



Easy[®] NRAS

User manual – version 2016/02

The “Easy[®] NRAS” Kit detects mutations of *NRAS* codons 12, 13, 59, 61, 117 e 146 by Real-Time PCR.

For *in vitro* diagnostic use



RT004



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Changes made since the previous version 2015/02:

- Substitution of **NRAS pos ctrl** with **Easy NRAS pos ctrl**.
- Update of section "Materials required but not provided" – Genomic DNA extraction.
- Validation of the kit on ABI 7500.

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® NRAS” Kit is intended for the qualitative detection by Real Time PCR of *NRAS* somatic mutations of codons 12, 13, 59, 61, 117 e 146 in the genomic DNA isolated from fresh, frozen or formalin fixed paraffin-embedded (FFPE) tumor tissue.

The “Easy® NRAS” 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** - 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:

NRAS codons 12-13 (not distinguishable between them) <ul style="list-style-type: none">▪ 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 codons 59-61 (not distinguishable between them) <ul style="list-style-type: none">▪ A59T (175G>A)▪ A59D (176C>A)▪ Q61H (183A>C)▪ Q61H (183A>T)
	NRAS codon 61 <ul style="list-style-type: none">▪ Q61K (181C>A)▪ Q61R (182A>G)▪ Q61L (182A>T)
	NRAS codon 117 (not distinguishable between them) <ul style="list-style-type: none">▪ K117R (350A>G)▪ K117N (351G>T)▪ K117N (351G>C)
	NRAS codon 146 <ul style="list-style-type: none">▪ A146T (436G>A)

PRINCIPLE OF THE ASSAY

The “Easy® NRAS” 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 by fluorescent probes labelled with FAM and HEX.

The “Easy® NRAS” Kit is composed of seven assays for the detection of the *NRAS* 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. **NRAS G12x-G13x**: 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) and G13V (38G>T) mutations but does not distinguish between them
2. **NRAS A59x-Q61H**: the assay detects the A59T (175G>A), A59D (176C>A), Q61H (183A>C) and Q61H (183A>T) mutations but does not distinguish between them
3. **NRAS Q61K**: the assay detects the Q61K (181C>A) mutation
4. **NRAS Q61R**: the assay detects the Q61R (182A>G) mutation
5. **NRAS Q61L**: the assay detects the Q61L (182A>T) mutation
6. **NRAS K117x**: the assay detects the K117R (350A>G), K117N (351G>T) and K117N (351G>C) mutations but does not distinguish between them
7. **NRAS A146x**: the assay detects the A146T (436G>A) mutation
8. **NRAS ctrl**: the assay detects a region of *NRAS* without any known polymorphism/mutation

The “Easy® NRAS” Kit includes a DNA reference standard **Horizon NRAS Q61K 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 3 runs (6 samples and two controls per run).

COMP	QUAN	Color	
NRAS G12x-G13x mix (1)	3 x 10 µl	BLUE	Mixture of specific primers and probes targeting both NRAS G12S, G12C, G12A, G12D, G12V, G13R, G13D, G13V mutations and the internal control.
NRAS A59x-Q61H mix (2)	3 x 10 µl	GREEN	Mixture of specific primers and probes targeting both NRAS A59T, A59D, Q61H (183A>C), Q61H (183A>T) mutations and the internal control.
NRAS Q61K mix (3)	3 x 10 µl	RED	Mixture of specific primers and probes targeting both NRAS Q61K mutation and the internal control.
NRAS Q61R mix (4)	3 x 10 µl	YELLOW	Mixture of specific primers and probes targeting both NRAS Q61R mutation and the internal control.
NRAS Q61L mix (5)	3 x 10 µl	PURPLE	Mixture of specific primers and probes targeting both NRAS Q61L mutation and the internal control.
NRAS K117x mix (6)	3 x 10 µl	BLACK	Mixture of specific primers and probes targeting both K117R, K117N (351G>T), K117N (351G>C) mutations and the internal control.
NRAS A146x mix (7)	3 x 10 µl	WHITE	Mixture of specific primers and probes targeting both NRAS A146T mutation and the internal control.
NRAS ctrl mix (8)	6 x 10 µl	GREY	Mixture of specific primers and probes targeting both a region of the NRAS gene free from any known polymorphism/mutation and the internal control.
Easy NRAS 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 NRAS Q61K 1%	1 x 12 µl		Horizon DNA reference standard NRAS Q61K 1% 1% to check analytical process.
WATER	CONTROL- 1 x 1.5 ml		DNase-, RNase-free water to be used 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 used exclusively as samples diluent.
Taq Premix 920	3 x 920 µl		Solution containing hot start Taq DNA polymerase, reaction buffer, Mg ²⁺ 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 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[®] NRAS 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® NRAS” 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)

