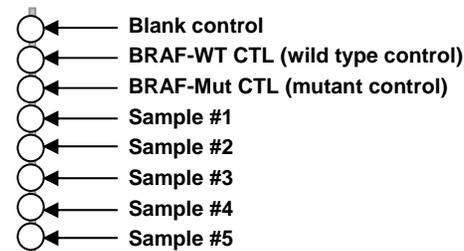
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Assay Procedure:

A. Reaction Preparation

Thaw all reagents to 2°C to 8°C when ready to use. Keep all reagents at 2°C to 8°C during preparations.

A.1. Collect PCR tubes and label the tubes as follows:



A.2. Prepare **Master Mix** for the total number of samples to be tested using the table below:

Reagents	Formulation	Volume
PCR Mix 4	$12.5 \mu\text{l} \times \left(\frac{\quad}{\text{sample\#}} + 3^* \right) \times 1.1^{**}$	
BRAF-TP	$1.0 \mu\text{l} \times \left(\frac{\quad}{\text{sample\#}} + 3^* \right) \times 1.1^{**}$	
NF water	$9.5 \mu\text{l} \times \left(\frac{\quad}{\text{sample\#}} + 3^* \right) \times 1.1^{**}$	
Total Volume		

* The "3" are the blank, positive and negative controls.

** 1.1 is the recommended volume correction factor for pipetting error during aliquoting.

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- A.3. Aliquot 23 µl of **Master Mix** to each tube.
- A.4. Add 2 µl of **NF Water** into the blank control tube.
- A.5. Add 2 µl of **BRAF-WT CTL** into the wild type control tube.
- A.6. Add 2 µl of **BRAF-Mut CTL** into the mutant control tube.
- A.7. Add 2 µl of sample DNA (10-50 ng/µl) into the sample tube.
- A.8. Cap the tubes and mix the samples well by tapping the tubes.
- A.9. Spin the tubes.
- A.10. Load the tube onto the Real-Time PCR System.

B. Run the PCR

The detector selection:

Allele	Detector
BRAF Mut	FAM
BRAF WT	VIC

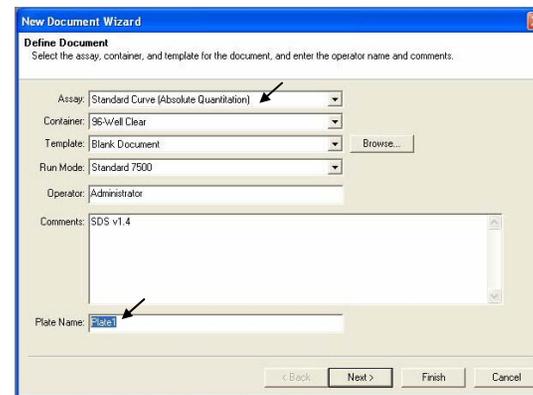
The thermal cycling conditions:

Stage 1	1 Cycle	95°C, 15 min
Stage 2	40 Cycles	95°C, 15 sec 62 °C, 30 sec 72 °C, 35 sec

The following procedure is an example of using Applied Biosystems Model 7500 Real-Time PCR System. When using other real-time PCR systems, users should set up their instrument based on instructions from the manufacturer.

- B.1. Click the 7500 System Software icon on the computer screen.
- B.2. Click "Create New Document" in the dialog box.

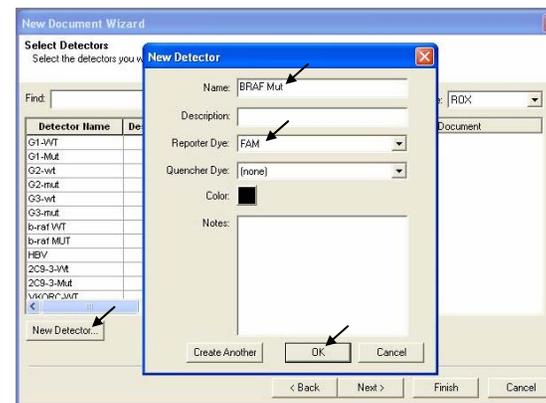
- B.3. In the New Document Wizard Dialog box, Select "Standard Curve (Absolute Quantification)" and **give a plate name**. Click "Next".



