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# EasyPGX® ready NRAS

User manual – version 2018/06

The “**EasyPGX® ready NRAS**” kit detects mutations of *NRAS* codons 12, 13, 59, 61, 117 and 146 by Real-Time PCR.

For *in vitro* diagnostic use

RT024

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Changes made since the previous version 2018/03:

- Section “Kit contents”: update of the number of reaction controls.
- Section “DNA extraction – use of **EasyPGX® Extraction reagents**”: update of DNA dilution volume
- Section “Amplification and mutation detection”: update of resuspension volume of dry **EasyPGX® NRAS strips.**
- Section “Data Analysis”: update of the analysis criteria.

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

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## INTENDED USE

The *in vitro* diagnostic "**EasyPGX® ready NRAS**" kit is intended for the qualitative detection by Real-Time PCR of *NRAS* somatic mutations in the genomic DNA isolated from tumor tissue (fresh, frozen or formalin fixed paraffin-embedded (FFPE)) or from plasma.

The "**EasyPGX® ready NRAS**" kit is validated for the use in combination with the following instrument:

- **EasyPGX® qPCR instrument 96** - Diatech Pharmacogenetics (96-well plate)

and accessories:

- **EasyPGX® dry block** - Diatech Pharmacogenetics
- **EasyPGX® centrifuge/vortex 1.5 ml** - Diatech Pharmacogenetics
- **EasyPGX® centrifuge/vortex 8-well strips** - Diatech Pharmacogenetics

List of detectable mutations:

<table>
<tr><td rowspan="4">NRAS codons 12-13<br>(not distinguishable between them)<br>▪ G12S (34G>A)<br>▪ G12C (34G>T)<br>▪ G12A (35G>C)<br>▪ G12D (35G>A)<br>▪ G12V (35G>T)<br>▪ G13R (37G>C)<br>▪ G13D (38G>A)<br>▪ G13V (38G>T)</td><td>NRAS codons 59-61<br>(not distinguishable between them)<br>▪ A59T (175G>A)<br>▪ A59D (176C>A)<br>▪ Q61H (183A>C)<br>▪ Q61H (183A>T)</td></tr>
<tr><td>NRAS codon 61<br>▪ Q61K (181C>A)<br>▪ Q61R (182A>G)<br>▪ Q61L (182A>T)</td></tr>
<tr><td>NRAS codon 117<br>(not distinguishable between them)<br>▪ K117R (350A>G)<br>▪ K117N (351G>T)<br>▪ K117N (351G>C)</td></tr>
<tr><td>NRAS codon 146<br>(not distinguishable between them)<br>▪ A146T (436G>A)<br>▪ A146V (437C>T)</td></tr>
</table>

## PRINCIPLE OF THE ASSAY

The "**EasyPGX® ready NRAS**" kit is delivered in 8-well strips preloaded with a complete amplification mix in a dry, room temperature stable format and it contains reagents for DNA extraction from formalin-fixed paraffin-embedded (FFPE) samples.

The "**EasyPGX® ready 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 fluorescent probes labelled with FAM and HEX.

The "**EasyPGX® ready 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) and A146V (437C>T) mutations mutations but does not distinguish between them
8. **NRAS ctrl**: the assay detects a region of *NRAS* without any known polymorphism/mutation

## CLINICAL RELEVANCE

Several studies on metastatic colorectal cancers (CRC) revealed that approximately 3-5% of patients carry somatic mutations in the *NRAS* gene[1,2]. These mutations in exon 2, 3, or 4 of the *NRAS* gene were negative predictive factors for treatments with monoclonal antibodies against the *Epidermal Growth Factor Receptor* (*EGFR*), such cetuximab and panitumumab[3-6]. So no benefit of anti-*EGFR* agents was observed in patients with metastatic CRC tumors with *NRAS* activating mutations. From these results, cetuximab and panitumumab have been used only in mCRC patients with *NRAS* wild type[7].
Therefore detection of the *NRAS* mutation status is an important element in choosing the most appropriate therapeutic regimen for cancer patients.