ⓘ 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)
- **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. Bio-Rad 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. 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:
 1. Qiagen, cat. no. 981103.
 2. LTF-Labortechnik GmbH & Co, no. 102.0170
- 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)		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		Global Trade Item Number
	Positive control		

PRODUCT USE LIMITATIONS

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

QUALITY CONTROL

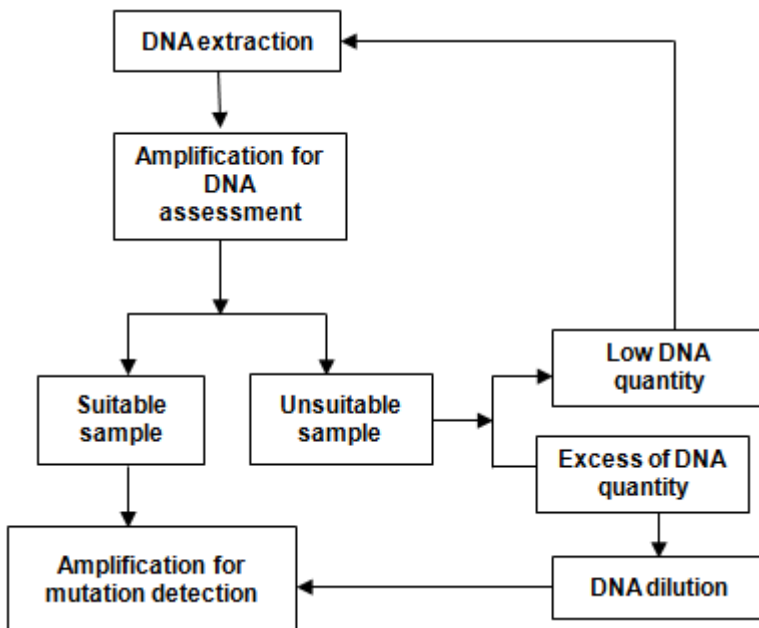
- The "**Easy**® **NRAS**" Kit was designed, developed and validated in accordance with D.Lgs n. 332 of 08/09/2000 ("Implementation of Directive 98/79 / EC on *in vitro* diagnostic medical devices") and subsequent legislative changes.
- In accordance with the company system of full quality assurance (certified according to European standards EN ISO 9001 and ISO 13485), to ensure consistent product quality, each batch of "**Easy**® **NRAS**" 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 work flow 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 of 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® NRAS” Kit are intended to be used solely with the other reagents in the same “Easy® NRAS” 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” - code 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 NRAS 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® NRAS” 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

- ① Perform this step in the area dedicated to DNA isolation and dilution, using dedicated materials and instruments.
 - The “**Easy® NRAS**” 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 **NRAS ctrl mix (8)**. The evaluation of quality and quantity of the DNA in the samples with the **NRAS ctrl mix (8)** before the mutation analysis is highly recommended. DNA assessment based on the **NRAS ctrl mix (8)** may differ from spectrophotometric quantification.
- ① All assays in the “**Easy® NRAS**” 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 wells of the plate to use.
 - Each run must include at least one amplification negative control (**WATER**) and one amplification positive control (**Easy NRAS 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” - “Gain Optimisation” 58°C Before 1st acquisition, “Tube Position”: 8 (**NRAS ctrl mix (8)**), “Target Sample Range” 20-30 FI
 - “Yellow 2”: source 530 nm – detector 555 nm – “Gain Optimisation” 58°C Before 1st acquisition, “Tube Position”:8 (**NRAS ctrl mix (8)**), “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 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.
- 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 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.