- B.4. In the "Select Detectors" Window, click "New Detectors". In the pop-up window, fill in the information following the table below. Click "OK".

(Note: Refer to the ABI 7500 User Manual for instructions on creating a new detector table.)

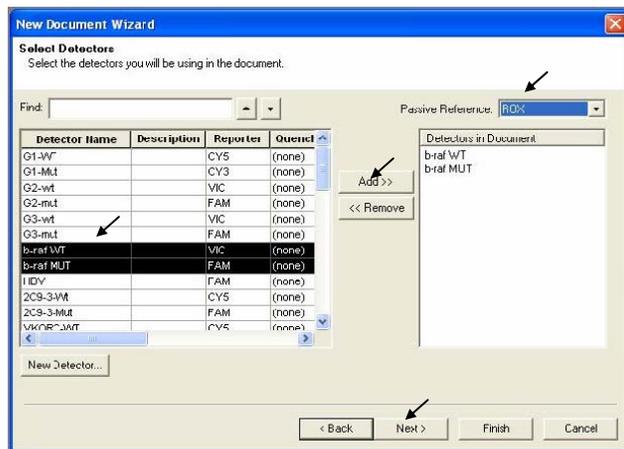
Detector Name	Reporter	Quencher
BRAF Mut	FAM	None
BRAF WT	VIC	None



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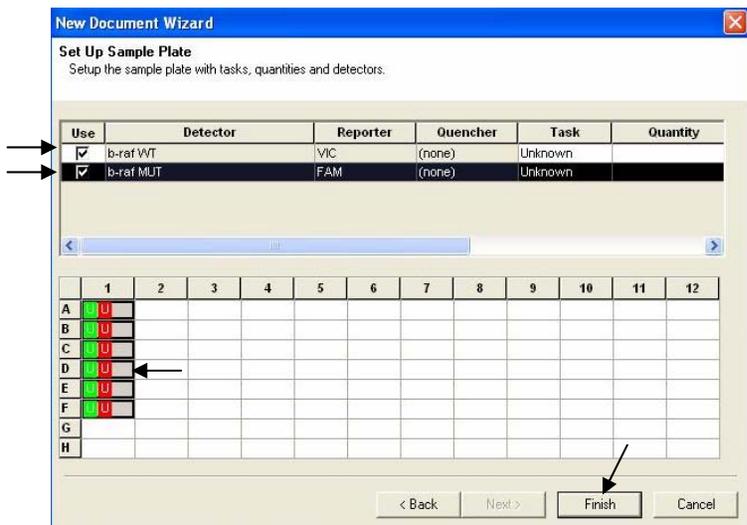
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B.5. In the same window, highlight the detectors created and then click “ADD” in order to add BRAF Mut and WT into the “Detectors in Document”. In the “Passive Reference” box, select “ROX”. Click “Next”.



