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Easy® THYROID

User manual – version 2017/02

The "**Easy® THYROID**" kit detects mutations of KRAS, NRAS, HRAS codons 12-13-61 and BRAF codons 600-601 by Real-Time PCR.

For *in vitro* diagnostic use

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INTENDED USE

The *in vitro* diagnostic "**Easy® THYROID**" kit is intended for the qualitative detection by Real-Time PCR of *HRAS, NRAS, KRAS* and *BRAF* somatic mutations in the genomic DNA isolated from thyroid cytology specimens and fresh, frozen or formalin fixed paraffin-embedded (FFPE) thyroid tumor tissue.

The "**Easy® THYROID**" 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 (con 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 601 (not distinguishable between them) ▪ K601E (1801A>G) ▪ V600_K601>E (1799_1801delTGA)
BRAF codon 600 (not distinguishable between them) ▪ V600E (1799T>A) ▪ V600Ecomplex (1799_1800TG>AA)
KRAS codons 12-13 (not distinguishable between them) ▪ G12C (34G>T) ▪ G12S (34G>A) ▪ G12R (34G>C) ▪ G12A (35G>C) ▪ G12D (35G>A) ▪ G12V (35G>T) ▪ G13D (38G>A)
KRAS codon 61 (not distinguishable between them) ▪ Q61K (181C>A) ▪ Q61R (182A>G) ▪ Q61L (182A>T) ▪ Q61H (183A>C) ▪ Q61H (183A>T)
NRAS codons 12-13 (not distinguishable between them) ▪ G12S (34G>A) ▪ G12C (34G>T) ▪ G12A (35G>C) ▪ G12D (35G>A) ▪ G12V (35G>T) ▪ G13R (37G>C) ▪ G13D (38G>A) ▪ G13V (38G>T)
NRAS codon 61 (not distinguishable between them) ▪ Q61K (181C>A) ▪ Q61R (182A>G) ▪ Q61L (182A>T) ▪ Q61H (183A>C) ▪ Q61H (183A>T)
HRAS codons 12-13 (not distinguishable between them) ▪ G12S (34G>A) ▪ G12C (34G>T) ▪ G12D (35G>A) ▪ G12V (35G>T) ▪ G13R (37G>C)
HRAS codon 61 (not distinguishable between them) ▪ Q61K (181C>A) ▪ Q61R (182A>G) ▪ Q61L (182A>T)

PRINCIPLE OF THE ASSAY

The "**Easy® THYROID**" 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® THYROID**" Kit is composed of 8 assays for the detection of the mutations. 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 quality and quantity of DNA, the amplification procedure and the possible presence of inhibitors, which may cause false negative results.

1. **BRAF K601E/V600_K601>E mix**: the assay detects the K601E (1801A>G), V600_K601>E (1799_1801delTGA) mutations but does not distinguish between them.
2. **BRAF V600E/Ec mix**: the assay detects the V600E (1799T>A), V600Ecomplex (1799_1800TG>AA) mutations but does not distinguish between them.
3. **KRAS G12x-G13D mix**: the assay detects the G13D (38G>A), G12A (35G>C), G12D (35G>A), G12V (35G>T), G12C (34G>T), G12S (34G>A), G12R (34G>C) mutations but does not distinguish between them.
4. **KRAS Q61x mix**: the assay detects the Q61H (183A>C), Q61H (183A>T), Q61K (181C>A), Q61R (182A>G), Q61L (182A>T) mutations but does not distinguish between them.
5. **NRAS G12x-G13x mix**: the assay detects the G12S (34G>A), G12C (34G>T), G12A (35G>C), G12D (35G>A), G12V (35G>T), G13R (37G>C), G13D (38G>A), G13V (38G>T) mutations but does not distinguish between them.
6. **NRAS Q61x mix**: the assay detects the Q61K (181C>A)**,** Q61R (182A>G)**,** Q61L (182A>T), Q61H (183A>C), Q61H (183A>T) mutations but does not distinguish between them.
7. **HRAS G12x-G13R mix**: the assay detects the G12S (34G>A), G12C (34G>T), G12D (35G>A), G12V (35G>T), G13R (37G>C) mutations but does not distinguish between them.
8. **HRAS Q61x mix**: the assay detects the Q61K (181C>A), Q61R (182A>G), Q61L (182A>T) mutations but does not distinguish between them.

The "**Easy® THYROID**" 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.

CLINICAL RELEVANCE

Thyroid cancer is the most common endocrine malignancy and typically originates in thyroid nodules that have a frequency of 19-68% in the adult population, in particular women. The majority of the thyroid nodules are benign and only a minority of them (5-15%) harbor malignancy. Fine needle aspiration (FNA) cytological analysis is the primary diagnostic intervention to classify benign or malignant nodules. Unfortunately nodule cytology remains indeterminate in nearly 25% of cases. Patients with indeterminate nodules are commonly referred for consideration of lobectomy or near-total thyroidectomy, however only the 10-40% of resected nodules are confirmed as malignant in subsequent histology. From the clinical point of view, the development of new diagnostics strategies that allow a more accurate classification of indeterminate nodules is crucial for a better estimation of the individual's thyroid cancer risk in order to reduce the rate of unnecessary surgical procedures and associated complications[1,6]. Many studies have demonstrated that the mutation analysis of BRAF, NRAS, KRAS and HRAS genes together with the detection of RET/PTC1, RET/PTC3 and PAX8/PPARγ rearrangements can be a valuable diagnostic tool for indeterminate nodules classification. The presence of these mutations or gene rearrangements is strongly correlated with malignancy. BRAF mutations, RET translocations, RAS mutation and PAX8/PPARγ fusions are present in the 45%, 20%, 40% and 35% of papillary thyroid tumors respectively. In particular the BRAF V600E mutation, RET/PTC and PAX8/PPARγ rearrangements are correlated with malignancy in 100% of cases, whereas RAS mutations are associated to a risk of 75-85%[1,2,3,4,7]. RAS and BRAF mutations are mutually exclusive in thyroid cancer and PAX8-PPAG fusions do not occur concomitantly with RAS mutations in papillary cancer[5].
BRAF somatic mutations are present in 40-45% of papillary, 30-40% of anaplastic and 20-30% of poorly differentiated thyroid cancers. The vast majority (98%) of BRAF mutations are V600E, but the K601E mutation has also been reported.
NRAS, HRAS and KRAS mutations are present in 6%, 4% and 3% of thyroid cancers. Among the RAS genes, mutations at codon 61 are the most common[5].

References:

1. Nikiforov YE et al. *Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples*. J.Clinical Endocrininol Metab, 96:3390-3397, 2011.
2. Tae Sook Hwang et al. *Preoperative RAS mutational analysis is of great value in predicting follicular variant of papillary thyroid carcinoma*. Biomed Research International, 2015.
3. Sang Ryung Lee et al. *Molecular genotyping of follicular variant of papillary thyroid carcinoma correlates with diagnostic category of fine-needle aspiration cytology: values of RAS mutation testing*. Thyroid vol.23, number 11, 2013.
4. Radkay L.A. *Thyroid nodules with KRAS mutations are different from nodules with NRAS and HRAS mutations with regard to cytopathologic and histopatologic outcome characteristics*. Cancer Cytophatolog, December 2014.
5. My Cancer Genome. Thyroid cancer. www.mycancergenome.org.
6. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer.
7. Michiya Nishino. *Molecular cytopathology for thyroid nodules: a review of methodology and test performance*. Cancer cytophatology, January 2016.

