



Easy[®] BRAF

User manual – version 2017/05

The “Easy[®] BRAF” Kit detects BRAF codon 600 mutations by Real-Time PCR.

For *in vitro* diagnostic use



RT002



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Changes made since the previous version 2016/02:

- Substitution of **Water (diluent)** with **Water**
- Update of section "Materials required but not provided". – Amplification, Materials
- Validation of the kit on ABI 7500 Fast.

For further details contact the technical support of the Diatech Pharmacogenetics (support@diatechpharmacogenetics.com).

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INTENDED USE

The *in vitro* diagnostic “Easy[®] BRAF” Kit is intended for the qualitative detection by Real-Time PCR of *BRAF* codon 600 somatic mutations (V600E/V600E*complex*, V600K, V600D e V600R) in the genomic DNA isolated from fresh, frozen or formalin fixed paraffin-embedded (FFPE) tumor tissue and thyroid fine-needle aspiration.

The “Easy[®] BRAF” Kit is validated on the following instruments:

- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7300** - Applied Biosystems (software v.1.4.1)
- **ABI 7500, 7500 Fast** - Applied Biosystems (software v. 2.0.5)
- **Stratagene Mx3000P, Mx3005P** - Agilent Technologies (software v.4.10 build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

List of detectable mutations:

BRAF codon 600

- V600E (1799T>A)*
- V600E*complex* (1799_1800TG>AA)*
- V600K (1798_1799GT>AA)
- V600D (1799_1800TG>AT)
- V600R (1798_1799GT>AG)

* not distinguishable between them

PRINCIPLE OF THE ASSAY

The “Easy[®] BRAF” Kit is designed to selectively amplify mutant specific sequences in samples that contain a mixture of wild-type and mutated DNA. The detection is achieved using fluorescent probes labelled with FAM and HEX.

The “Easy[®] BRAF” Kit is composed of four assays for the detection of the *BRAF* mutations and a control assay for the assessment of DNA content in the sample.

Each assay contains primers and probes for the detection of the target (FAM) as well as an endogenous control gene (HEX).

The amplification of the endogenous control gene enables to verify the amplification procedure and the possible presence of inhibitors, which may cause false negative results.

1. **BRAF V600E**: the assay detects V600E (1799T>A) and V600E*complex* (1799_1800TG>AA) mutations but does not distinguish between them
2. **BRAF V600K**: the assay detects the V600K (1798_1799GT>AA) mutation
3. **BRAF V600D**: the assay detects the V600D (1799_1800TG>AT) mutation
4. **BRAF V600R**: the assay detects the V600R (1798_1799GT>AG) mutation
5. **BRAF ctrl**: the assay detects a *BRAF* region without any known polymorphism/mutation

The “Easy[®] BRAF” Kit includes a DNA reference standard **Horizon BRAF V600E 1%** containing a defined ratio between wild-type and mutant DNA to check the analytical process and the assay performances.

KIT CONTENTS

The kit contains sufficient reagents to carry out 24 tests for each assay for a maximum of 2 runs (10 samples and two controls per run).

COMP	QUAN	Color	
BRAF V600E mix (1)	2 x 14 µl	RED	Mixture of specific primers and probes targeting both BRAF V600E and V600E <i>complex</i> mutations and the internal control.
BRAF V600K mix (2)	2 x 14 µl	GREEN	Mixture of specific primers and probes targeting the BRAF V600K mutation and the internal control.
BRAF V600D mix (3)	2 x 14 µl	BLUE	Mixture of specific primers and probes targeting the BRAF V600D mutation and the internal control.
BRAF V600R mix (4)	2 x 14 µl	YELLOW	Mixture of specific primers and probes targeting the BRAF V600R mutation and the internal control.
BRAF ctrl mix (5)	4 x 14 µl	GREY	Mixture of specific primers and probes targeting a BRAF region free from any known polymorphism or mutation and the internal control.
Easy BRAF pos ctrl	CONTROL+ 3 x 100 µl		Positive control DNA containing a mixture of synthetic DNA sequences that correspond each mutation detected by this kit in a background of wild-type genomic DNA.
Horizon BRAF V600E 1%	1 x 12 µl		Horizon DNA reference standard BRAF V600E 1% to check the analytical process.
WATER	CONTROL- 1 x 1.5 ml		DNase-, RNase-free water, 1 aliquot to be used exclusively for the preparation of the PCR mix and as negative control, and 1 aliquot to be used exclusively as samples diluent
Taq Premix 920	2 x 920 µl		Solution containing hot start Taq DNA polymerase, reaction buffer, Mg ²⁺ and dNTP Mixture.
Dye R-I	2 x 40 µl		Inert fluorophore to be used for the amplification on the ABI 7300 instrument.
Dye R-II	2 x 40 µl		Inert fluorophore to be used for the amplification on the ABI 7500 and ABI7500 Fast instrument.
8-strip tubes & caps	1 x 5 strips		0.2 ml 8-tube strip DNase-, RNase-free to be used for the preparation of reaction mixture.

DOCUMENTS AVAILABLE ON-LINE

Easy[®] BRAF User Manual and Material Safety Data Sheet (MSDS) are available at www.diatechpharmacogenetics.com/en/reserved-area.

MATERIALS REQUIRED BUT NOT PROVIDED

Genomic DNA extraction

The “Easy[®] BRAF” Kit does not contain reagents for DNA extraction.

Recommended kits:

- “QIAamp[®] DNA FFPE Tissue kit” (cod. 56404, Qiagen)
- “QIAamp[®] DNA Mini kit” (cod. 51304, Qiagen)
- “Genomic DNA FFPE One-Step Kit” (cod. MGF-03, RBC); to use with MagCore Automated Nucleic Acid Extractor (RBC Bioscience) automatic systems
- “Genomic DNA Tissue Kit” (cod. MGT-02, RBC); to use with MagCore Automated Nucleic Acid Extractor (RBC Bioscience) automatic systems
- In case you are using FFPE tissues, you will also need:
 - Xylene (e.g.: “Xylenes, histological grade” – cod. 534056, Sigma Aldrich)
 - Absolute Ethanol (quality of analytical degree)

① In case you employ kits which are different from those recommended, it is the user's responsibility to use standardized samples (e.g.: VEQ – EQAS quality schemes, Horizon Diagnostics samples) to verify that this does not imply a reduction of the performance of the system under analysis.

Amplification

Real-Time PCR instruments:

- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7300** - Applied Biosystems (software v.1.4.1)
- **ABI 7500**- Applied Biosystems (software v. 2.0.5)
- **ABI 7500 Fast**- Applied Biosystems (software v. 2.0.5)
- **Stratagene Mx3000P, Mx3005P** - Agilent Technologies (software v.4.10 build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

Detection channels for FAM and HEX fluorescence. Range of environmental temperature: 15-30°C.