ABI 7500

- 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		
Taq Premix 920		Reagent volume for 1 reaction (µl)
WATER	<input type="checkbox"/> CONTROL -	4
NRAS ctrl mix (8)		1
Total Volume		15

ABI 7300		
Amp-Mix		
Taq Premix 920		Reagent volume for 1 reaction (µl)
Dye R-I		0,4
WATER	<input type="checkbox"/> CONTROL -	3,6
NRAS ctrl mix (8)		1
Total Volume		15

ABI 7500		
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	
NRAS ctrl mix (8)	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 NRAS 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 8 different mixes: **NRAS G12x-G13x mix (1)**, **NRAS A59x-Q61H mix (2)**, **NRAS Q61K mix (3)**, **NRAS Q61R mix (4)**, **NRAS Q61L mix (5)**, **NRAS K117x mix (6)**, **NRAS A146x mix (7)** e **NRAS ctrl mix (8)**.
- ① The DNA reference standard **Horizon NRAS Q61K 1%** must be amplified only with the mixes **NRAS Q61K mix (3)** and **NRAS ctrl mix (8)**.
- ① The kit content is optimized to analyze six clinical samples and two controls **Easy NRAS pos ctrl** and **WATER** in each run:

Mx3000P/3005P, ABI 7300/7500, CFX96 (RT004 sample grid A)

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

1	G12x-G13x mix (1)
2	A59x-Q61H mix (2)
3	Q61K mix (3)
4	Q61R mix (4)
5	Q61L mix (5)
6	A146x mix (6)
7	K117x mix (7)
8	NRAS ctrl mix (8)

Rotor-Gene (RT004 sample grid B)

DNA1		DNA2		DNA3		DNA4		DNA5		DNA6		POS CTRL		WATER			
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, eight different amplification mixtures (**Amp-Mix**), one for each assay, as indicated in the following scheme, where N is the number of samples and controls to be tested.

① 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 **Amp-Mixes** 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	
NRAS G12x-G13x mix (1) or	1	
NRAS A59x-Q61H mix (2) or		
NRAS Q61K mix (3) or		
NRAS Q61R mix (4) or		
NRAS Q61L mix (5) or		
NRAS K117x mix (6) or		
NRAS A146x mix (7) or		
NRAS ctrl mix (8)		
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	
NRAS G12x-G13x mix (1) or	1	
NRAS A59x-Q61H mix (2) or		
NRAS Q61K mix (3) or		
NRAS Q61R mix (4) or		
NRAS Q61L mix (5) or		
NRAS K117x mix (6) or		
NRAS A146x mix (7) or		
NRAS ctrl mix (8)		
Total Volume	15	

ABI 7500		
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	
NRAS G12x-G13x mix (1) or	1	
NRAS A59x-Q61H mix (2) or		
NRAS Q61K mix (3) or		
NRAS Q61R mix (4) or		
NRAS Q61L mix (5) or		
NRAS K117x mix (6) or		
NRAS A146x mix (7) or		
NRAS ctrl mix (8)		
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 NRAS pos ctrl	CONTROL+