KIT CONTENTS

The kit contains sufficient reagents to carry out 24 tests for each assay for a maximum of 3 runs (6 samples and 2 controls per run).

COMP	QUAN	Color	
BRAF K601E/V600_K601>E mix (1)	3 x 20 µl	BLUE	Mixture of specific primers and probes targeting both BRAF K601E, V600_K601>E mutations and the internal control.
BRAF V600E/Ec mix (2)	3 x 20 µl	GREEN	Mixture of specific primers and probes targeting both BRAF V600E e V600Ecomplex mutations and the internal control.
KRAS G12x-G13D mix (3)	3 x 20 µl	RED	Mixture of specific primers and probes targeting both KRAS G13D , G12A , G12D , G12V , G12C , G12S , G12R mutations and the internal control.
KRAS Q61x mix (4)	3 x 20 µl	YELLOW	Mixture of specific primers and probes targeting both KRAS Q61H (183A>C), Q61H (183A>T), Q61K, Q61R, Q61L mutations and the internal control.
NRAS G12x-G13x mix (5)	3 x 20 µl	ORANGE	Mixture of specific primers and probes targeting both NRAS G12S, G12C, G12A, G12D, G12V, G13R, G13D, G13V mutations and the internal control.
NRAS Q61x mix (6)	3 x 20 µl	BLACK	Mixture of specific primers and probes targeting both NRAS Q61H (183A>C), Q61H (183A>T), Q61K, Q61R, Q61L mutations and the internal control.
HRAS G12x-G13R mix (7)	3 x 20 µl	WHITE	Mixture of specific primers and probes targeting both HRAS G12S, G12C, G12D, G12V, G13R and the internal control.
HRAS Q61x mix (8)	3 x 20 µl	BROWN	Mixture of specific primers and probes targeting both HRAS Q61K, Q61R, Q61L and the internal control.
Easy THYROID pos ctrl CONTROL +	3 x 150 µl		Positive control DNA containing a mixture of synthetic DNA sequences that correspond to 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 to be use exclusively for the preparation of the PCR mix and as negative control.
Water (diluent)	1 x 1.5 ml		DNase-, RNase-free water to be use exclusively as samples diluent.
Taq Premix 920	3 x 920 µl		Solution containing hot start Taq DNA polymerase, reaction buffer, Mg2+ and dNTP Mixture.
Dye R-I	3 x 40 µl		Inert fluorophore to be used for the amplification on the ABI 7300 instrument.
Dye R-II	3 x 40 µl		Inert fluorophore to be used for the amplification on the ABI 7500/7500 Fast instrument.
8-strip tubes & caps	1 x 5 unità		0.2 ml 8-tube strip DNase-, RNase-free to be used for the preparation of the reaction mixture.

DOCUMENTS AVAILABLE ON-LINE

Easy® THYROID 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® THYROID**" 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:
    - Xilene (e.g:. "Xylenes, histological grade" – cod. 534056, Sigma Aldrich)
    - Absolute Ethanol (quality of analytical degree)

ⓘ 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, 7500 Fast** - Applied Biosystems (con 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 7300/7500: 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

REF	Catalog number (product code)	CONTROL -	Negative control
LOT	Lot number		Consult the instruction
	Content sufficient for <n> tests		User manual (handbook)
IVD	For *in vitro* diagnostic use		Expiration date
CONT	Content		Storage conditions
COMP	Components		Manufactured by
NUM	Number of aliquots		Important Note
QUAN	Quantity per aliquot	GTIN	Global Trade Item Number
CONTROL +	Positive control		

PRODUCT USE LIMITATIONS

- The "**Easy® THYROID**" 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® THYROID**" kit is designed to be used with the instruments "**Rotor-Gene Q**" (Qiagen), "**Rotor-Gene™ 6000**" (Corbett Research), **CFX96** (Bio-Rad), **ABI 7300/7500/7500 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 speciments, 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.

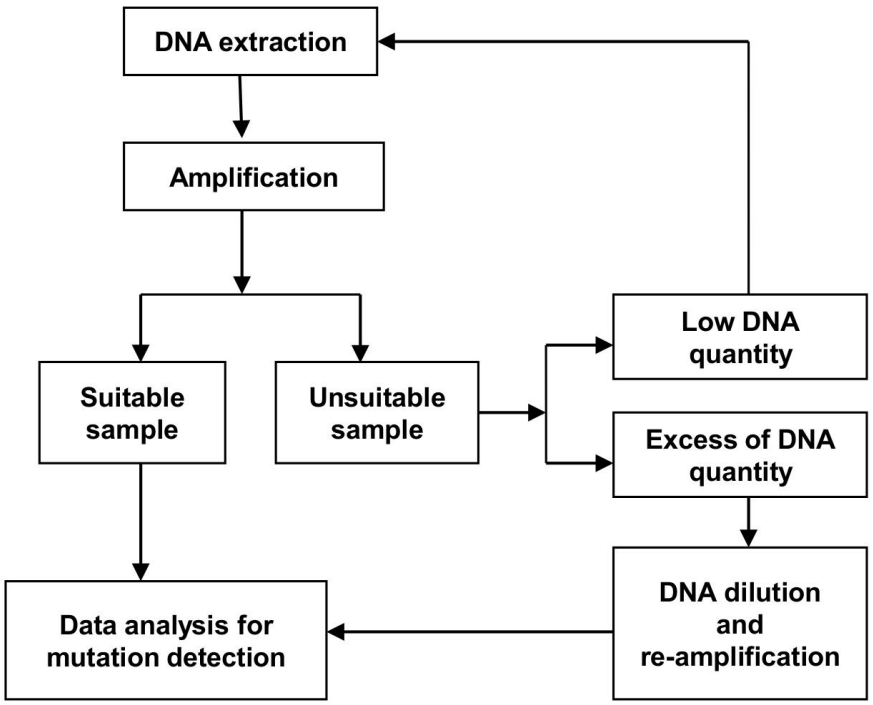
QUALITY CONTROL

- The "**Easy® THYROID**" Kit was designed, developed and validated in accordance with D.Lgs n. 332 of 08/09/2000 ("Implementation of Directive 98/79 / EC on in vitro diagnostic medical devices") and subsequent legislative changes.
- In accordance with the company system of full quality assurance (certified according to European standards EN ISO 9001 and ISO 13485), to ensure consistent product quality, each batch of "**Easy® THYROID**" is subjected to functional quality control according to technical specifications and is released only if it is compliant with the quality control criteria.