Materials:

- 1.5 ml polypropylene twist-lock tubes (DNase-, RNase-, DNA-, PCR inhibitor-free)
- Micropipettes (volumes from 1 to 1.000 µl)
- Sterile filter tips DNase-, RNase-free (volumes from 1 to 1.000 µl)
- 96 well plates and foil or caps compatibles with the thermal-cycler used (check the compatibility in the user manual of the instrument) for instance:
 1. CFX96: Hard Shell PCR plates 96-well WHT/CLR cod. HSP 9601; MICROSEAL B SEALS cod. MSB 1001
 2. ABI 7300/ABI 7500: MICROAMP OPTICAL 96 WELL RNX PLATE cod. N8010560; OPTICAL ADHESIVE COVERS cod. 4360954
 3. ABI 7500 Fast: MICROAMP FAST OPTICAL 96 WELL RNX PLATE cod. 4346907; OPTICAL ADHESIVE COVERS cod. 4360954
 4. Stratagene Mx3000P/Mx3005P: Optical caps (8x strip) cod. 401425; QPCR 96-Well Plates, Non-Skirted cod. 401333
- DNase- and RNase-free, thin-wall, PCR tubes with flat cap or 0.1 ml tubes in strip, suitable for use on Rotor-Gene, for instance:
 - **PCR tubes 0.1 (1000)** - cod. DIA-PL1, Diatech Pharmacogenetics
- Powder-free disposable gloves

STABILITY AND STORAGE

Store all the reagents according to the instructions on the packages, in particular:

- Store all the reagents at -35/-20°C in the original package immediately upon receipt.
- After thawing, store **Taq PreMix 920** at +2/+8°C and use it within 6 months or within the expiration date.
- Avoid thawing and re-freezing the reagents more than twice, as this could lead to poor performance.
- Protect all mixes containing probes from light to avoid degradation of the fluorescent dyes.
- If properly stored, the reagents remain stable until the expiration date displayed on the individual label.

SYMBOLS

	Catalog number (product code)		Positive control
	Global Trade Item Number		Negative control
	Lot number		Consult the instruction
	Content sufficient for <n> tests		User manual (handbook)
	For <i>in vitro</i> diagnostic use		Expiration date
	Content		Storage conditions
	Components		Manufactured by
	Number of aliquots		Important Note
	Quantity per aliquot		

PRODUCT USE LIMITATIONS

- The “**Easy® BRAF**” Kit can only be used by specialized personnel, properly instructed and trained.
- It is necessary to operate in compliance with the general guidelines of Good Laboratory Practice (GLP) and the instructions contained in this manual.
- Do not use expired or incorrectly stored reagents.
- The “**Easy® BRAF**” Kit is designed to be used with the instruments “**Rotor-Gene Q**” (Qiagen), “**Rotor-Gene™ 6000**” (Corbett Research), **CFX96** (Bio-Rad), **ABI 7300/ABI 7500/ABI 7500 Fast** (Applied Biosystems) and **Stratagene Mx3000P, Mx3005P** (Agilent Technologies).
- The reliability of the results also depends on the procedures carried out in the pre-amplification stages, including the selection of starting biological specimens, the preservation of the samples and DNA extraction.
- Any diagnostic results generated by this procedure must be interpreted with reference to other clinical or laboratory findings.
- “**Easy® BRAF**” is a CE-IVD marked product under the European Directive 98/79/EC on IVD medical devices only into those countries which are accepting the handbook translated into the languages listed at www.diatechpharmacogenetics.com/area-riservata
- Diatech Pharmacogenetics does not respond to the quality of the result obtained using accessories other than those recommended in this manual

QUALITY CONTROL

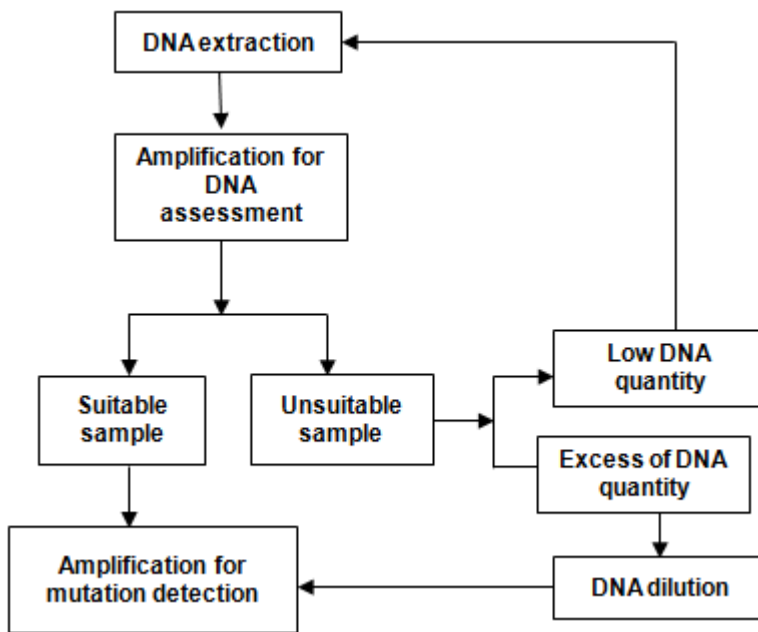
- The “**Easy® BRAF**” Kit was designed, developed and validated in accordance with the Directive 98/79/EC on *in vitro* diagnostic medical devices, transposed at national level with the D.Lgs n. 332/2000 and the subsequent legislative changes, and in accordance with the company system of full quality assurance certified in accordance to the European standards EN ISO 9001 and ISO 13485
- The consistent product quality of the “**Easy® BRAF**” kit is guaranteed by the application of a tight quality control process on operating procedures for the realization of the product and subsequent management to the customer. The quality of each lot is attested in the Certificate of Analysis available upon request to the Customer Service (support@diatechpharmacogenetics.com)

WARNINGS AND PRECAUTIONS

1. The kit may only be used by specialist personnel, properly instructed and trained to perform *in vitro* laboratory techniques.
2. Carefully read this User Manual.
3. Check that the version of this User Manual corresponds to the one described on the “**Easy® BRAF**” Kit box label.
4. Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
5. Follow the laboratory safety procedures described in “*Biosafety in Microbiological and Biomedical Laboratories*” (Richmond, JY and McKinney, RW (eds) - 5th edition (2009) and in the NCCLS (National Committee for Clinical Laboratory Standards) Document M29-T. Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative guidelines. – Villanova, PA:NCCLS, 1989).

6. Do not eat, drink or smoke in the laboratory. When handling biological samples, disposable gloves, gowns and goggles or face masks should be worn to protect against biological agents.
7. ① Constantly check that the gloves are free from contamination by the biological material being treated. If not, replace them immediately to avoid the possibility of cross-contamination between samples and contamination of the workplace. Wash hands thoroughly after handling samples and reagents.
8. The Material Safety Data Sheet (MSDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com.
9. Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
10. It is recommended to ensure that the laboratory work flow proceeds in a unidirectional manner, preparing, if possible, two separate work areas for:
 - a. extraction of nucleic acids;
 - b. amplification reaction;
11. Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
12. Use sterile filter tips. Avoid aerosols.
13. Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
14. During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by only opening the reagents within the hood.
15. Change the pipette tip before each extraction of reagents and every time you move from one sample to another in any stage of the procedure.
16. The precision pipettes used should have an accuracy of within 3% of the set volume.
17. Periodically check the calibration status of the dispensing instruments.
18. Do not use reagents after the expiration date shown on each container.
19. All reagents supplied in the “**Easy® BRAF**” Kit are intended to be used solely with the other reagents in the same “**Easy® BRAF**” Kit. Do not substitute or mix reagents in the kit from different batches, in order to maintain optimal performance.
20. Only use the **Taq PreMix 920** that is provided in the kit. Do not substitute with **Taq PreMix 920** from other kits or with similar reagents from other suppliers.
21. Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
22. Extraction area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which worked has been carried out, by cleaning with appropriate products (e.g. FD 322, Dürr Dental, Germany) and UV irradiate the work surface of the biological cabinet where the pipettes should be carefully placed after decontamination.
23. Amplification area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products to eliminate nucleic acids and amplicons (e.g. “DNA Cleaner” - code DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
24. Avoid contamination of samples and reagents.
25. Store reagents and samples separately.
26. In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.
27. Before use all reagents need to be thawed at room temperature, mixed by inverting 10 times and centrifuged briefly.
28. All reagents contained in the kit are ready-to-use and don't need to be diluted. The reagent dilution may result in a loss of performance.
29. Include in each run at least 1 negative control (**WATER**) and 1 positive control (**Easy BRAF pos ctrl**).
30. In order to avoid any mixing up of samples pay particular attention to samples dispensation, placement of tubes into the instrument, editing the sample name in the software.
31. The right to contest the kit before the expiration date becomes void if the product is used in violation of GLP guidelines and the manufacturer's recommendations.
32. The registered names and trademarks indicated in this document are to be considered protected by law, even when not explicitly stated.