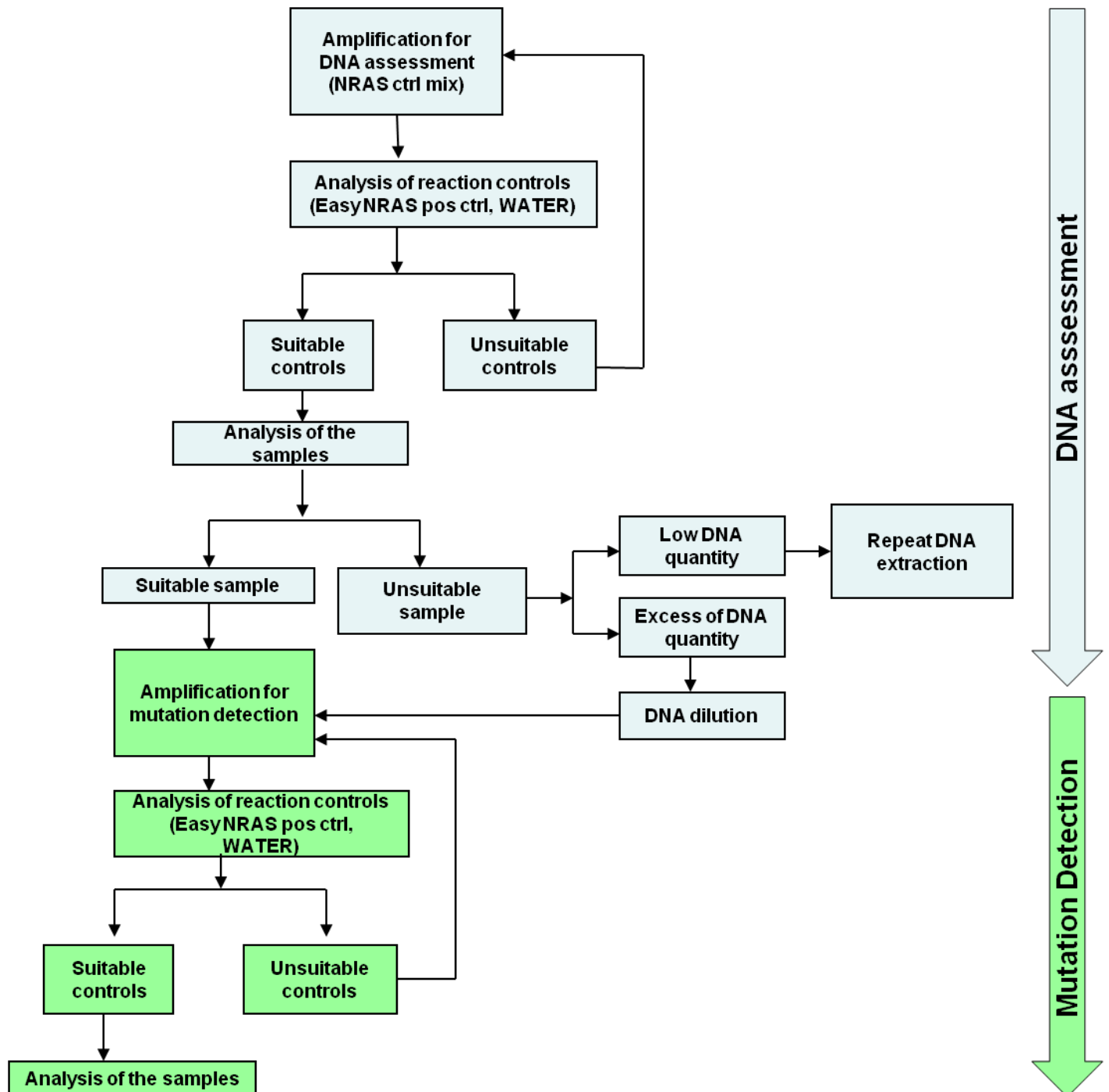
- Reaction volume: 20 µl.
 - Briefly centrifuge the plate.
 - Check that the thermal profile is setted up correctly and start the run.
- ① Before starting the run, please pay attention to the plate orientation (well A1 on the upper left position) or to the Rotor-Gene 0.1ml strips of tubes orientation (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 NRAS 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 (**NRAS ctrl mix (8)**) 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 channels Green and Yellow.

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

	NRAS ctrl mix	G12x-G13x, A59x-Q61H, Q61K, Q61R, Q61, K117x, A146x mix	All mixes	
	Green	Green	Yellow	Results
WATER	Ct > 35	Ct > 35	Ct > 32	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy NRAS pos ctrl	24 ≤ Ct ≤ 28	16 ≤ Ct ≤ 22	19 ≤ Ct ≤ 23	Proceed with analysis of the samples.
	Ct < 24 or Ct > 28	Ct < 16 or Ct > 22	Ct < 19 or Ct > 23	Possible error in the set up of the reaction /run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of NRAS ctrl mix for the DNA assessment