**References**
1. Smith et al., Activat-ing K-Ras mutations outwith 'hotspot' codons in sporadic colorec-tal tumours: implications for personalised cancer medicine. Br J Cancer 2010; 102: 693-703.
2. Vaughn CP et al., Fre-quency of KRAS, BRAF, and NRASmutations in colorectal cancer. Genes Chromosomes Cancer 2011; 50: 307-12.
3. De Roock W et al., Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010 Aug;11(8):753-62.
4. Lambrechts D et al., The role of KRAS, BRAF, NRAS, and PIK3CA mutations as markers of resistance to cetuximab in chemorefractory metastatic colorectal cancer. ASCO Annual Meeting (2009)
5. Douillard JY et al., Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 2013; 369: 1023-1034
6. Schwartzberg LS et al., PEAK: a randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. J Clin Oncol 2014; 32: 2240-2247.
7. AIOM-SIAPEC-IAP Recommendations for mutagenic analysis of RAS mutations in colorectal cancer. 2015.

## KIT CONTENTS

The "**EasyPGX® ready NRAS**" kit is delivered in 8-well strips preloaded with a complete amplification mix in a dry, room temperature stable format and it contains reagents for DNA extraction from formalin-fixed paraffin-embedded (FFPE) samples.
The kit contains sufficient reagents to carry out 48 tests.

| Destination **EXTRACTION AREA**<br>Storage temperature **+2/+25°C** | | | |
|---|---|---|---|
| **EasyPGX® Extraction reagents** | | | |
| COMP | | QUAN | |
| **EasyPGX Dep solution** | | 1 x 6 ml | Solution for deparaffinization of formalin-fixed paraffin-embedded (FFPE) samples |
| **EasyPGX Buffer** | | 1 x 6 ml | Buffer for DNA extraction from formalin-fixed paraffin-embedded (FFPE) samples |
| **EasyPGX Enzyme** | | 1 x 600 µl | Enzyme for DNA extraction from formalin-fixed paraffin-embedded (FFPE) samples |
| Destination **AMPLIFICATION AREA**<br>Storage temperature **+2/+25°C** | | | |
| COMP | | QUAN | |
| **EasyPGX NRAS strips** | | 4 x 12 strips | BLUE 8-well strips: 8 dry complete mixtures containing specific primers and probes targeting the following NRAS mutations and the internal control:<br>**Position 1: G12x-G13x**<br>**Position 2: A59x-Q61H**<br>**Position 3: Q61K**<br>**Position 4: Q61R**<br>**Position 5: Q61L**<br>**Position 6: K117x**<br>**Position 7: A146x**<br>**Position 8: control region**<br>For the list of the detectable mutation refer to "Intended Use" page 4 |
| **8-strip flat optical caps** | | 2 x 25 strips | 0.2 ml 8-tube cap strips DNase-, RNase-free to be used to recap the **EasyPGX NRAS strips** |
| **EasyPGX® Pos & Neg Controls** | | | |
| COMP | | QUAN | |
| **EasyPGX NRAS pos ctrl** | CONTROL + | 5 tubes | DNA positive control in a dry format containing a mixture of synthetic DNA sequences that corresponds to each mutation detected by this kit in a background of wild-type genomic DNA. Every aliquot must be resuspended with 700 µl of **WATER** before the use. |
| **WATER** | CONTROL - | 8 x 1.5 ml | DNase-, RNase-free water, 2 aliquots to be used exclusively to resuspend the dry positive controls, 2 aliquots to be used exclusively as negative control in the PCR reaction and 4 aliquots to be used exclusively as samples diluent. |

## DOCUMENTS AVAILABLE ON-LINE

The following documents are available at www.diatechpharmacogenetics.com/area-riservata:

- "**EasyPGX® ready NRAS**" - User Manual
- Safety Data Sheets (SDSs)

ⓘ For further details please contact the Diatech Pharmacogenetics technical support:
email: support@diatechpharmacogenetics.com, tel. +39 0731 213243

## MATERIALS REQUIRED BUT NOT PROVIDED

Genomic DNA extraction

The "**EasyPGX® ready NRAS**" kit contains reagents for DNA extraction from formalin-fixed paraffin-embedded (FFPE) samples.

*Required accessories*:
- **EasyPGX® dry block** (code RT801, Diatech Pharmacogenetics)
- **EasyPGX® centrifuge/vortex 1.5 ml** (code RT802, Diatech Pharmacogenetics)

Other recommended options for DNA extraction and purification:

a. Tissue:

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

b. Plasma:

- "Helix Circulating Nucleic Acid" (code H8040, Diatech Pharmacogenetics)
- "QIAamp® Circulating Nucleic Acid" (code 55114, Qiagen)

ⓘ For each of the above options, DNA extraction and purification shall be done following the related user manual indications and prescriptions.