ANALYTICAL PROCEDURE



DNA EXTRACTION

① Perform this step in the area dedicated to DNA isolation and dilution, using dedicated materials and instruments.

- The “**Easy® BRAF**” Kit does not include the reagents for DNA extraction.
- The quantity of biological material required for the DNA extraction depends on protocols.
- Refer also to the extraction kit manual for selection and treatment of FFPE slides.
- Commercial kits working with silica filters or magnetic beads are recommended. Avoid methods that use phenol or boiling in basic solution without purification.
- Perform the DNA extraction following the instructions of the extraction kit in use.
- If the extraction protocol involves the use of wash buffers containing ethanol, it is advisable to perform a further centrifugation before final elution to remove any possible traces of ethanol. This will prevent inhibition of the reaction by the ethanol.
- After the extraction, proceed immediately with the quali-quantitative evaluation of the DNA and the amplification reaction, or store the extracted DNA at $\leq -20^{\circ}\text{C}$, divided into aliquots in order to maintain the experimental conditions constant in case of repetition.
- Just as an indication, for non-paraffin embedded samples like fresh/frozen tissue, plasma, blood, the recommended DNA amount in each test tube is 5-10 ng; for paraffin embedded samples, the recommended DNA amount in each reaction tube is 15-20 ng.

① As absorbance reading cannot distinguish between fragmented and not fragmented DNA and therefore it can overestimate the concentration of template, DNA assessment should be based on the **BRAF ctrl mix (5)**. The evaluation of quality and quantity of the DNA in the samples with the **BRAF ctrl mix (5)** before the mutation analysis is highly recommended. DNA assessment based on the **BRAF ctrl mix (5)** may differ from spectrophotometric quantification.

① All assays in the “**Easy® BRAF**” Kit amplify short DNA sequences. However heavily fragmented DNA can generate no amplification product.

AMPLIFICATION

General recommendations for amplification (valid for all instruments)

- ① Perform this step in the area dedicated to amplification mixes preparation, using dedicated materials and instruments. Before starting decontaminate pipettes, benches and wood in order to degrade any trace of DNA and possibly radiate with UV light for at least 30 minutes.
- Switch on the instrument and the software at least 20-30 minutes before starting the reaction to allow the heating of the lamps where necessary.
 - Thaw all necessary reagents before use.
 - Thoroughly mix the reagents in a vortex, or inverting each tube ten times, and spin them briefly before use.
 - Prepare and mark an appropriate number of tubes or well of the plate to use.
 - Each run must include at least one amplification negative control (**WATER**) and one amplification positive control (**Easy BRAF pos ctrl**) for each mix.

INSTRUMENT SETUP

Rotor-Gene Q, Rotor-Gene 6000

- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:
 - “Green”: source 470 nm – detector 510 nm – Gain 10
 - “Yellow”: source 530 nm – detector 555 nm – Gain 10
 - “Green 2” - source 470 nm – detector 510 nm – “Gain Optimisation” 58°C Before 1st acquisition, “Tube Position”:49 (**BRAF ctrl mix (5)**), “Target Sample Range” 20-30 FI
 - “Yellow 2”: source 530 nm – detector 555 nm – “Gain Optimisation” 58°C Before 1st acquisition, “Tube Position”:49 (**BRAF ctrl mix (5)**), “Target Sample Range” 20-30 FI

Thermal Profile	
Hold	95°C for 2 minutes
40 cycles	95°C for 10 seconds / 58°C for 60 seconds (acquire fluorescence in channels “Green”, “Green 2”, “Yellow”, Yellow 2”)

- Reaction volume: 20 µl.

Stratagene Mx3000P, Mx3005P

- Select “New” – “Quantitative PCR (Multiple standards)”, then “OK”.
- Check for the presence of the message “Lamp – Warm-up”.
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:
 - “FAM”: source 492 nm – detector 516 nm – Filter gain factor x8
 - “HEX”: source 535 nm – detector 555 nm – Filter gain factor x8

Thermal Profile	
Hold	95°C for 2 minutes
40 cycles	95°C for 10 seconds / 58°C for 60 seconds (acquire fluorescence in channels “FAM” and “HEX” setting up END 1)

- Reaction volume: 20 µl.

CFX96

- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channel and thermal profile:

Thermal Profile	
Step	
1	95°C for 2 minutes
2	95°C for 10 seconds
3	58°C for 60 seconds (Plate read – All Channels)
4	GO TO 2 39 more times

- Reaction volume: 20 µl.

① “Select the option “All Channels” to acquire the signal in both FAM and HEX.

ABI 7300

- Select “Create new document” – On Assay: “Standard Curve (Absolute Quantitation)”, then “Next”.
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channel and thermal profile:

Thermal Profile	
Hold	95°C for 2 minutes
40 cycles	95°C for 10 seconds / 58°C for 60 seconds (acquire fluorescence in channels FAM, JOE; Quencer – None; Passive Reference Dye - ROX)

- Reaction volume: 20 µl.

ABI 7500/ABI 7500 Fast

- Select “New Experiment” then “7500 (96 Wells)”, “Quantitation – Standard Curve”, “TaqMan® Reagents”, “Standard (2 hours to complete a run)”.
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:

Thermal Profile	
Hold	95°C for 2 minutes
40 cicli	95°C for 10 seconds / 58°C for 60 seconds (acquire fluorescence in channels FAM, JOE; Quencer – None; Passive Reference Dye - ROX)

- Reaction volume: 20 µl.

DNA ASSESSMENT

- For each sample and control prepare an amplification mixture (**Amp-Mix**), according to the following table, where N is the total number of samples and controls to be tested:

Rotor-Gene, Stratagene, CFX96		
Amp-Mix	Reagent volume for 1 reaction (µl)	Reagent volume for N reactions +1 (µl)
Taq Premix 920	10	
WATER CONTROL -	4	
BRAF ctrl mix (5)	1	
Total Volume	15	

ABI 7300		
Amp-Mix	Reagent volume for 1 reaction (µl)	Reagent volume for N reactions +1 (µl)
Taq Premix 920	10	
Dye R-I	0.4	
WATER CONTROL -	3.6	
BRAF ctrl mix (5)	1	
Total Volume	15	

ABI 7500, ABI 7500 Fast		
Amp-Mix	Reagent volume for 1 reaction (µl)	Reagent volume for N reactions +1 (µl)
Taq Premix 920	10	
Dye R-II	0.2	
WATER CONTROL -	3.8	
BRAF ctrl mix (5)	1	
Total Volume	15	

- Mix the **Amp-Mix** thoroughly by repeated pipetting or rapid vortexing, then centrifuge briefly.
- Pipette 15 µl of the **Amp-Mix** in all the marked reaction test tubes or wells.
- Add to the respective test tubes or wells:

<u>negative control</u>	5 µl WATER	CONTROL -
<u>samples</u>	5 µl DNA	
<u>positive control</u>	5 µl Easy BRAF pos ctrl	CONTROL +

- Final volume: 20 µl.
- Briefly centrifuge the plate.
- Check that the thermal profile is setted up correctly and start the run.