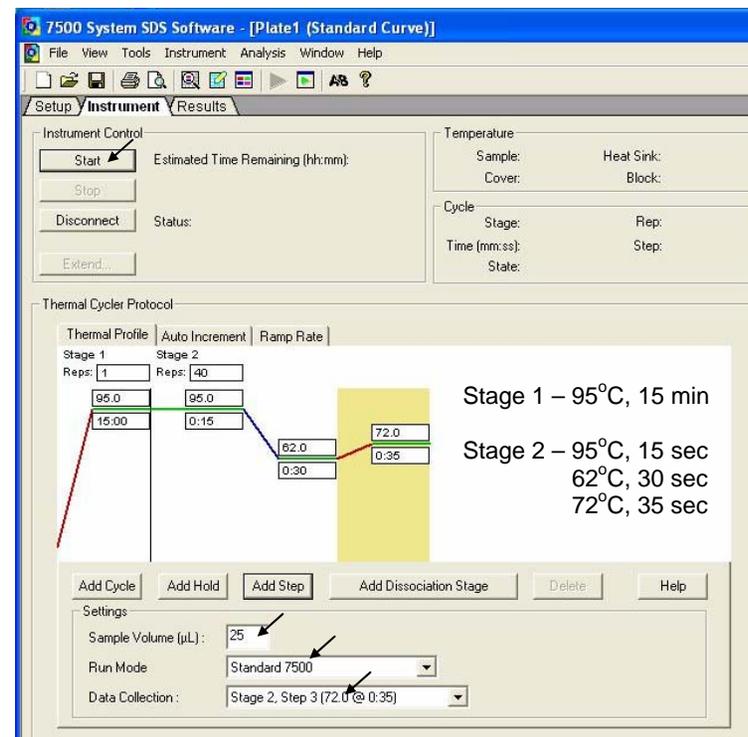
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B.6. At the “Set up Sample Plate” window, specify the “Detector” and “Task” (from top window options) for each selected well (from lower window). For example, assign “BRAF Mut” and “BRAF WT” to the sample wells. Click “Finish” to create the plate document.



B.7. At the next screen, click the “Instrument” tab.

B.8. Set up the thermal cycle conditions, under the “Thermal Profile” tab, as follows:



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B.9. Enter “25” in the “Sample Volume (µL)” box.

B.10. Set “Data Collection” at “Stage 2, step 3 (72°C for 0:35)”.

B.11. Click “Start” to run the PCR amplification.

B.12. Click on “Save and Continue”.

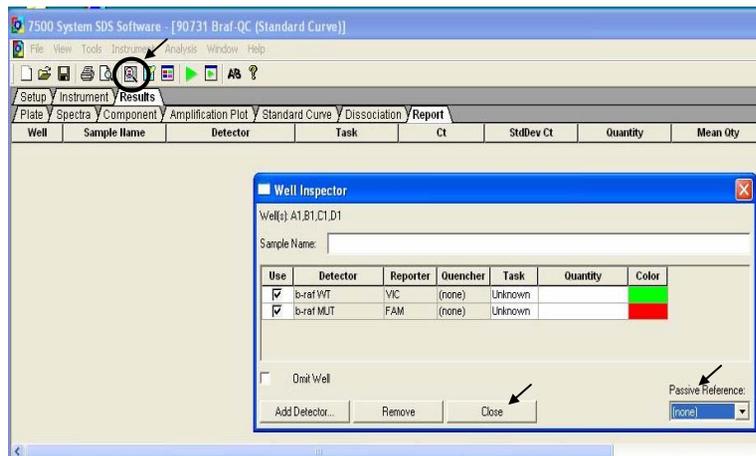
B.13. Save file under new file name.

C. Interpretation of Results

C.1. After the PCR program has finished, locate the data file and open it.

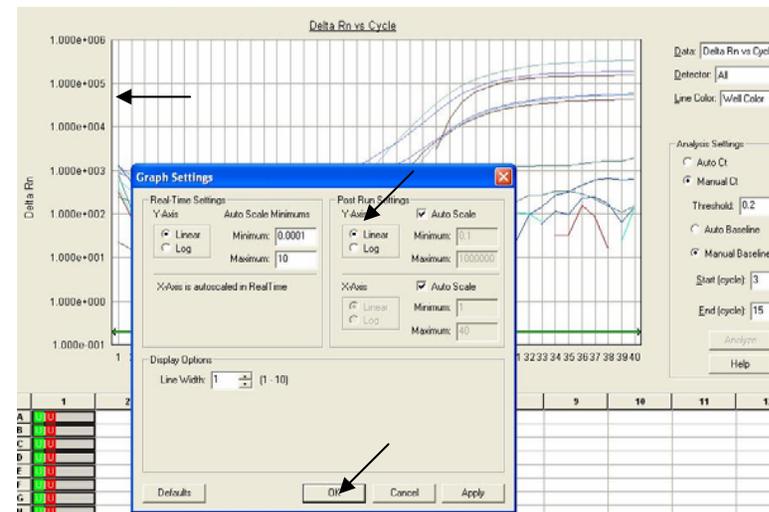
C.2. Click “Results” tab. Go to the “Amplification Plot”.