WARNINGS AND PRECAUTIONS

- The kit may only be used by specialist personnel, properly instructed and trained to perform *in vitro* laboratory techniques.
- Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
- 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).
- 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.
- 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.
- The Material Safety Data Sheet (MSDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com.
- Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
- It is recommended to ensure that the laboratory workflow proceeds in a unidirectional manner, preparing, if possible, two separate work areas for:
  - extraction of nucleic acids;
  - amplification reaction;
- Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
- Use sterile filter tips. Avoid aerosols.
- Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
- During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by only opening the reagents within the hood.
- 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.
- The precision pipettes used should have an accuracy within 3% of the set volume.
- Periodically check the calibration status of the dispensing instruments.
- Do not use reagents after the expiration date shown on each container.
- All reagents supplied in the "**Easy® THYROID**" kit are intended to be used solely with the other reagents in the same "**Easy® THYROID**" kit. Do not substitute or mix reagents in the kit from different batches, in order to maintain optimal performance.
- 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.
- Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
- Extraction 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 (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.
- 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**" – cod. DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
- Avoid contamination of samples and reagents.
- Store reagents and samples separately.
- In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.
- Before use all reagents need to be thawed at room temperature, mixed by inverting 10 times and centrifuged briefly.
- 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.
- Include in each run at least 1 negative control (**WATER**) and 1 positive control (**Easy THYROID pos ctrl**).
- 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.
- Carefully read this User Manual.
- Check that the version of this User Manual corresponds to the one described on the "**Easy® THYROID**" kit box label.
- 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.
- 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

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

- The "**Easy® THYROID**" 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 the biological material.
- 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 the inhibition of the reaction by ethanol.
- After the extraction, proceed immediately with the quali-quantitative evaluation of the DNA and the amplification reaction, or store the extracted DNA at ≤-20°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 FNA, 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 15-30 ng.

ⓘ 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 internal control amplification (HEX channel). DNA assessment based on the internal control may differ from spectrophotometric quantification.

ⓘ All assays in the "**Easy® THYROID**" kit amplify short DNA sequences. However heavily fragmented DNA can generate no amplification product.

AMPLIFICATION

General recommendations for amplification *(valid for all instruments)*

ⓘ Perform this step in the area dedicated to amplification mixes preparation, using dedicated materials and instruments. Before starting decontaminate pipettes, benches and hood 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 wells of the plate to use.
- Each run must include at least one amplification negative control (**WATER**) and one amplification positive control (**Easy THYROID 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 8
- "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": 5 **NRAS G12x-G13x mix (5)**, "Target Sample Range" 20-30 Fl
- "Yellow 2": source 530 nm – detector 555 nm – "Gain Optimisation" 58°C Before 1st acquisition, "Tube Position": 5 **NRAS G12x-G13x mix (5)**, "Target Sample Range" 20-30 Fl

Thermal Profile	
Hold	95°C for 2 minutes
40 cycles	95°C for 10 seconds / 58°C for 60 seconds (acquire fluorescense 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 channels 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.

ⓘ Select the options "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 channels 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 7500/7500 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 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.

MUTATION DETECTION

ⓘ Each sample must be amplified with 8 different mixes: **BRAF K601E/V600_K601>E mix (1)**, **BRAF V600E/Ec mix (2)**, **KRAS G12x-G13D mix (3)**, **KRAS Q61x mix (4)**, **NRAS G12x-G13x mix (5)**, **NRAS Q61x mix (6)**, **HRAS G12x-G13R mix (7)**, **HRAS Q61x mix (8)**

ⓘ The DNA reference standard **Horizon BRAF V600E 1%** must be amplified with the mix **BRAF V600E/Ec mix (2)** only.

ⓘ The kit content is optimized to analyze six clinical samples and two controls (**Easy THYROID pos ctrl** e **WATER**) in each run.

Mx3000P/3005P, ABI 7300/7500/7500 Fast, CFX96 (Easy® THYROID sample grid A)

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

1	BRAF K601E/V600_K601>E mix (1)
2	BRAF V600E/Ec mix (2)
3	KRAS G12x-G13D mix (3)
4	KRAS Q61x mix (4)
5	NRAS G12x-G13x mix (5)
6	NRAS Q61x mix (6)
7	HRAS G12x-G13R mix (7)
8	HRAS Q61x mix (8)

Rotor-Gene (Easy® THYROID sample grid B)

WATER		POS CTRL		DNA1		DNA2		DNA3		DNA4		DNA5		DNA6			
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	•

- Prepare, for each sample and control, 8 different amplification mixtures (**Amp-Mix**), one for each assay, as indicated in the following scheme, where N is the numer of samples and controls 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 the 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 -	3	
BRAF K601E/V600_K601>E mix (1) o **BRAF V600E/Ec mix (2)** o **KRAS G12x-G13D mix (3)** o **KRAS Q61x mix (4)** o **NRAS G12x-G13x mix (5)** o **NRAS Q61x mix (6)** o **HRAS G12x-G13R mix (7)** o **HRAS Q61x mix (8)**	2	
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 -	2.6	
BRAF K601E/V600_K601>E mix (1) o **BRAF V600E/Ec mix (2)** o **KRAS G12x-G13D mix (3)** o **KRAS Q61x mix (4)** o **NRAS G12x-G13x mix (5)** o **NRAS Q61x mix (6)** o **HRAS G12x-G13R mix (7)** o **HRAS Q61x mix (8)**	2	
Total Volume	15	

ABI 7500/7500 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 -	2.8	
BRAF K601E/V600_K601>E mix (1) o **BRAF V600E/Ec mix (2)** o **KRAS G12x-G13D mix (3)** o **KRAS Q61x mix (4)** o **NRAS G12x-G13x mix (5)** o **NRAS Q61x mix (6)** o **HRAS G12x-G13R mix (7)** o **HRAS Q61x mix (8)**	2	
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:

negative control	5 µl **WATER**	CONTROL -
sample	5 µl DNA	
positive control	5 µl **Easy THYROID pos ctrl**	CONTROL +

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

ⓘ 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).