① Before starting the run, please pay attention to the plate orientation (well A1 on the upper left position) or to the Rotor-Gene 0.1ml strips of tubes orientation (mark the first tube of each strip).

① Proceed with the analysis following the instructions of the section “Data analysis”.

MUTATION DETECTION

① Only samples that after DNA assessment are suitable can be analyzed for mutations detection.

① Each sample must be amplified with 5 different mixes: **BRAF V600E mix (1)**, **BRAF V600K mix (2)**, **BRAF V600D mix (3)**, **BRAF V600R mix (4)** e **BRAF ctrl mix (5)**.

① The DNA reference standard **Horizon BRAF V600E 1%** must be amplified only with the mixes **BRAF V600E mix (1)** e **BRAF ctrl mix (5)**.

① The kit content is optimized to analyze 10 clinical samples and two controls **Easy BRAF pos ctrl** and **WATER** in each run.

Mx3000P/3005P, ABI 7300, ABI 7500, ABI 7500 Fast. CFX96 (RT002 sample grid A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA1	DNA2	DNA3	DNA4	DNA5	DNA6	DNA7	DNA8	DNA9	DNA10	POS CTRL	WATER
B	DNA1	DNA2	DNA3	DNA4	DNA5	DNA6	DNA7	DNA8	DNA9	DNA10	POS CTRL	WATER
C	DNA1	DNA2	DNA3	DNA4	DNA5	DNA6	DNA7	DNA8	DNA9	DNA10	POS CTRL	WATER
D	DNA1	DNA2	DNA3	DNA4	DNA5	DNA6	DNA7	DNA8	DNA9	DNA10	POS CTRL	WATER
E	DNA1	DNA2	DNA3	DNA4	DNA5	DNA6	DNA7	DNA8	DNA9	DNA10	POS CTRL	WATER
F												
G												
H												

1	V600E mix (1)
2	V600K mix (2)
3	V600D mix (3)
4	V600R mix (4)
5	ctrl mix (5)

Rotor-Gene (RT002 sample grid B)

DNA1		DNA2		DNA3		DNA4		DNA5		DNA6		ctrl mix		ctrl mix			
1	●	9	●	17	●	25	●	33	●	41	●	49	● DNA1	57	● DNA9	65	●
2	●	10	●	18	●	26	●	34	●	42	●	50	● DNA2	58	● DNA10	66	●
3	●	11	●	19	●	27	●	35	●	43	●	51	● DNA3	59	● POS CTRL	67	●
4	●	12	●	20	●	28	●	36	●	44	●	52	● DNA4	60	● WATER	68	●
DNA7		DNA8		DNA9		DNA10		POS CTRL		WATER		ctrl mix					
5	●	13	●	21	●	29	●	37	●	45	●	53	● DNA5	61	●	69	●
6	●	14	●	22	●	30	●	38	●	46	●	54	● DNA6	62	●	70	●
7	●	15	●	23	●	31	●	39	●	47	●	55	● DNA7	63	●	71	●
8	●	16	●	24	●	32	●	40	●	48	●	56	● DNA8	64	●	72	●

- Prepare, for each sample and control, five different amplification mixtures (**Amp-Mix**), one for each assay, as indicated in the following scheme, where N is the number of samples and control to be tested.
- If you are working with a 96 well plate, it is possible to prepare the **Amp-Mix** in the strip provided with the kit, so you can dispense all **Amp-Mix** with a multichannel pipette.

Rotor-Gene, Stratagene, CFX96		
Amp-Mix	Reagent volume for 1 reaction (µl)	Reagent volume for N reactions +1 (µl)
Taq Premix 920	10	
WATER CONTROL -	4	
BRAF V600E mix (1) or BRAF V600K mix (2) or BRAF V600D mix (3) or BRAF V600R mix (4) or BRAF ctrl mix (5)	1	
Total Volume	15	

ABI 7300		
Amp-Mix	Reagent volume for 1 reaction (µl)	Reagent volume for N reactions +1 (µl)
Taq Premix 920	10	
Dye R-I	0.4	
WATER CONTROL -	3.6	
BRAF V600E mix (1) or BRAF V600K mix (2) or BRAF V600D mix (3) or BRAF V600R mix (4) or BRAF ctrl mix (5)	1	
Total Volume	15	

ABI 7500, ABI 7500 Fast		
Amp-Mix	Reagent volume for 1 reaction (µl)	Reagent volume for N reactions +1 (µl)
Taq Premix 920	10	
Dye R-II	0.2	
WATER CONTROL -	3.8	
BRAF V600E mix (1) or BRAF V600K mix (2) or BRAF V600D mix (3) or BRAF V600R mix (4) or BRAF ctrl mix (5)	1	
Total Volume	15	

- Mix all **Amp-Mix** thoroughly by repeated pipetting or rapid vortexing, then centrifuge briefly.
- Pipette 15 µl of each **Amp-Mix** in all the tubes/wells previously marked.
- Add to the respective tubes/wells for each of the eight assays:

<u>negative control</u>	5 µl WATER	CONTROL -
<u>sample</u>	5 µl DNA	
<u>positive control</u>	5 µl Easy BRAF pos ctrl	CONTROL +

- Reaction volume: 20 µl.
- Briefly centrifuge the plate.
- Check that the thermal profile is setted up correctly and start the run.

① Before starting the run, please pay attention to the plate orientation (well A1 on the upper left position) or to the Rotor-Gene 0.1ml strips of tubes orientation (mark the first tube of each strip).