NRAS ctrl mix	Green	Yellow	Results
DNA samples	21 ≤ Ct ≤ 31	17 ≤ Ct ≤ 28	Suitable sample. Proceed with the mutation analysis ¹ .
	Ct < 21	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 > 31	Ct > 28	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: <ul style="list-style-type: none"> ▪ 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 decreases also the concentration of the target DNA.
1. If the results of the Yellow channel are not in the expected range, consider only the Green/Green2 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:

$$\Delta Ct = Ct \text{ Green mutation} - Ct \text{ Green NRAS ctrl mix}$$

Assay	Amplification of Internal Control (HEX)	ΔCt	Results
G12x-G13x	OK (similar Ct value for all the mixes)	≤ 6.5	G12x-G13x positive
A59x-Q61H		≤ 6	A59x-Q61H positive
Q61K		≤ 8	Q61K positive
Q61R		≤ 6.5	Q61R positive
Q61L		≤ 8	Q61L positive
K117x		≤ 4.5	K117x positive
A146x		≤ 3	A146x positive
G12x-G13x	OK (similar Ct value for all the mixes)	> 6.5	Wild-type sample or beneath the LOD ²
A59x-Q61H		> 6	
Q61K		> 8	
Q61R		> 6.5	
Q61L		> 8	
K117x		> 4.5	
A146x		> 3	
G12x-G13x	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
A59x-Q61H		\	
Q61K		\	
Q61R		\	
Q61L		\	
K117x		\	
A146x		\	
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 enter the samples name.
- In the section Analysis, Analysis Selection/Setup, deselect the function Amplification-based threshold and select only the reactions controls **WATER** and **Easy NRAS 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 modify).
- 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 result in both channels with their respective threshold and Ct values.
- Click save.

1. Analysis of reaction control

	NRAS ctrl mix	G12x-G13x, A59x-Q61H, Q61K, Q61R, Q61, K117x, A146x mix	All mixes	
	FAM	FAM	HEX	Results
WATER	Ct > 35	Ct > 35	Ct > 32	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy NRAS pos ctrl	24 ≤ Ct ≤ 28	17 ≤ Ct ≤ 22	19 ≤ Ct ≤ 23	Proceed with analysis of the samples.
	Ct < 24 or Ct > 28	Ct < 17 or Ct > 22	Ct < 19 or Ct > 23	Possible error in the set up of the reaction /run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of NRAS ctrl mix for the DNA assessment

NRAS ctrl mix	FAM ¹	HEX ¹	Results
DNA samples	$(x-3.5) \leq Ct \leq (x+5.5)$	$(x-3.5) \leq Ct \leq (x+5.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Ct < (x-3.5)	Ct < (x-3.5)	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 > (x+5.5)	Ct > (x+5.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: <ul style="list-style-type: none"> 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 decreases also the concentration of the target DNA.

1. $x = Ct$ Easy NRAS 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 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 FAM for the mutation and the equivalent for the control assay belong to the same sample:

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

Assay	Amplification of Internal Control (HEX)	ΔCt	Results
G12x-G13x	OK (Ct comparabile per tutte le mix)	≤ 7	G12x-G13x positive
A59x-Q61H		≤ 6.2	A59x-Q61H positive
Q61K		≤ 8	Q61K positive
Q61R		≤ 6.5	Q61R positive
Q61L		≤ 8.5	Q61L positive
K117x		≤ 4.5	K117x positive
A146x		≤ 3.7	A146x positive
G12x-G13x	OK (Ct comparabile per tutte le mix)	> 7	Wild type sample or beneath the LOD ¹
A59x-Q61H		> 6.2	
Q61K		> 8	
Q61R		> 6.5	
Q61L		> 8.5	
K117x		> 4.5	
A146x		> 3.7	
G12x-G13x	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
A59x-Q61H		\	
Q61K		\	
Q61R		\	
Q61L		\	
K117x		\	
A146x		\	
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 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
- In the page Quantification data, the results are displayed for both channels with the corresponding values of Cq.
- Click Save.