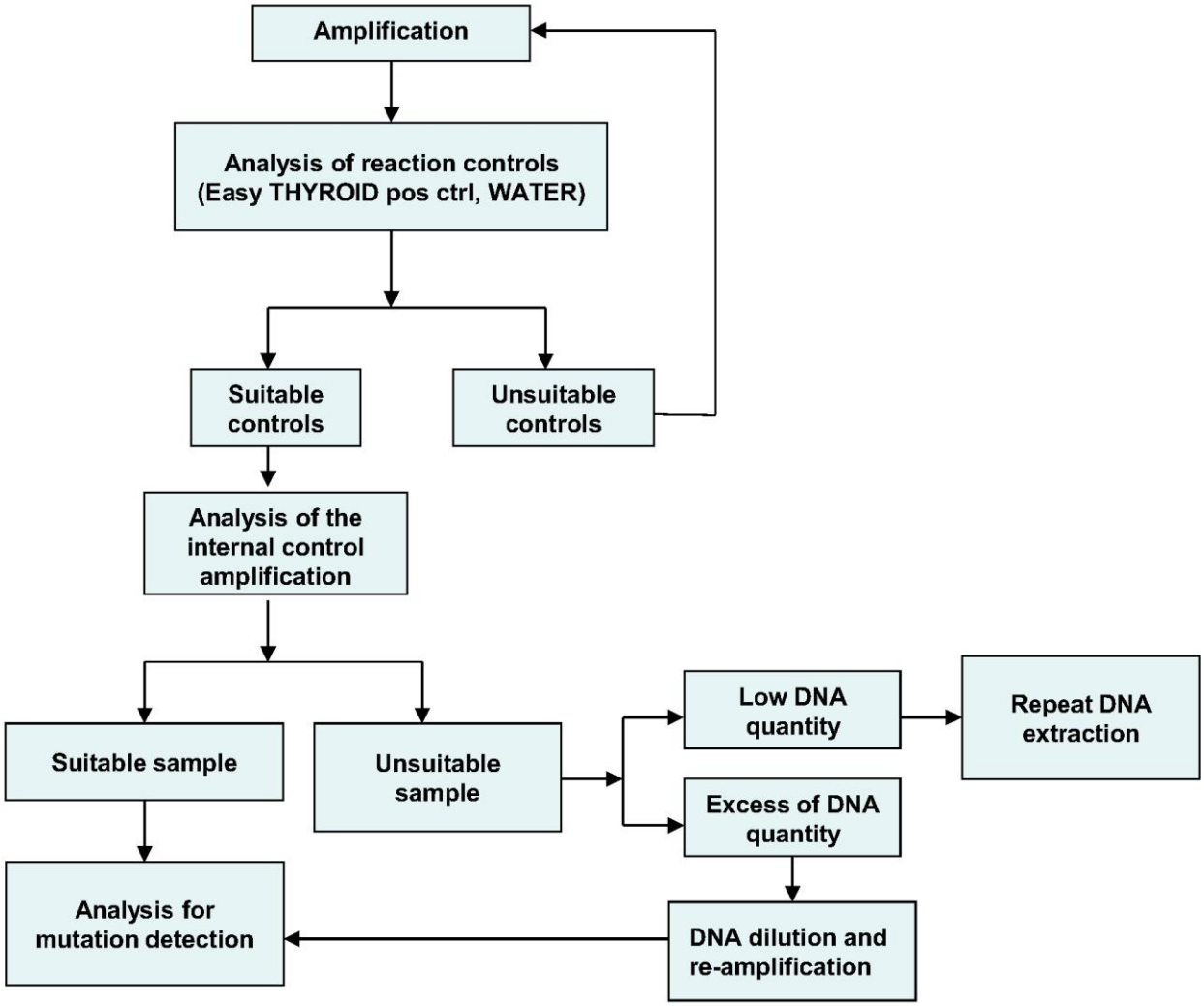
ⓘ Proceed with the analysis following the instructions of the section “Data analysis”.

DATA ANALYSIS

General reccomandations pertain to all the instruments

- ⓘ Analyze first the negative control **WATER** and the positive control **Easy THYROID 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.
- ⓘ 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.

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.

ⓘ 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

	Assay	Green	Yellow	Results
WATER	All mixes	Ct > 32	Ct > 32	Proceed with analysis of the samples.
		Ct ≤ 32	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy THYROID pos ctrl	BRAF K601E/V600_K601>E	27 ≤ Ct ≤ 31	17 ≤ Ct ≤ 23	Proceed with analysis of the samples.
	BRAF V600E/Ec	25 ≤ Ct ≤ 29		
	KRAS G12x-G13D	25 ≤ Ct ≤ 29		
	KRAS Q61x	25 ≤ Ct ≤ 29		
	NRAS G12x-G13x	27 ≤ Ct ≤ 31		
	NRAS Q61x	25 ≤ Ct ≤ 29		
	HRAS G12x-G13R	25 ≤ Ct ≤ 29		
	HRAS Q61x	24 ≤ Ct ≤ 28		
	BRAF K601E/V600_K601>E	Ct < 27 o Ct > 31	Ct < 17 o Ct >23	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	BRAF V600E/Ec	Ct < 25 o Ct > 29		
	KRAS G12x-G13D	Ct < 25 o Ct > 29		
	KRAS Q61x	Ct < 25 o Ct > 29		
	NRAS G12x-G13x	Ct < 27 o Ct > 31		
	NRAS Q61x	Ct < 25 o Ct > 29		
	HRAS G12x-G13R	Ct < 25 o Ct > 29		
	HRAS Q61x	Ct < 24 o Ct > 28		

2. Analysis of the internal control for the DNA assessment

	Yellow	Results
DNA samples	17 ≤ Ct ≤ 29	Suitable sample. Proceed with the mutation analysis.
	Ct < 17	Excess of DNA. Samples must be diluted with **Water (diluent)** 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 > 29	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 (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target DNA.

3. Analysis of the mutation assays

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

- For each sample compare the Ct values of the Green and Yellow channels with those reported in the following table. If the Ct of the Green channel is below the corresponding cut-off value, the presence of the mutation is confirmed using the ΔΔCt method as follows:

  - Calculate the ΔCt values for both the sample and the **Easy THYROID pos ctrl**, taking care that the Ct value in Green for the mutation and the equivalent Ct value in Yellow belong to the same sample:

  ΔCt (Sample) = Ct Green (Sample) – Ct Yellow (Sample)

  ΔCt (**Easy THYROID pos ctrl**) = Ct Green (**Easy THYROID pos ctrl**) – Ct Yellow (**Easy THYROID pos ctrl**)

  - Calculate the ΔΔCt value using the formula:

  ΔΔCt = ΔCt (Sample) – ΔCt (**Easy THYROID pos ctrl**)

  - Compare the obtained ΔΔCt value with those reported in the table.

Assay	Green	Yellow	ΔΔCt	Results
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	≤ 0.0	BRAF K601E/V600_K601>E positive
BRAF V600E/Ec			≤ 3.5	BRAF V600E/Ec positive
KRAS G12x-G13D			≤ 3.0	KRAS G12x-G13D positive
KRAS Q61x			≤ 4.0	KRAS Q61x positive
NRAS G12x-G13x			≤ 1.0	NRAS G12x-G13x positive
NRAS Q61x			≤ 2.5	NRAS Q61x positive
HRAS G12x-G13R	Ct < 33		≤ 2.0	HRAS G12x-G13R positive
HRAS Q61x			≤ 1.0	HRAS Q61x positive
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	> 0.0	Wild-type sample or beneath the LOD[1]
BRAF V600E/Ec			> 3.5	
KRAS G12x-G13D			> 3.0	
KRAS Q61x			> 4.0	
NRAS G12x-G13x			> 1.0	
NRAS Q61x			> 2.5	
HRAS G12x-G13R	Ct < 33		> 2.0	
HRAS Q61x			> 1.0	
BRAF K601E/V600_K601>E	No Ct o Ct ≥ 35	OK (similar Ct value for all the mixes)	\	
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R	No Ct o Ct ≥ 33		\	
HRAS Q61x			\	
BRAF K601E/V600_K601>E	Any value	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x			\	

Notes
1. LOD = Limit Of Detection
\ ΔΔCt calculation not required

ⓘ If multiple assays show a ΔΔCt equal to or below the cut-off value, the signal giving the higher ΔΔCt 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 ΔΔCt.