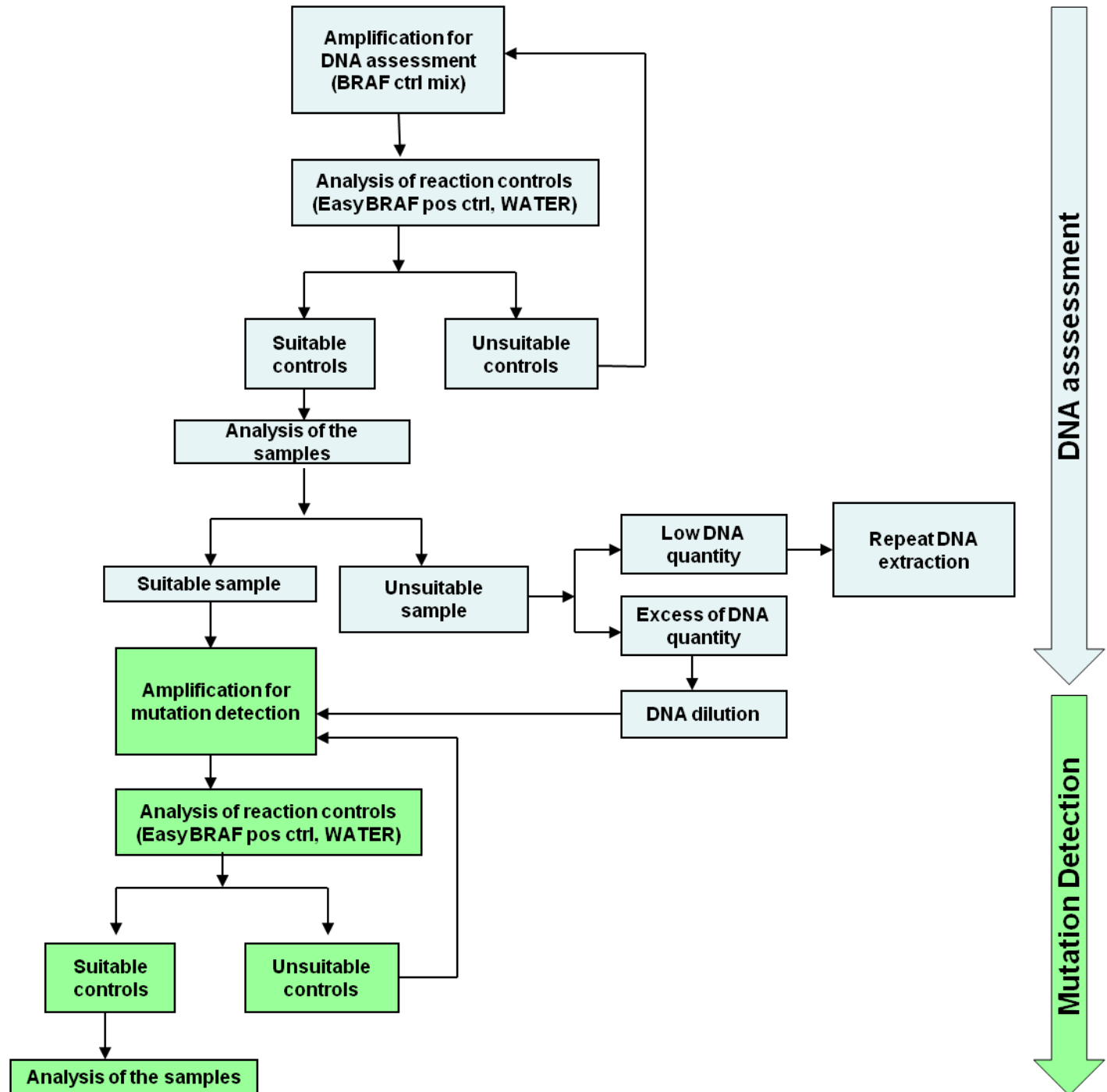
① Proceed with the analysis following the instructions of the section "Data analysis".

DATA ANALYSIS

General recommendations pertain to all the instruments

- ① Analyze first the negative control **WATER** and the positive control **Easy BRAF pos ctrl** . If they are in the range of expected values, proceed with the analysis of the samples, otherwise the session should be considered invalid and the results of the samples should be rejected.
- ① It is necessary to verify that the Ct values obtained are generated from a real amplification reaction (sigmoidal fluorescence curve) and not from an artifact (linear fluorescence curve), checking the normalized fluorescence graphs.
- ① Only the samples that after the DNA assessment reaction (**BRAF ctrl mix (5)**) are suitable can be analyzed for the presence of mutations.

Proceed with the analysis as indicated by the following scheme:



Rotor-Gene

- At the end of the run, click [Analysis](#) and select [Quantitation](#).
- Highlight [Dynamic tube](#) and [Slope correct](#) for all the channels.
- Set [Threshold](#) 0.04 for the Green and Yellow channels.

① If it is not possible to perform the analysis in the Green and/or Yellow channel because of the fluorescence level is too high, see Troubleshooting section.

1. Analysis of the reaction controls

	BRAF ctrl mix	V600E, V600K, V600D and V600R mix	All mixes	
	Green	Green	Yellow	Results
WATER	Ct > 33	Ct > 33	Ct > 33	Proceed with analysis of the samples.
	Ct ≤ 33	Ct ≤ 33	Ct ≤ 33	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy BRAF pos ctrl	23 ≤ Ct ≤ 28	19 ≤ Ct ≤ 27	21 ≤ Ct ≤ 29	Proceed with analysis of the samples.
	Ct < 23 or Ct > 28	Ct < 19 or Ct > 27	Ct < 21 or Ct > 29	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of BRAF ctrl mix for the DNA assessment

BRAF ctrl mix	Green	Yellow	Results
DNA samples	18 ≤ Ct ≤ 28	17 ≤ Ct ≤ 28	Suitable sample. Proceed with the mutation analysis ¹ .
	Ct < 18	Ct < 17	Excess of DNA. Samples must be diluted with Water (aliquot to be used for sample dilution) so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Ct of 1 unit.
	Ct > 28	Ct > 28	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: - If it is possible, amplify a DNA volume > 5µl (max 9µl) or proceed with a new DNA extraction to obtain an higher concentration of template and/or a DNA of higher quality. - If the presence of inhibitors is suspected, dilute the sample with Water (aliquot to be used for sample dilution). Consider that the dilution also reduces the presence of inhibitor, but decreases the concentration of the target DNA.

1. If the results of the Yellow channel are not in the expected range, consider only the Green channel results to value the sample suitability.

3. Analysis of the mutation assays

- ① Only samples that following the assessment of DNA are suitable can be analyzed to search for mutations.
- Compare the ΔCt values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔCt values should be calculated with the following formula, taking care that the Ct value in Green for the mutation and the equivalent for the control assay belong to the same sample:

$$\Delta Ct = Ct \text{ Green Mutation} - Ct \text{ Green BRAF ctrl mix}$$

Assay	Amplification of Internal Control (Yellow)	ΔCt	Results
V600E	OK (similar Ct value for all the mixes)	≤ 12.5	V600E/V600E <i>complex</i> positive
V600K		≤ 12.5	V600K positive
V600D		≤ 14	V600D positive
V600R		≤ 11.5	V600R positive
V600E	OK (similar Ct value for all the mixes)	> 12.5	Wild-type sample or beneath the LOD ¹
V600K		> 12.5	
V600D		> 14	
V600R		> 11.5	
V600E	NO	\	Failed: not sufficient template/PCR inhibition / mistake during sample dispensation
V600K		\	
V600D		\	
V600R		\	
1. LOD = Limit Of Detection			
\ Any value			

- ① If multiple assays show a ΔCt equal to or below the cut-off value, the signal giving the higher ΔCt is probably due to cross-reactivity. Although double mutants have been observed, these are rare. In this case the sample should be considered positive only for the mutation with the lowest ΔCt .

Stratagene Mx3000P, Mx3005P

- At the end of the run click Set-up, Plate Setup, and set the names of the samples.
- In the section Analysis, Analysis Selection/Setup, deselect the function Amplification-based threshold and select only the reactions controls **WATER** and **Easy BRAF pos ctrl**.
- In the section Analysis, Results, click on the icon Padlock in the box Threshold Fluorescence (in this way the automatically selected values cannot be modified).
- Go Back to the section Analysis, Analysis Selection/Setup, and select the all the samples.
- In the section Analysis, Results, select Text report to see the results in both channels with their respective threshold and Ct.
- Click Save.