1. Analysis of reaction controls

	NRAS ctrl mix	G12x-G13x, A59x-Q61H, Q61K, Q61R, Q61, K117x, A146x mix	All mixes	Results
	FAM	FAM	HEX	
WATER	Cq > 35	Cq > 35	Cq > 32	Proceed with analysis of the samples.
	Cq ≤ 35	Cq ≤ 35	Cq ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy NRAS pos ctrl	24 ≤ Cq ≤ 28	17 ≤ Cq ≤ 22	21 ≤ Cq ≤ 25	Proceed with analysis of the samples.
	Cq < 24 o Cq > 28	Cq < 17 o Cq > 22	Cq < 21 o Cq > 25	Possible error in the set up of the reaction /run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of NRAS ctrl mix for the DNA assessment

NRAS ctrl mix	FAM ¹	HEX ¹	Results
DNA samples	$(x-3.5) \leq Cq \leq (x+5.5)$	$(x-3.5) \leq Cq \leq (x+5.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Cq <(x-3.5)	Cq <(x-3.5)	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 Ct of 1 unit.
	Cq > (x+5.5)	Cq > (x+5.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: <ul style="list-style-type: none"> ▪ 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 decreases also the concentration of the target DNA.

1. x = Cq Easy NRAS 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 ΔCq values 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:

$$\Delta Cq = Cq \text{ FAM Mutations} - Cq \text{ FAM NRAS ctrl mix}$$

Assay	Amplification of Internal Control (HEX)	ΔCq	Results
G12x-G13x	OK (Cq comparabile per tutte le mix)	≤ 6.5	G12x-G13x positive
A59x-Q61H		≤ 5	A59x-Q61H positive
Q61K		≤ 10	Q61K positive
Q61R		≤ 7.5	Q61R positive
Q61L		≤ 10	Q61L positive
K117x		≤ 4	K117x positive
A146x		≤ 3.3	A146x positive
G12x-G13x	OK (Cq comparabile per tutte le mix)	> 6.5	Wild-type sample or beneath the LOD ¹
A59x-Q61H		> 5	
Q61K		> 10	
Q61R		> 7.5	
Q61L		> 10	
K117x		> 4	
A146x		> 3.3	
G12x-G13x	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
A59x-Q61H		\	
Q61K		\	
Q61R		\	
Q61L		\	
K117x		\	
A146x		\	
1. LOD = Limit Of Detection, limite di sensibilità del kit \ 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 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 reaction controls

	NRAS ctrl mix	G12x-G13x, A59x-Q61H, Q61K, Q61R, Q61, K117x, A146x mix	All mixes	Results
	FAM	FAM	JOE	
WATER	Ct > 35	Ct > 35	Ct > 32	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy NRAS pos ctrl	23 ≤ Ct ≤ 27	16 ≤ Ct ≤ 21	22 ≤ Ct ≤ 29	Proceed with analysis of the samples.
	Ct < 23 o Ct > 27	Ct < 16 o Ct > 21	Ct < 22 o 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 NRAS ctrl mix for the DNA assessment

NRAS ctrl mix	FAM ¹	JOE ¹	Results
DNA samples	$(x-3.5) \leq Ct \leq (x+5.5)$	$(x-3.5) \leq Ct \leq (x+5.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Ct < (x-3.5)	Ct < (x-3.5)	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 > (x+5.5)	Ct > (x+5.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: <ul style="list-style-type: none"> ▪ 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 decreases also the concentration of the target DNA.

1. x = Ct **Easy NRAS 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 mutations assays

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

- Compare the ΔC_t values 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:

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

Assay	Amplification of Internal Control (JOE)	ΔC_t	Results
G12x-G13x	OK (similar C_t value for all the mixes)	≤ 6.1	G12x-G13x positive
A59x-Q61H		≤ 6.6	A59x-Q61H positive
Q61K		≤ 10	Q61K positive
Q61R		≤ 7.5	Q61R positive
Q61L		≤ 10	Q61L positive
K117x		≤ 4	K117x positive
A146x		≤ 3.2	A146x positive
G12x-G13x	OK (similar C_t value for all the mixes)	> 6.1	Wild-type sample or beneath the LOD ¹
A59x-Q61H		> 6.6	
Q61K		> 10	
Q61R		> 7.5	
Q61L		> 10	
K117x		> 4	
A146x		> 3.2	
G12x-G13x	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
A59x-Q61H		\	
Q61K		\	
Q61R		\	
Q61L		\	
K117x		\	
A146x		\	
1. LOD = Limit Of Detection \ Any value			

- ① If multiple assays show a ΔC_q equal to or below the cut-off value, the signal giving the higher ΔC_q 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_q .