Stratagene Mx3000P, Mx3005P

- At the end of the run click Set-up, Plate Setup, and enter the sample names.
- In the section Analysis, Analysis Selection/Setup, deselect the function Amplification-based threshold and select only the reactions controls **WATER** and **Easy THYROID pos ctrl**.
- In the section Analysis, Results, click on the lock icon 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 all the samples.
- In the section Analysis, Results, select Text report to see the results in both channels with their respective threshold and Ct values.
- Click save.

1. Analysis of the reaction controls

	Assay	FAM	HEX	Results
WATER	All mixes	Ct > 32	Ct > 32	Proceed with analysis of the samples.
		Ct ≤ 32	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy THYROID pos ctrl	BRAF K601E/V600_K601>E	28 ≤ Ct ≤ 32	17 ≤ Ct ≤ 23	Proceed with analysis of the samples.
	BRAF V600E/Ec	27 ≤ Ct ≤ 31		
	KRAS G12x-G13D	25 ≤ Ct ≤ 31		
	KRAS Q61x	25 ≤ Ct ≤ 30		
	NRAS G12x-G13x	26 ≤ Ct ≤ 31		
	NRAS Q61x	25 ≤ Ct ≤ 29		
	HRAS G12x-G13R	25 ≤ Ct ≤ 30		
	HRAS Q61x	24 ≤ Ct ≤ 29		
	BRAF K601E/V600_K601>E	Ct < 28 o Ct > 32	Ct < 17 o Ct >23	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	BRAF V600E/Ec	Ct < 27 o Ct > 31		
	KRAS G12x-G13D	Ct < 25 o Ct > 31		
	KRAS Q61x	Ct < 25 o Ct > 30		
	NRAS G12x-G13x	Ct < 26 o Ct > 31		
	NRAS Q61x	Ct < 25 o Ct > 29		
	HRAS G12x-G13R	Ct < 25 o Ct > 30		
	HRAS Q61x	Ct < 24 o Ct > 29		

2. Analysis of the internal control for the DNA assessment

	HEX	Results
DNA samples	17 ≤ Ct ≤ 30	Suitable sample. Proceed with the mutation analysis.
	Ct < 17	Excess of DNA. Samples must be diluted with **Water (diluent)** 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 > 30	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 (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target DNA.

3. Analysis of the mutation assays

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

- For each sample compare the Ct values of the FAM and HEX channels with those reported in the following table. If the Ct of the FAM channel is below the corresponding cut-off value, the presence of the mutation is confirmed using the ΔΔCt method as follows:

  - Calculate the ΔCt values for both the sample and the **Easy THYROID pos ctrl**, taking care that the Ct value in FAM for the mutation and the equivalent Ct value in HEX belong to the same sample:

  ΔCt (Sample) = Ct FAM (Sample) – Ct HEX (Sample)

  ΔCt (**Easy THYROID pos ctrl**) = Ct FAM (**Easy THYROID pos ctrl**) – Ct HEX (**Easy THYROID pos ctrl**)

  - Calculate the ΔΔCt value using the formula:

  ΔΔCt = ΔCt (Sample) – ΔCt (**Easy THYROID pos ctrl**)

  - Compare the obtained ΔΔCt value with those reported in the table.

Assay	**FAM**	**HEX**	**ΔΔCt**	**Results**
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	≤ 2.5	BRAF K601E/V600_K601>E positive
BRAF V600E/Ec			≤ 3.0	BRAF V600E/Ec positive
KRAS G12x-G13D			≤ 3.5	KRAS G12x-G13D positive
KRAS Q61x			≤ 5.0	KRAS Q61x positive
NRAS G12x-G13x			≤ 2.5	NRAS G12x-G13x positive
NRAS Q61x			≤ 3.5	NRAS Q61x positive
HRAS G12x-G13R			≤ 4.0	HRAS G12x-G13R positive
HRAS Q61x			≤ 2.5	HRAS Q61x positive
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	> 2.5	Wild-type sample or beneath the LOD[1]
BRAF V600E/Ec			> 3.0	
KRAS G12x-G13D			> 3.5	
KRAS Q61x			> 5.0	
NRAS G12x-G13x			> 2.5	
NRAS Q61x			> 3.5	
HRAS G12x-G13R			> 4.0	
HRAS Q61x			> 2.5	
BRAF K601E/V600_K601>E	No Ct o Ct ≥ 35	OK (similar Ct value for all the mixes)	\	
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x			\	
BRAF K601E/V600_K601>E	Any value	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x			\	

Notes
1. LOD = Limit Of Detection
\ ΔΔCt calculation not required

ⓘ If multiple assays show a ΔΔCt equal to or below the cut-off value, the signal giving the higher ΔΔCt 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 ΔΔCt.

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 samples name.
- 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

ⓘ Always check the automatic threshold values for the FAM and HEX channels. If the FAM or HEX threshold value is below 10, open the section Settings – Baseline Threshold and set the threshold value manually by changing the Single Threshold setting from Auto Calculated to User defined.

- In the page Quantification data, the results are displayed for both channels with the corresponding values of Cq.
- Click Save.

1. Analysis of the reaction controls

	Assay	FAM	HEX	Results
WATER	All mixes	Cq > 32	Cq > 32	Proceed with analysis of the samples.
		Cq ≤ 32	Cq ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy THYROID pos ctrl	BRAF K601E/V600_K601>E	28 ≤ Cq ≤ 32	18 ≤ Cq ≤ 23	Proceed with analysis of the samples.
	BRAF V600E/Ec	25 ≤ Cq ≤ 30		
	KRAS G12x-G13D	24 ≤ Cq ≤ 30		
	KRAS Q61x	24 ≤ Cq ≤ 29		
	NRAS G12x-G13x	25 ≤ Cq ≤ 30		
	NRAS Q61x	24 ≤ Cq ≤ 29		
	HRAS G12x-G13R	24 ≤ Cq ≤ 29		
	HRAS Q61x	24 ≤ Cq ≤ 29		
	BRAF K601E/V600_K601>E	Cq < 28 o Cq > 32	Cq < 18 o Cq >23	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	BRAF V600E/Ec	Cq < 25 o Cq > 30		
	KRAS G12x-G13D	Cq < 24 o Cq > 30		
	KRAS Q61x	Cq < 24 o Cq > 29		
	NRAS G12x-G13x	Cq < 25 o Cq > 30		
	NRAS Q61x	Cq < 24 o Cq > 29		
	HRAS G12x-G13R	Cq < 24 o Cq > 29		
	HRAS Q61x	Cq < 24 o Cq > 29		

2. Analysis of the internal control for the DNA assessment

	HEX	Results
DNA samples	18 ≤ Cq ≤ 31	Suitable sample. Proceed with the mutation analysis.
	Cq < 18	Excess of DNA. Samples must be diluted with **Water (diluent)** so that Cq fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Cq of 1 unit.
	Cq > 31	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 (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target DNA.