- C.3. Highlight the wells to be analyzed at the bottom of the screen.
- C.4. Go to the top of the menu bar and select "Well Inspector". At the bottom of the menu, select "**none**" in the "Passive Reference" box. Click the "Close" button.



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- C.9. In the next window, double click the Y axis to open the "Graph Setting" dialog box and select "Liner", then click "OK".

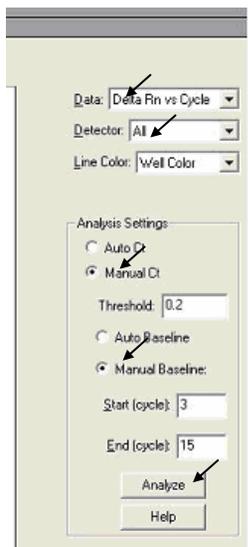


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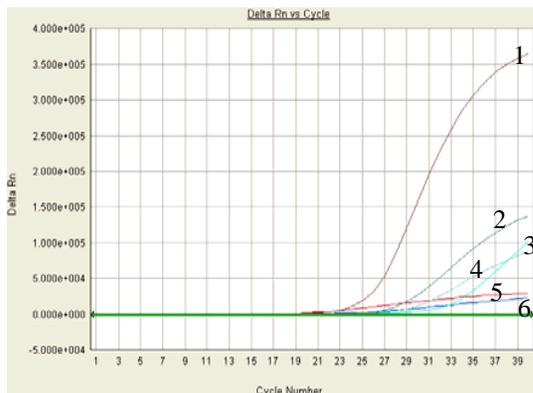
- C.10. Click on each sample position (from the lower window) to highlight the sample that you would like to analyze. Each sample must be analyzed on one of the two "Detector" windows (BRAF Mut or BRAF WT). Only one allele type, Wild type (WT) or Mut (V600E) can be analyzed per "Detector" window.

Whether the individual carries a mutant allele is determined by the end-point fluorescence value of the amplification curve. For example, if the sample does carry a BRAF mutation, its end-point fluorescence value should be greater than that of the BRAF-WT CTL or your wild type control sample.

- C.11. Select "Mut" in the "Detector" window
- C.12. Make sure one has highlighted the wells (samples #1-#6) to be analyzed at the bottom of the screen



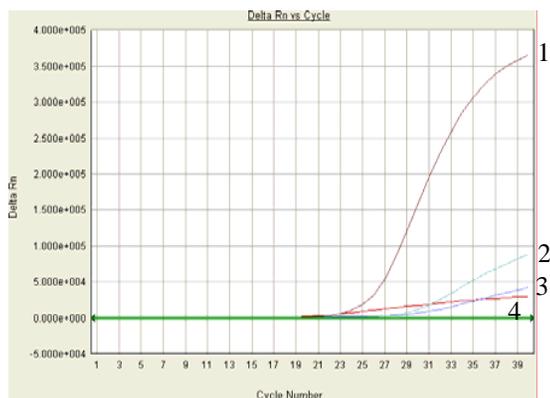
- C.5. Then, select "Delta Rn vs Cycle" in the Data drop-down list.
- C.6. In the Detector drop-down list, select a detector, either wild type (WT), mutant (Mut) or All.
- C.7. Under Analysis Settings, select "Manual Ct" and "Manual Baseline".
- C.8. Click on the "Analyze" button.