ABI 7500

- 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 reaction controls

	NRAS ctrl mix	G12x-G13x, A59x-Q61H, Q61K, Q61R, Q61, K117x, A146x mix	All mixes	Results
	FAM	FAM	JOE	
WATER	Ct > 33	Ct > 33	Ct > 32	Proceed with analysis of the samples.
	Ct ≤ 33	Ct ≤ 33	Ct ≤ 32	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Easy NRAS pos ctrl	23 ≤ Ct ≤ 28	15 ≤ Ct ≤ 22	20 ≤ Ct ≤ 26	Proceed with analysis of the samples.
	Ct < 23 o Ct > 28	Ct < 15 o Ct > 22	Ct < 20 o Ct > 26	Possible error in the set up of the reaction /run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of NRAS ctrl mix for the DNA assessment

NRAS ctrl mix	FAM ¹	JOE ¹	Results
DNA samples	$(x-3.5) \leq Ct \leq (x+5.5)$	$(x-3.5) \leq Ct \leq (x+5.5)$	Suitable sample. Proceed with the mutation analysis ² .
	Ct < (x-3.5)	Ct < (x-3.5)	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 > (x+5.5)	Ct > (x+5.5)	Suboptimal amount of starting DNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: <ul style="list-style-type: none"> 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 decreases also the concentration of the target DNA.

3. x = Ct **Easy NRAS pos ctrl**

4. 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 mutations 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 FAM for the mutation and the equivalent for the control assay belong to the same sample:

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

Assay	Amplification of Internal Control (JOE)	ΔCt	Results
G12x-G13x	OK (similar Ct value for all the mixes)	≤ 8.2	G12x-G13x positive
A59x-Q61H		≤ 5.9	A59x-Q61H positive
Q61K		≤ 8	Q61K positive
Q61R		≤ 7.6	Q61R positive
Q61L		≤ 10.1	Q61L positive
K117x		≤ 3.1	K117x positive
A146x		≤ 3.5	A146x positive
G12x-G13x	OK (similar Ct value for all the mixes)	> 8.2	Wild-type sample or beneath the LOD ¹
A59x-Q61H		> 5.9	
Q61K		> 8	
Q61R		> 7.6	
Q61L		> 10.1	
K117x		> 3.1	
A146x		> 3.5	
G12x-G13x	NO	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
A59x-Q61H		\	
Q61K		\	
Q61R		\	
Q61L		\	
K117x		\	
A146x		\	
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 .

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 NRAS pos ctrl and samples.	Incorrect selection of the fluorescence acquisition channels.	<ul style="list-style-type: none"> Check the fluorescence acquisition channels and repeat amplification with the settings described in this manual.
	Incorrect selection of the Gain on the Rotor-Gene.	<ul style="list-style-type: none"> Repeat amplification of Rotor-Gene setting the gain properly, as described in this manual.
	Incorrect setting of the thermal-profile.	<ul style="list-style-type: none"> Check the temperature profile and repeat amplification with the settings described in this manual.
	Incorrect dispensation or manipulation of the reagents.	<ul style="list-style-type: none"> 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.	<ul style="list-style-type: none"> 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 NRAS pos ctrl is within the expected values.	Insufficient amount of starting DNA and / or presence of PCR inhibitors.	<ul style="list-style-type: none"> 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 (diluent).
	Incorrect or no dispensation of the samples.	<ul style="list-style-type: none"> 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 NRAS pos ctrl	Degradation of the Easy NRAS pos ctrl .	<ul style="list-style-type: none"> Repeat the amplification testing a new aliquot of Easy NRAS pos ctrl .
	Incorrect or failure in the dispensation of the Easy NRAS pos ctrl .	<ul style="list-style-type: none"> Repeat the amplification by pipetting the appropriate volume of Easy NRAS pos ctrl .
You can not perform the analysis on Rotor-Gene in Cycling A. Green and/or Yellow because of a fluorescence intensity too high or out of scale for one or more than one assay.	Rotor-Gene gain too high.	<ul style="list-style-type: none"> 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: $\Delta Ct = Ct \text{ Green 2 Mutation} - Ct \text{ Green 2 NRAS ctrl mix}$.
The positive control Easy NRAS 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.	<ul style="list-style-type: none"> Repeat the amplification after marking unambiguously the reaction tubes for samples and controls.
	Incorrect dispensing of the samples.	<ul style="list-style-type: none"> Repeat the amplification paying attention to the dispensation of the DNA in reaction tubes/wells.
	Incorrect samples names set-up in the software .	<ul style="list-style-type: none"> Check samples names set-up.
One sample shows HEX/Yellow Ct values different from each other for the assays.	DNA dispensation error	<ul style="list-style-type: none"> 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 FAM/Green and HEX/Yellow channels.	Contamination	<ul style="list-style-type: none"> 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.	<ul style="list-style-type: none"> Wear gloves.
<ul style="list-style-type: none"> 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: <ul style="list-style-type: none"> e-mail support@diatechpharmacogenetics.com telephone +39 0731 213243 fax +39 0731 213239 		