3. Analysis of the mutation assays

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

- For each sample compare the Cq values of the FAM and HEX channels with those reported in the following table. If the Cq of the FAM channel is below the corresponding cut-off value, the presence of the mutation is confirmed using the ΔΔCq method as follows:

  - Calculate the ΔCq values for both the sample and the **Easy THYROID pos ctrl**, taking care that the Cq value in FAM for the mutation and the equivalent Cq value in HEX belong to the same sample:

  ΔCq (Sample) = Cq FAM (Sample) – Cq HEX (Sample)

  ΔCq (**Easy THYROID pos ctrl**) = Cq FAM (**Easy THYROID pos ctrl**) – Cq HEX (**Easy THYROID pos ctrl**)

  - Calculate the ΔΔCq value using the formula:

  ΔΔCq = ΔCq (Sample) – ΔCq (**Easy THYROID pos ctrl**)

  - Compare the obtained ΔΔCq value with those reported in the table.

Assay	**FAM**	**HEX**	**ΔΔCq**	**Results**
BRAF K601E/V600_K601>E	Cq < 35	OK (similar Cq value for all the mixes)	≤ 2.0	BRAF K601E/V600_K601>E positive
BRAF V600E/Ec			≤ 3.0	BRAF V600E/Ec positive
KRAS G12x-G13D			≤ 2.5	KRAS G12x-G13D positive
KRAS Q61x			≤ 4.5	KRAS Q61x positive
NRAS G12x-G13x			≤ 2.0	NRAS G12x-G13x positive
NRAS Q61x			≤ 3.0	NRAS Q61x positive
HRAS G12x-G13R			≤ 2.5	HRAS G12x-G13R positive
HRAS Q61x	Cq < 33		≤ 1.0	HRAS Q61x positive
BRAF K601E/V600_K601>E	Cq < 35	OK (similar Cq value for all the mixes)	> 2.0	Wild-type sample or beneath the LOD[1]
BRAF V600E/Ec			> 3.0	
KRAS G12x-G13D			> 2.5	
KRAS Q61x			> 4.5	
NRAS G12x-G13x			> 2.0	
NRAS Q61x			> 3.0	
HRAS G12x-G13R			> 2.5	
HRAS Q61x	Cq < 33		> 1.0	
BRAF K601E/V600_K601>E	No Cq o Cq ≥ 35	OK (similar Cq value for all the mixes)	\	
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x	No Cq o Cq ≥ 33		\	
BRAF K601E/V600_K601>E	Any value	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x			\	

Notes
1. LOD = Limit Of Detection
\ ΔΔCq calculation not required

ⓘ If multiple assays show a ΔΔCq equal to or below the cut-off value, the signal giving the higher ΔΔCq 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 ΔΔCq.

ABI 7300

- At the end of the run, click Results - Plate and enter samples name.
- 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 the reaction controls

	Assay	FAM	JOE	Results
WATER	All mixes	Ct > 32	Ct > 32	Proceed with analysis of the samples.
		Ct ≤ 32	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy THYROID pos ctrl	BRAF K601E/V600_K601>E	28 ≤ Ct ≤ 32	19 ≤ Ct ≤ 26	Proceed with analysis of the samples.
	BRAF V600E/Ec	26 ≤ Ct ≤ 30		
	KRAS G12x-G13D	25 ≤ Ct ≤ 30		
	KRAS Q61x	24 ≤ Ct ≤ 28		
	NRAS G12x-G13x	25 ≤ Ct ≤ 29		
	NRAS Q61x	24 ≤ Ct ≤ 28		
	HRAS G12x-G13R	24 ≤ Ct ≤ 28		
	HRAS Q61x	24 ≤ Ct ≤ 28		
	BRAF K601E/V600_K601>E	Ct < 28 o Ct > 32	Ct < 19 o Ct >26	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	BRAF V600E/Ec	Ct < 26 o Ct > 30		
	KRAS G12x-G13D	Ct < 25 o Ct > 30		
	KRAS Q61x	Ct < 24 o Ct > 28		
	NRAS G12x-G13x	Ct < 25 o Ct > 29		
	NRAS Q61x	Ct < 24 o Ct > 28		
	HRAS G12x-G13R	Ct < 24 o Ct > 28		
	HRAS Q61x	Ct < 24 o Ct > 28		

2. Analysis of the internal control for the DNA assessment

	JOE	Results
DNA samples	18 ≤ Ct ≤ 31	Suitable sample. Proceed with the mutation analysis.
	Ct < 18	Excess of DNA. Samples must be diluted with **Water (diluent)** 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 > 31	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 (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target DNA.

3. Analysis of the mutation assays

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

- For each sample compare the Ct values of the FAM and JOE channels with those reported in the following table. If the Ct of the FAM channel is below the corresponding cut-off value, the presence of the mutation is confirmed using the ΔΔCt method as follows:

  - Calculate the ΔCt values for both the sample and the **Easy THYROID pos ctrl**, taking care that the Ct value in FAM for the mutation and the equivalent Ct value in JOE belong to the same sample:

  ΔCt (Sample) = Ct FAM (Sample) – Ct JOE (Sample)

  ΔCt (**Easy THYROID pos ctrl**) = Ct FAM (**Easy THYROID pos ctrl**) – Ct JOE (**Easy THYROID pos ctrl**)

  - Calculate the ΔΔCt value using the formula:

  ΔΔCt = ΔCt (Sample) – ΔCt (**Easy THYROID pos ctrl**)

  - Compare the obtained ΔΔCt value with those reported in the table.

Assay	**FAM**	**JOE**	**ΔΔCt**	**Results**
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	≤ 1.5	BRAF K601E/V600_K601>E positive
BRAF V600E/Ec			≤ 3.5	BRAF V600E/Ec positive
KRAS G12x-G13D			≤ 3.0	KRAS G12x-G13D positive
KRAS Q61x			≤ 5.0	KRAS Q61x positive
NRAS G12x-G13x			≤ 1.0	NRAS G12x-G13x positive
NRAS Q61x			≤ 4.0	NRAS Q61x positive
HRAS G12x-G13R			≤ 2.5	HRAS G12x-G13R positive
HRAS Q61x	Ct < 33		≤ 2.0	HRAS Q61x positive
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	> 1.5	Wild-type sample or beneath the LOD[1]
BRAF V600E/Ec			> 3.5	
KRAS G12x-G13D			> 3.0	
KRAS Q61x			> 5.0	
NRAS G12x-G13x			> 1.0	
NRAS Q61x			> 4.0	
HRAS G12x-G13R			> 2.5	
HRAS Q61x	Ct < 33		> 2.0	
BRAF K601E/V600_K601>E	No Ct o Ct ≥ 35	OK (similar Ct value for all the mixes)	\	
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x	No Ct o Ct ≥ 33		\	
BRAF K601E/V600_K601>E	Any value	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x			\	

Notes
1. LOD = Limit Of Detection
\ ΔΔCt calculation not required

ⓘ If multiple assays show a ΔΔCt equal to or below the cut-off value, the signal giving the higher ΔΔCt 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 ΔΔCt.