1. Analysis of reaction control

	BRAF ctrl mix	V600E, V600K, V600D and V600R mix	All mixes	
	FAM	FAM	HEX	Results
WATER	Ct > 33	Ct > 33	Ct > 33	Proceed with analysis of the samples.
	Ct ≤ 33	Ct ≤ 33	Ct ≤ 33	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy BRAF pos ctrl	23 ≤ Ct ≤ 26	20 ≤ Ct ≤ 25	21 ≤ Ct ≤ 25	Proceed with analysis of the samples.
	Ct < 23 or Ct > 26	Ct < 20 or Ct > 25	Ct < 21 or Ct > 25	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of BRAF ctrl mix for the DNA assessment

BRAF ctrl mix	FAM ¹	HEX ¹	Results
DNA samples	$(x-4.5) \leq Ct \leq (x+4.5)$	$(x-4.5) \leq Ct \leq (x+4.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Ct < (x-4.5)	Ct < (x-4.5)	Excess of DNA. Samples must be diluted with Water (aliquot to be used for sample dilution) so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Ct of 1 unit.
	Ct > (x+4.5)	Ct > (x+4.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: - If it is possible, amplify a DNA volume > 5µl (max 9µl) or proceed with a new DNA extraction to obtain an higher concentration of template and/or a DNA of higher quality. - If the presence of inhibitors is suspected, dilute the sample with Water (aliquot to be used for sample dilution). Consider that the dilution also reduces the presence of inhibitor, but decreases the concentration of the target DNA.

1. x = Ct **Easy BRAF pos ctrl**

2. If the results of the HEX channel are not in the expected range, consider only the FAM channel results to value the sample suitability.

3. Analysis of the mutation assays

① Only samples that following the assessment of DNA are suitable can be analyzed to search for mutations.

- Compare the values ΔCt of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔCt values should be calculated with the following formula, taking care that the Ct value in FAM for the mutation and the equivalent for the control assay belong to the same sample:

$$\Delta Ct = Ct \text{ FAM Mutation} - Ct \text{ FAM BRAF ctrl mix}$$

Assay	Amplification of Internal Control (HEX)	ΔCt	Results
V600E	OK (similar Ct value for all the mixes)	≤ 13.5	V600E/V600Ecomplex positive
V600K		≤ 12.5	V600K positive
V600D		≤ 14	V600D positive
V600R		≤ 12.5	V600R positive
V600E	OK (similar Ct value for all the mixes)	> 13.5	Wild-type sample or beneath the LOD ¹
V600K		> 12.5	
V600D		> 14	
V600R		> 12.5	
V600E	NO	\	Failed: not sufficient template/PCR inhibition / mistake during sample dispensation
V600K		\	
V600D		\	
V600R		\	
1. LOD = Limit Of Detection			
\ Any value			

- ① If multiple assays show a ΔCt equal to or below the cut-off value, the signal giving the higher ΔCt is probably due to cross-reactivity. Although double mutants have been observed, these are rare. In this case the sample should be considered positive only for the mutation with the lowest ΔCt .

CFX96

- At the end of the run, click [Plate Setup – View/Edit Plate](#), select the wells used, pick the FAM and HEX fluorophores and enter the sample names.
- In the section [Settings](#) set up the following analysis criteria as default:
 - Cq determination mode: Single Threshold
 - Baseline settings: Baseline Subtracted Curve fit
 - Analysis Mode: Fluorophore
 - Baseline Threshold: Auto Calculated for every channel
- In the page [Quantification data](#), the results are displayed for both channels with the corresponding values of Cq.
- Click [Save](#).

1. Analysis of reaction control

	BRAF ctrl mix	V600E, V600K, V600D and V600R mix	All mixes	
	FAM	FAM	HEX	Results
WATER	Cq > 33	Cq > 33	Cq > 33	Proceed with analysis of the samples.
	Cq ≤ 33	Cq ≤ 33	Cq ≤ 33	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy BRAF pos ctrl	24 ≤ Cq ≤ 27	20 ≤ Cq ≤ 26	23 ≤ Cq ≤ 27	Proceed with analysis of the samples.
	Cq < 24 or Cq > 27	Cq < 20 or Cq > 26	Cq < 23 or Cq > 27	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of BRAF ctrl mix for the DNA assessment

BRAF ctrl mix	FAM ¹	HEX ¹	Results
DNA samples	$(x-4.5) \leq Cq \leq (x+4.5)$	$(x-4.5) \leq Cq \leq (x+4.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Cq < (x-4.5)	Cq < (x-4.5)	Excess of DNA. Samples must be diluted with Water (aliquot to be used for sample dilution) so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Cq of 1 unit.
	Cq > (x+4.5)	Cq > (x+4.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: - If it is possible, amplify a DNA volume > 5µl (max 9µl) or proceed with a new DNA extraction to obtain an higher concentration of template and/or a DNA of higher quality. - If the presence of inhibitors is suspected, dilute the sample with Water (aliquot to be used for sample dilution). Consider that the dilution also reduces the presence of inhibitor, but decreases the concentration of the target DNA.
1. x = Cq Easy BRAF pos ctrl 2. If the results of the HEX channel are not in the expected range, consider only the FAM channel results to value the sample suitability.			

3. Analysis of the mutation assays

- ① Only samples that following the assessment of DNA are suitable can be analyzed to search for mutations.
- Compare the values ΔCq of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔCq values should be calculated with the following formula, taking care that the Cq value in FAM for the mutation and the equivalent for the control assay belong to the same sample:

$$\Delta Cq = Cq \text{ FAM Mutation} - Cq \text{ FAM BRAF ctrl mix}$$

Assay	Amplification of Internal Control (HEX)	ΔCq	Results
V600E	OK (similar Cq value for all the mixes)	≤ 12.5	V600E/V600E <i>complex</i> positive
V600K		≤ 12.5	V600K positive
V600D		≤ 14	V600D positive
V600R		≤ 12	V600R positive
V600E	OK (similar Cq value for all the mixes)	> 12.5	Wild-type sample or beneath the LOD ¹
V600K		> 12.5	
V600D		> 14	
V600R		> 12	
V600E	NO	\	Failed: not sufficient template/PCR inhibition / mistake during sample dispensation
V600K		\	
V600D		\	
V600R		\	
1. LOD = Limit Of Detection \ Any value			

- ① If multiple assays show a ΔCq equal to or below the cut-off value, the signal giving the higher ΔCq is probably due to cross-reactivity. Although double mutants have been observed, these are rare. In this case the sample should be considered positive only for the mutation with the lowest ΔCq .

ABI 7300

- At the end of the run, click Results - Plate and enter the sample names.
- In the page Results – Amplification plot select all the samples and controls.
- Select Detector – All.
- In Analysis settings select Auto Ct then Analyze.
- Select Manual Ct, Manual baseline Start cycle 3, End cycle 15 then Analyze.
- In the page Report Ct values are visualized for both channels.
- Click Save.

1. Analysis of reaction control

	BRAF ctrl mix	V600E, V600K, V600D and V600R mix	All mixes	
	FAM	FAM	JOE	Results
WATER	Ct > 33	Ct > 33	Ct > 33	Proceed with analysis of the samples.
	Ct ≤ 33	Ct ≤ 33	Ct ≤ 33	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
BRAF pos ctrl II	18 ≤ Ct ≤ 26	18 ≤ Ct ≤ 25	19 ≤ Ct ≤ 29	Proceed with analysis of the samples.
	Ct < 18 or Ct > 26	Ct < 18 or Ct > 25	Ct < 19 or Ct > 29	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of BRAF ctrl mix for the DNA assessment

BRAF ctrl mix	FAM ¹	JOE ¹	Results
DNA samples	$(x-4.5) \leq Ct \leq (x+4.5)$	$(x-4.5) \leq Ct \leq (x+4.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Ct < (x-4.5)	Ct < (x-4.5)	Excess of DNA. Samples must be diluted with Water (aliquot to be used for sample dilution) so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Ct of 1 unit.
	Ct > (x+4.5)	Ct > (x+4.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: - If it is possible, amplify a DNA volume > 5µl (max 9µl) or proceed with a new DNA extraction to obtain an higher concentration of template and/or a DNA of higher quality. - If the presence of inhibitors is suspected, dilute the sample with Water (aliquot to be used for sample dilution). Consider that the dilution also reduces the presence of inhibitor, but decreases the concentration of the target DNA.