PERFORMANCE VALIDATION

The “Easy® NRAS” Kit 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** - 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 “Easy® NRAS” 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 “Easy® NRAS” Kit, and have been already genotyped through pyrosequencing technology (kit “Anti-EGFR MoAb response® (NRAS status)”, cod. UP038 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 FFPE samples were available, Horizon Diagnostics standards, cell lines or plasmids have been tested.

Easy® NRAS	Rotor-Gene		Stratagene Mx3000P, Mx3005P		CFX96		ABI 7300		ABI 7500	
	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
NRAS G12x-G13x	10	10/10	11	11/11	10	10/10	10	10/10	2	2/2
NRAS A59x-Q61H	1	1/1	*	*	*	*	*	*	*	*
NRAS Q61K	8	8/8	11	11/11	9	9/9	8	8/8	3	3/3
NRAS Q61R	9	9/9	11	11/11	9	9/9	9	9/9	2	2/2
NRAS Q61L	*	*	1	1/1	1	1/1	3	3/3	*	*
NRAS K117x	*	*	*	*	*	*	*	*	*	*
NRAS A146x	*	*	1	1/1	*	*	*	*	*	*
NRAS wild-type	2	2/2	8	8/8	2	2/2	2	2/2	23	23/23
Totale	30	30/30	43	43/43	31	31/31	32	32/32	30	30/30

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

Limit of detection (LOD)

The LOD of “Easy® NRAS” 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/3005P, ABI 7300/7500, 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® NRAS” Kit, considering all the instruments tested is:

Assay	LOD C ₉₅ (at medium DNA input concentration)
G12x-G13x	1-5%
A59x-Q61H	1-5%
Q61K	0.5-1%
Q61R	1%
Q61L	0.5%
K117x	2-7.5%
A146x	1-5%

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.

DATE												RUN NAME	
	1	2	3	4	5	6	7	8	9	10	11	12	
A													G12x-G13x (1)
B													A59x-Q61H (2)
C													Q61K (3)
D													Q61R (4)
E													Q61L (5)
F													K117x (6)
G													A146x (7)
H													ctrl mix (8)
INSTRUMENT n.									USER MANUAL version				
CODE		LOT						EXPIRY DATE					
NOTES													
OPERATOR									SIGN				

DATE		RUN NAME	
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G12x-G13x (1)	1 ●	9 ●	17 ●	25 ●	33 ●	41 ●	49 ●	57 ●	65 ●
A59x-Q61H (2)	2 ●	10 ●	18 ●	26 ●	34 ●	42 ●	50 ●	58 ●	66 ●
Q61K (3)	3 ●	11 ●	19 ●	27 ●	35 ●	43 ●	51 ●	59 ●	67 ●
Q61R (4)	4 ●	12 ●	20 ●	28 ●	36 ●	44 ●	52 ●	60 ●	68 ●
Q61L (5)	5 ●	13 ●	21 ●	29 ●	37 ●	45 ●	53 ●	61 ●	69 ●
K117x (6)	6 ●	14 ●	22 ●	30 ●	38 ●	46 ●	54 ●	62 ●	70 ●
A146x (7)	7 ●	15 ●	23 ●	31 ●	39 ●	47 ●	55 ●	63 ●	71 ●
ctrl mix (8)	8 ●	16 ●	24 ●	32 ●	40 ●	48 ●	56 ●	64 ●	72 ●

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

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