ABI 7500/7500 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 Ct values are visualized for both channels.
- Click Save.

1. Analysis of the reaction controls

	Assay	FAM	JOE	Results
WATER	All mixes	Ct > 32	Ct > 32	Proceed with analysis of the samples.
		Ct ≤ 32	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy THYROID pos ctrl	BRAF K601E/V600_K601>E	28 ≤ Ct ≤ 32	19 ≤ Ct ≤ 24	Proceed with analysis of the samples.
	BRAF V600E/Ec	26 ≤ Ct ≤ 30		
	KRAS G12x-G13D	24 ≤ Ct ≤ 30		
	KRAS Q61x	25 ≤ Ct ≤ 29		
	NRAS G12x-G13x	26 ≤ Ct ≤ 30		
	NRAS Q61x	24 ≤ Ct ≤ 28		
	HRAS G12x-G13R	24 ≤ Ct ≤ 28		
	HRAS Q61x	24 ≤ Ct ≤ 28		
	BRAF K601E/V600_K601>E	Ct < 28 o Ct > 32	Ct < 19 o Ct >24	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	BRAF V600E/Ec	Ct < 26 o Ct > 30		
	KRAS G12x-G13D	Ct < 24 o Ct > 30		
	KRAS Q61x	Ct < 25 o Ct > 29		
	NRAS G12x-G13x	Ct < 26 o Ct > 30		
	NRAS Q61x	Ct < 24 o Ct > 28		
	HRAS G12x-G13R	Ct < 24 o Ct > 28		
	HRAS Q61x	Ct < 24 o Ct > 28		

2. Analysis of the internal control for the DNA assessment

	JOE	Results
DNA samples	19 ≤ Ct ≤ 31	Suitable sample. Proceed with the mutation analysis.
	Ct < 19	Excess of DNA. Samples must be diluted with **Water (diluent)** 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 > 31	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 (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target DNA.

3. Analysis of the mutation assays

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

- For each sample compare the Ct values of the FAM and JOE channels with those reported in the following table. If the Ct of the FAM channel is below the corresponding cut-off value, the presence of the mutation is confirmed using the ΔΔCt method as follows:

  - Calculate the ΔCt values for both the sample and the **Easy THYROID pos ctrl**, taking care that the Ct value in FAM for the mutation and the equivalent Ct value in JOE belong to the same sample:

  ΔCt (Sample) = Ct FAM (Sample) – Ct JOE (Sample)

  ΔCt (**Easy THYROID pos ctrl**) = Ct FAM (**Easy THYROID pos ctrl**) – Ct JOE (**Easy THYROID pos ctrl**)

  - Calculate the ΔΔCt value using the formula:

  ΔΔCt = ΔCt (Sample) – ΔCt (**Easy THYROID pos ctrl**)

  - Compare the obtained ΔΔCt value with those reported in the table.

Assay	**FAM**	**JOE**	**ΔΔCt**	**Results**
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	≤ 1.5	BRAF K601E/V600_K601>E positive
BRAF V600E/Ec			≤ 3.5	BRAF V600E/Ec positive
KRAS G12x-G13D			≤ 3.0	KRAS G12x-G13D positive
KRAS Q61x			≤ 5.0	KRAS Q61x positive
NRAS G12x-G13x			≤ 2.0	NRAS G12x-G13x positive
NRAS Q61x			≤ 5.0	NRAS Q61x positive
HRAS G12x-G13R			≤ 3.0	HRAS G12x-G13R positive
HRAS Q61x	Ct < 33		≤ 3.0	HRAS Q61x positive
BRAF K601E/V600_K601>E	Ct < 35	OK (similar Ct value for all the mixes)	> 1.5	Wild-type sample or beneath the LOD[1]
BRAF V600E/Ec			> 3.5	
KRAS G12x-G13D			> 3.0	
KRAS Q61x			> 5.0	
NRAS G12x-G13x			> 2.0	
NRAS Q61x			> 5.0	
HRAS G12x-G13R			> 3.0	
HRAS Q61x	Ct < 33		> 3.0	
BRAF K601E/V600_K601>E	No Ct o Ct ≥ 35	OK (similar Ct value for all the mixes)	\	
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x	No Ct o Ct ≥ 33		\	
BRAF K601E/V600_K601>E	Any Value	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
BRAF V600E/Ec			\	
KRAS G12x-G13D			\	
KRAS Q61x			\	
NRAS G12x-G13x			\	
NRAS Q61x			\	
HRAS G12x-G13R			\	
HRAS Q61x			\	

Notes
1. LOD = Limit Of Detection
\ ΔΔCt calculation not required

ⓘ If multiple assays show a ΔΔCt equal to or below the cut-off value, the signal giving the higher ΔΔCt 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 ΔΔCt.

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 THYROID 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 expired reagents.
No amplification signal in both "Green/FAM" and "Yellow/HEX" channels for all assays. **Easy THYROID 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 > 5µl DNA (max volume 9µl), 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 (diluent)**.
	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 THYROID pos ctrl**.	Degradation of the **Easy THYROID pos ctrl**.	▪ Repeat the amplification testing a new aliquot of **Easy THYROID pos ctrl**.
	Incorrect or failure in the dispensation of the **Easy THYROID pos ctrl**.	▪ Repeat the amplification by pipetting the appropriate volume of **Easy THYROID 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: ΔCt = Ct Green 2 – Ct Yellow 2.
The positive control **Easy THYROID 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 an amplification signal 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 THYROID pos ctrl** in reaction tubes/wells.
	Incorrect samples names set-up in the software.	▪ Check samples names set-up.
One sample shows “Yellow/HEX” Ct values different from each other for the assays.	DNA dispensation error.	▪ Repeat the amplification paying attention to the dispensation of the DNA in reaction tubes/wells.

The negative control **WATER**, shows an amplification signal in both "Green/FAM" and "Yellow/HEX" 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 reaction 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 7500/7500 Fast** - Applied Biosystems (con 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 FNA or FFPE specimens 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 real-Time PCR ("**Easy® KRAS**", cod. RT001; "**Easy® BRAF**", cod. RT002; "**Easy® NRAS**", cod. RT004, Diatech Pharmacogenetics), or with Mass Spectrometry using MassArray® platform ("**Myriapod® Colon Status**" cod. SQ010, "**Myriapod® Lung Status**" cod. SQ011, Diatech Pharmacogenetics), or with direct sequencing. If no FNA or FFPE samples were available, Horizon Diagnostics standards, cell lines or plasmids have been tested.