1. x = Ct **Easy BRAF pos ctrl**

2. If the results of the JOE channel are not in the expected range, consider only the FAM channel results to value the sample suitability.

3. Analysis of the mutation assays

① Only samples that following the assessment of DNA are suitable can be analyzed to search for mutations.

- Compare the values ΔC_t of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔC_t values should be calculated with the following formula, taking care that the C_t value in FAM for the mutation and the equivalent for the control assay belong to the same sample:

$$\Delta C_t = C_t \text{ FAM Mutation} - C_t \text{ FAM BRAF ctrl mix}$$

Assay	Amplification of Internal Control (JOE)	ΔCt	Results
V600E	OK (similar Ct value for all the mixes)	≤ 14	V600E/V600Ecomplex positive
V600K		≤ 13	V600K positive
V600D		≤ 14	V600D positive
V600R		≤ 12	V600R positive
V600E	OK (similar Ct value for all the mixes)	> 14	Wild-type sample or beneath the LOD ¹
V600K		> 13	
V600D		> 14	
V600R		> 12	
V600E	NO	\	Failed: not sufficient template/PCR inhibition / mistake during sample dispensation
V600K		\	
V600D		\	
V600R		\	
1. LOD = Limit Of Detection \ Any value			

① If multiple assays show a ΔC_t equal to or below the cut-off value, the signal giving the higher ΔC_t is probably due to cross-reactivity. Although double mutants have been observed, these are rare. In this case the sample should be considered positive only for the mutation with the lowest ΔC_t .

ABI 7500, ABI 7500 Fast

- At the end of the run, click Setup – Plate setup – Assign Targets and Samples and enter samples name.
- In the section Analysis – Amplification plot in the page View Plate layout select all the samples and controls and omit all empty wells.
- Select Reanalyse.
- In Analysis settings, for each channel, deselect Use Default Settings, Automatic Threshold and Automatic Baseline.
- Click on Apply Analysis Settings and then Reanalyse.
- In the page View Well Table C_t values are visualized for both channels.
- Click Save.

1. Analysis of reaction controls

	BRAF ctrl mix	V600E, V600K, V600D and V600R mix	All mixes	
	FAM	FAM	JOE	Results
WATER	$C_t > 30$	$C_t > 33$	$C_t > 33$	Proceed with analysis of the samples.
	$C_t \leq 30$	$C_t \leq 33$	$C_t \leq 33$	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
BRAF pos ctrl II	$22 \leq C_t \leq 29$	$18 \leq C_t \leq 28$	$22 \leq C_t \leq 28$	Proceed with analysis of the samples.
	$C_t < 22$ or $C_t > 29$	$C_t < 18$ or $C_t > 28$	$C_t < 22$ or $C_t > 28$	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of **BRAF ctrl mix** for the DNA assessment

BRAF ctrl mix	FAM ¹	JOE ¹	Results
DNA samples	$(x-4.5) \leq Ct \leq (x+4.5)$	$(x-4.5) \leq Ct \leq (x+4.5)$	Suitable sample. Proceed with the mutation analysis ² .
	$Ct < (x-4.5)$	$Ct < (x-4.5)$	Excess of DNA. Samples must be diluted with Water (aliquot to be used for sample dilution) so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Ct of 1 unit.
	$Ct > (x+4.5)$	$Ct > (x+4.5)$	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: - If it is possible, amplify a DNA volume > 5µl (max 9µl) or proceed with a new DNA extraction to obtain an higher concentration of template and/or a DNA of higher quality. - If the presence of inhibitors is suspected, dilute the sample with Water (aliquot to be used for sample dilution). Consider that the dilution also reduces the presence of inhibitor, but decreases the concentration of the target DNA.

1. $x = Ct$ Easy BRAF pos ctrl

2. If the results of the JOE channel are not in the expected range, consider only the FAM channel results to value the sample suitability.

3. Analysis of the mutation assays

① Only samples that following the assessment of DNA are suitable can be analyzed to search for mutations.

- Compare the values ΔCt of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔCt values should be calculated with the following formula, taking care that the Ct value in FAM for the mutation and the equivalent for the control assay belong to the same sample:

$$\Delta Ct = Ct \text{ FAM Mutation} - Ct \text{ FAM BRAF ctrl mix}$$

Assay	Amplification of Internal Control (JOE)	ΔCt	Results
V600E	OK (similar Ct value for all the mixes)	≤ 13.5	V600E/V600Ecomplex positive
V600K		≤ 16	V600K positive
V600D		≤ 16	V600D positive
V600R		≤ 16	V600R positive
V600E	OK (similar Ct value for all the mixes)	> 13.5	Wild-type sample or beneath the LOD ¹
V600K		> 16	
V600D		> 16	
V600R		> 16	
V600E	NO	\	Failed: not sufficient template/PCR inhibition / mistake during sample dispensation
V600K		\	
V600D		\	
V600R		\	

2. LOD = Limit Of Detection

\ Any value

① If multiple assays show a ΔCt equal to or below the cut-off value, the signal giving the higher ΔCt is probably due to cross-reactivity. Although double mutants have been observed, these are rare. In this case the sample should be considered positive only for the mutation with the lowest ΔCt .