- Sample #1: Kit mutation control (showing exponential curve)
- Samples #2-4: > 5% mutation (showing exponential curve)
- Sample #5: Kit wild type control (no amplification)
- Sample #6: Wild type patient sample control (no amplification)

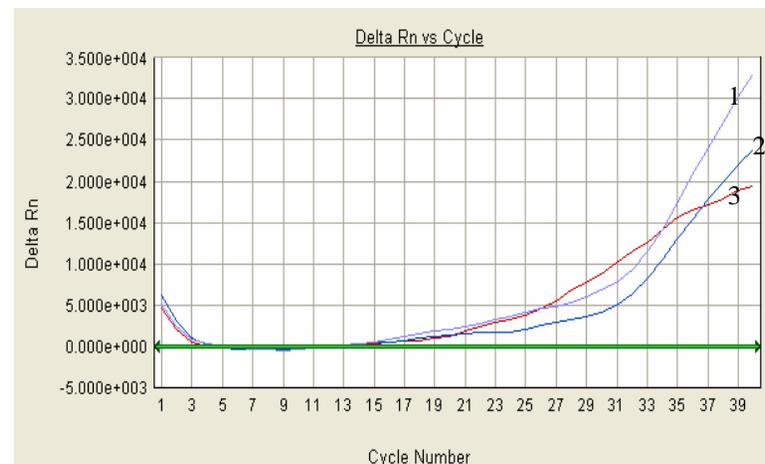
C.13. For samples containing less than 5% mutation, such as sample #3 (in Fig. K1) and samples #1 & #2 (in Fig. K2), it is recommended to repeat the test to double confirm the low level mutation.

Fig. K1



- Sample #1: Kit mutation control (showing exponential curve)
- Sample #2: About 5% mutation (showing exponential curve)
- Sample #3: < 5% mutation (showing exponential curve)
- Sample #4: Wild type kit control (no amplification)

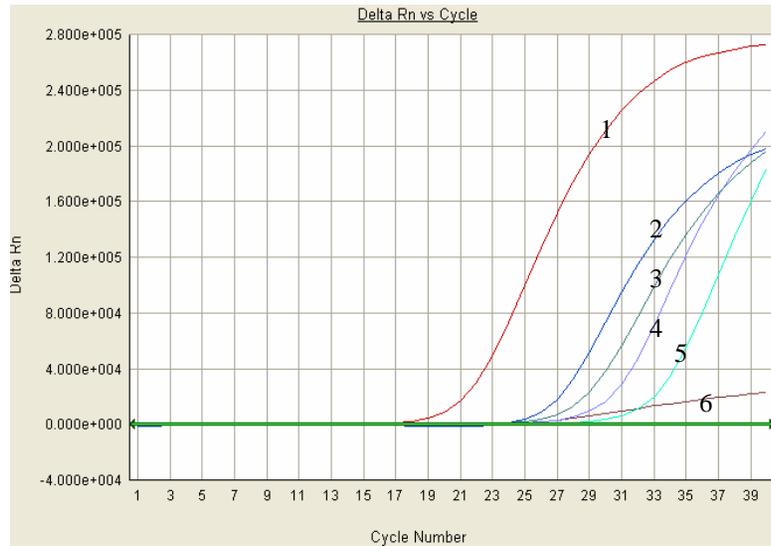
Fig. K2



- Sample #1: < 5% mutation (showing exponential curve)
- Sample #2: < 5% mutation (showing exponential curve)
- Sample #3: Wild type patient sample control (no amplification)

NOTES:

C.14. All test samples should contain the wild type allele. To view the wild type allele, select "WT" in the "Detector" window



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Sample #1: Kit wild type control (showing exponential curve)

Samples #2-#5: Containing wild type allele (showing exponential curve)

Sample #6: Kit mutation control (no amplification)