	Rotor-Gene		**Stratagene Mx3000P, Mx30005P**		**CFX96**		**ABI 7300**		**ABI 7500/7500 Fast**	
	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 K601E/V600_K601>E	9	9/9	3	3/3	3	3/3	2	2/2	3	3/3
BRAF V600E/Ec	8	8/8	4	4/4	5	5/5	5	5/5	6	6/6
KRAS G12x-G13D	5	5/5	2	2/2	2	2/2	1	1/1	1	1/1
KRAS Q61x	4	4/4	3	3/3	3	3/3	2	2/2	2	2/2
NRAS G12x-G13x	4	4/4	1	1/1	2	2/2	1	1/1	1	1/1
NRAS Q61x	5	5/5	5	5/5	5	5/5	5	5/5	4	4/4
HRAS G12x-G13R	*	*	*	*	*	*	*	*	*	*
HRAS Q61x	11	11/11	3	3/3	4	4/4	7	7/7	5	5/5
Wild-type	8	8/8	6	6/6	6	6/6	7	7/7	7	7/7
Total	54	54/54	27	27/27	30	30/30	30	30/30	29	29/29
* FNA or 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 7500/7500 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 the "**Easy® THYROID**" Kit, considering all the instruments tested is:

Assay	**LOD C_{95} at a medium input DNA concentration**
BRAF K601E/V600_K601>E	1%
BRAF V600E/Ec	1%
KRAS G12x-G13D	2-5%
KRAS Q61x	0.5-2%
NRAS G12x-G13x	2-5%
NRAS Q61x	1-3%
HRAS G12x-G13R	7.5%
HRAS Q61x	7.5%

Reproducibility

System reproducibility (*inter-assay* variability) has been evaluated analyzing Horizon Diagnostics standards, cell lines or plasmids at the concentration of 12.5 ng/reaction. Each sample has been tested in duplicate in at least three independent experiments performed by two different operators in different days. Results, expressed as median ΔΔCt value ± standard deviation, are reproducible in terms of genotyping for all assays and samples analyzed in all instruments.

		Inter-assay variability ($\Delta\Delta Ct_{mean}$ ± SD)				
Sample	**Assay**	**Rotor-Gene 6000/Q**	**Stratagene Mx3000P/3005P**	**CFX96**	**ABI 7300**	**ABI 7500/ 7500 Fast**
BRAF K601E 1%	BRAF K601E/V600_K601>E	-2.4 ± 0.2	-2.4 ± 0.4	-1.4 ± 0.5	-3.0 ± 0.7	-3.0 ± 0.6
BRAF V600E 1%	BRAF V600E/Ec	1.3 ± 0.6	1.2 ± 0.3	0.7 ± 0.3	1.0 ± 0.1	0.6 ± 0.7
KRAS G13D 2%	KRAS G12x-G13D	1.9 ± 0.5	1.8 ± 0.3	0.9 ± 0.4	1.4 ± 0.4	1.5 ± 0.7
KRAS Q61L 2%	KRAS Q61x	1.5 ± 0.2	2.2 ± 0.2	1.6 ± 0.3	1.5 ± 0.2	1.7 ± 0.4
NRAS G12D 2%	NRAS G12x-G13x	0.0 ± 0.5	1.4 ± 0.5	-0.5 ± 0.3	-0.5 ± 0.5	-0.4 ± 0.2
NRAS Q61R 1%	NRAS Q61x	0.9 ± 0.5	1.5 ± 0.4	1.0 ± 0.2	1.7 ± 0.6	1.3 ± 0.5
HRAS G12V 7.5%	HRAS G12x-G13R	1.3 ± 0.3	3.2 ± 0.4	1.2 ± 0.7	0.9 ± 0.6	0.9 ± 0.6
HRAS Q61L 7.5%	HRAS Q61x	0.4 ± 0.3	1.6 ± 0.4	0.3 ± 0.4	0.6 ± 0.7	0.7 ± 0.3

Repeatability

System repeatability (*intra-assay* variability) has been evaluated analyzing Horizon Diagnostics standards, cell lines or plasmids in triplicate at the concentration of 12.5 ng/reaction. Results, expressed as median ΔΔCt value ± standard deviation, are reproducible in terms of genotyping for all assays and samples analyzed in all instruments.

		Intra-assay variability ($\Delta\Delta Ct_{mean}$ ± SD)				
Sample	**Assay**	**Rotor-Gene 6000/Q**	**Stratagene Mx3000P/3005P**	**CFX96**	**ABI 7300**	**ABI 7500/ 7500 Fast**
BRAF K601E 1%	BRAF K601E/V600_K601>E	-2.2 ± 0.1	-2.5 ± 0.1	-1.1 ± 0.1	-3.6 ± 0.3	-3.6 ± 0.1
BRAF V600E 1%	BRAF V600E/Ec	0.8 ± 0.3	1.5 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	1.4 ± 0.3
KRAS G13D 2%	KRAS G12x-G13D	2.1 ± 0.1	1.6 ± 0.2	1.1 ± 0.3	1.6 ± 0.2	2.3 ± 0.3
KRAS Q61L 2%	KRAS Q61x	1.5 ± 0.2	2.1 ± 0.3	1.7 ± 0.1	1.6 ± 0.2	1.6 ± 0.2
NRAS G12D 2%	NRAS G12x-G13x	-0.1 ± 0.1	1.6 ± 0.3	-0.4 ± 0.2	-0.9 ± 0.2	-0.5 ± 0.1
NRAS Q61R 1%	NRAS Q61x	0.6 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	0.6 ± 0.2
HRAS G12V 7.5%	HRAS G12x-G13R	1.1 ± 0.1	3.1 ± 0.4	0.7 ± 0.5	0.3 ± 0.1	0.3 ± 0.1
HRAS Q61L 7.5%	HRAS Q61x	0.1 ± 0.1	1.2 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.5 ± 0.2

Robustness

Lot to lot consistency

Different batches of **Taq Premix 920** 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.

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RT008 sample grid A – Easy® THYROID

DATE		RUN NAME	

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

BRAF K601E/V600_K601>E mix (1)
BRAF V600E/Ec mix (2)
KRAS G12x-G13D mix (3)
KRAS Q61x mix (4)
NRAS G12x-G13x mix (5)
NRAS Q61x mix (6)
HRAS G12x-G13R mix (7)
HRAS Q61x mix (8)

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

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RT008 sample grid B – Easy® THYROID

DATE		RUN NAME	

BRAF K601E/V600_K601>E mix (1)	1 ●	9 ●	17 ●	25 ●	33 ●	41 ●	49 ●	57 ●	65 ●
BRAF V600E/Ec mix (2)	2 ●	10 ●	18 ●	26 ●	34 ●	42 ●	50 ●	58 ●	66 ●
KRAS G12x-G13D mix (3)	3 ●	11 ●	19 ●	27 ●	35 ●	43 ●	51 ●	59 ●	67 ●
KRAS Q61x mix (4)	4 ●	12 ●	20 ●	28 ●	36 ●	44 ●	52 ●	60 ●	68 ●
NRAS G12x-G13x mix (5)	5 ●	13 ●	21 ●	29 ●	37 ●	45 ●	53 ●	61 ●	69 ●
NRAS Q61x mix (6)	6 ●	14 ●	22 ●	30 ●	38 ●	46 ●	54 ●	62 ●	70 ●
HRAS G12x-G13R mix (7)	7 ●	15 ●	23 ●	31 ●	39 ●	47 ●	55 ●	63 ●	71 ●
HRAS Q61x mix (8)	8 ●	16 ●	24 ●	32 ●	40 ●	48 ●	56 ●	64 ●	72 ●

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

OPERATOR NOTES

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