TROUBLESHOOTING

Problem	Possible reason	Recommendation
Low or absent amplification signal in the channel "Green/FAM" and/or in the channel "Yellow/HEX" for both Easy BRAF pos ctrl and samples.	Incorrect selection of the fluorescence acquisition channels.	Check the fluorescence acquisition channels and repeat amplification with the settings described in this manual.
	Incorrect selection of the Gain on the Rotor-Gene.	Repeat amplification of Rotor-Gene setting the gain properly, as described in this manual.
	Incorrect setting of the thermal-profile.	Check the temperature profile and repeat amplification with the settings described in this manual.
	Incorrect dispensation or manipulation of the reagents.	- Mix reagents by vortexing or inverting the tubes ten times and briefly spin before use. - Keep reagents on ice or refrigerated blocks during the preparation of the Amp-Mix.
	Reagents improperly stored or expired.	- Protect all mixes from light. - Store all reagents at -35/-20°C and avoid thawing and refreezing more than twice. - Do not store the mixes containing primers and probes at +2/+8°C for more than 5 hours. - Do not store the Taq Premix 920 at +2/+8°C for more than 6 months. - Do not use expyred reagents.
No amplification signal in both "Green / FAM" and "Yellow / HEX" channels for all assays. Easy BRAF pos ctrl is within the expected values.	Insufficient amount of starting DNA and / or presence of PCR inhibitors.	- Check the quantity and quality of the extracted DNA and, if appropriate, repeat the extraction faithfully following the instructions of the extraction kit. - If the extraction protocol involves the use of washing buffers containing ethanol, it is advisable to carry out a further centrifugation prior to final elution to remove any possible trace of alcohol. - If it is assumed that the amount of starting DNA is insufficient, repeat the reaction amplifying > 5ul DNA (max volume 9 ul), reducing the corresponding volume of WATER in the Amp-Mix. Otherwise repeat the DNA extraction by reducing the volume of elution. - If you suspect the presence of inhibitors, repeat the amplification diluting sample 1:5 or 1:10 with Water.
	Incorrect or no dispensation of the samples.	Repeat the amplification dispensing the correct volume of DNA and including positive and negative controls.
Amplification signal weak or absent in "Green / FAM" and "Yellow / HEX" only for the positive control Easy BRAF pos ctrl .	Degradation of the Easy BRAF pos ctrl .	Repeat the amplification testing a new aliquot of Easy BRAF pos ctrl.
	Incorrect or failure in the dispensation of the Easy BRAF pos ctrl .	Repeat the amplification by pipetting the appropriate volume of Easy BRAF pos ctrl.
You can not perform the analysis on Rotor-Gene in Cycling A. Green and/or Cycling A. Yellow because of a fluorescence intensity too high or out of scale for one or more than one assay.	Rotor-Gene gain too high.	Perform analysis on Rotor-Gene selecting the channel Green 2 and/or Yellow 2 and the threshold value at 0.04. The ΔCt values should be calculated with the following formula: $\Delta Ct = Ct \text{ Green 2 Mutation} - Ct \text{ Green 2 BRAF ctrl mix}$.
The positive Easy BRAF pos ctrl shows no amplification signal in the channel "Green / FAM" for assays of mutations or the signal is detectable only for some mixes; while one or more samples show a signal amplification in all assays of mutations.	Wrong tubes identification.	Repeat the amplification after marking unambiguously the reaction tubes for samples and controls.
	Incorrect dispensing of the samples.	Repeat the amplification paying attention to the dispensation of the DNA and the Easy BRAF pos ctrl in reaction tubes/wells.
	Incorrect samples names set-up in the software.	Check samples names set-up.
One sample shows HEX/Yellow Ct values different from each other for the assays.	DNA dispensation error.	Repeat the amplification paying attention to the dispensation of the DNA and the Easy BRAF pos ctrl in reaction tubes/wells.
The negative control WATER , shows an amplification signal in both FAM/Green and HEX/Yellow channels.	Contamination.	- The results should be rejected and samples must be reamplified using new reagents. - Prepare Amp-Mix in a dedicated area. Carefully decontaminate benches, pipettes and instruments.
Fluorescence intensity variable.	Cutaneous fat on the tube.	Wear gloves.
- If the problems persist despite the implementation of the recommendations given and for any other questions or problems, please contact technical support of Diatech Pharmacogenetics: - e-mail support@diatechpharmacogenetics.com - telephone +39 0731 213243 - fax +39 0731 213239		

PERFORMANCE VALIDATION

The performance validation has been performed using all the reagents supplied with the Kit.

The experiments have been performed according to the instructions reported in this user manual on the following real-time instruments:

- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7300** - Applied Biosystems (software v.1.4.1)
- **ABI 7500, ABI 7500 Fast** - Applied Biosystems (software v. 2.0.5)
- **Stratagene Mx3000P, Mx3005P** - Agilent Technologies (software v.4.10 build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

Clinical specificity

In order to evaluate the Kit specificity DNA samples isolated from FFPE tumor tissue have been tested. Samples were suitable in terms of starting DNA amount and for the presence of mutations detected by the Kit, and have been already genotyped through pyrosequencing technology (kit “**Anti-EGFR MoAb response® (BRAF status)**”, cod. UP033 Diatech Pharmacogenetics), or with Mass Spectrometry using MassArray® platform (“**Myriapod® Colon Status**” cod. SQ010, “**Myriapod® Lung Status**” cod. SQ011, “**Myriapod® Cancer Status**” cod. SQ020 Diatech Pharmacogenetics), or with direct sequencing. If no FFPE samples were available, Horizon Diagnostics standards, cell lines or plasmids have been tested.

	Rotor-Gene		Stratagene Mx3000P, Mx3005P		CFX96		ABI 7300		ABI 7500/ABI 7500 Fast	
Easy® BRAF	N° samples tested	N° samples correctly genotyped	N° samples tested	N° samples correctly genotyped	N° samples tested	N° samples correctly genotyped	N° samples tested	N° samples correctly genotyped	N° samples tested	N° samples correctly genotyped
BRAF V600E	10	10/10	14	14/14	10	10/10	13/13	13/13	2	2/2
BRAF V600K	2	2/2	1	1/1	1	1/1	*	*	1	1/1
BRAF V600D	*	*	*	*	*	*	*	*	*	*
BRAF V600R	2	2/2	2	2/2	1	1/1	2	2/2	1	1/1
BRAF wild-type	9	9/9	8	8/8	8	8/8	9	9/9	29	29/29
Totale	23	23/23	25	25/25	20	20/20	24/24	24/24	33	33/33

* FFPE sample not available: Horizon Diagnostics standard, cell lines or plasmids have been tested.

Limit of detection (LOD)

The LOD of the Kit is 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 tests.

To determine the LOD, samples with different percentage of mutation and a medium input DNA concentration have been tested. For each real-time instrument (Rotor-Gene, Mx3000P/Mx3005P, ABI 7300, ABI 7500, ABI 7500 Fast CFX96) three independent experiments have been performed. In each experiment mutated samples have been tested in duplicates. If available Horizon Diagnostics standards have been tested to determinate the LOD, otherwise cell lines or plasmids have been used.

The LOD of “**Easy® BRAF**” Kit, considering all the instruments tested is:

Assay	LOD C ₉₅ (at medium DNA input concentration)
V600E	1%
V600K	0.5-1%
V600D	1%
V600R	0.5%

Reproducibility

System reproducibility (*inter-assay* variability) has been evaluated analyzing the data deriving from three independent runs with standard DNA samples. Results were reproducible in terms of genotyping for all assays and samples analyzed.

Repeatability

System repeatability (*intra-assay* variability) has been evaluated analyzing the data deriving from three independent runs with standard DNA samples. Results were reproducible in terms of genotyping for all assays and samples analyzed.

Robustness

Lot to lot consistency

Different batches of Taq PreMix have been tested with the same DNA samples. Results from the different lots are comparable. Two different batches of primers and probes have been tested with the same DNA samples. Results from the different lots are comparable.

APPENDIX A -RT002 sample grid A – Easy® BRAF

DATE						RUN NAME							
	1	2	3	4	5	6	7	8	9	10	11	12	
A													V600E mix (1)
B													V600K mix (2)
C													V600D mix (3)
D													V600R mix (4)
E													ctrl mix (5)
F													
G													
H													

INSTRUMENT n.						USER MANUAL version			
CODE		LOT				CODE			
NOTES									
OPERATOR						SIGN			

APPENDIX B - RT002 sample grid B – Easy® BRAF

DATE		RUN NAME	
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1	•	9	•	17	•	25	•	33	•	41	•	49	•	57	•	65	•
2	•	10	•	18	•	26	•	34	•	42	•	50	•	58	•	66	•
3	•	11	•	19	•	27	•	35	•	43	•	51	•	59	•	67	•
4	•	12	•	20	•	28	•	36	•	44	•	52	•	60	•	68	•
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	•

V600E mix (1)
V600K mix (2)
V600D mix (3)
V600R mix (4)
ctrl mix (5)

INSTRUMENT n.		USER MANUAL version	
CODE		LOT	
NOTES			
OPERATOR		SIGN	

OPERATOR NOTES

[illegible]

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