ⓘ 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 instrument*:
- **EasyPGX® qPCR instrument 96** code RT800-96, Diatech Pharmacogenetics (Agilent Aria Sofware v1.4)

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

*Required accessory:*
- **EasyPGX® centrifuge/vortex 8-well strips** (code RT803, Diatech Pharmacogenetics)

*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)
- 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 +2/+25°C in the original package. If in the storage environment there isn't a temperature data-logger for temperature monitoring, it is recommended to store all the reagents at +2/+8°C.

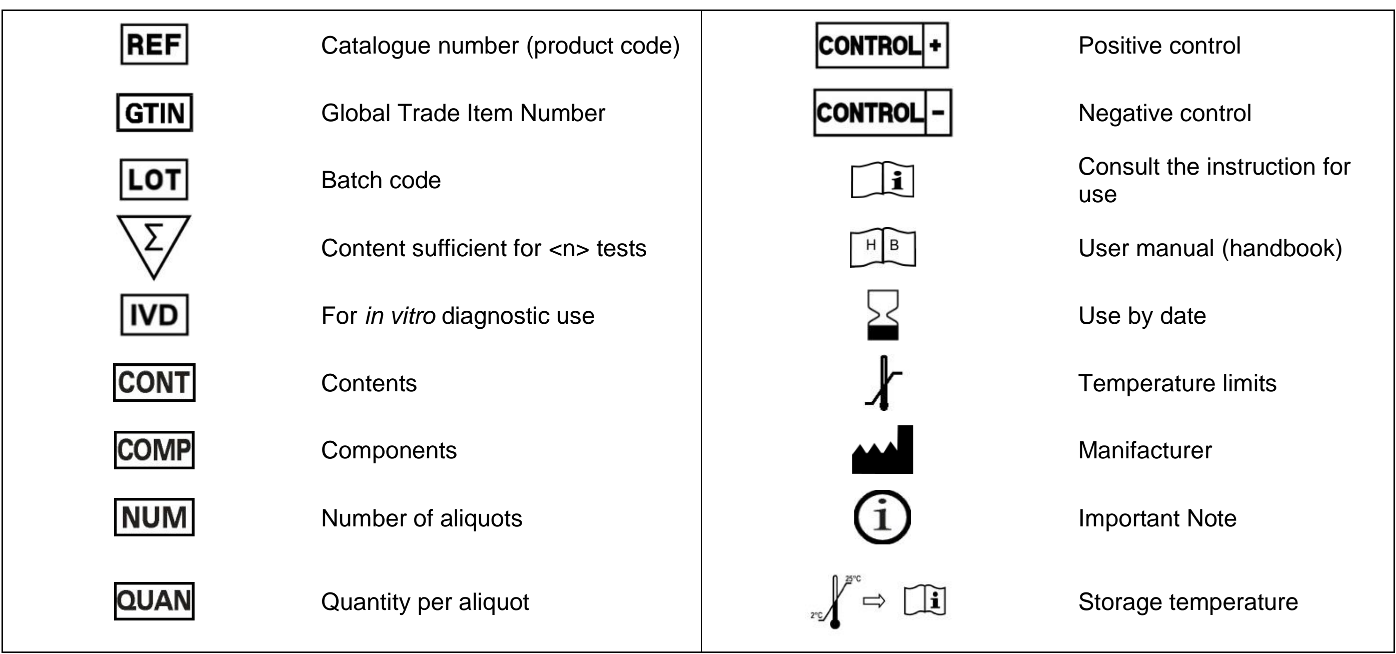
*EasyPGX® Extraction reagents*

- After the first use, store the **EasyPGX Enzyme** at +2/+8°C and use it within the expiration date.

*EasyPGX® Amplification reagents*

- Once a **EasyPGX NRAS strips** package is opened, store it at +2/+8°C and use the contained strips within 2 months and within the expiration date.
- Once resuspended, store the **EasyPGX NRAS pos ctrl** at -20/-35°C and use it within the expiration date. Avoid thawing and re-freezing more than four times, as this could lead to poor performance.
- Protect all the dry mixes 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



| Symbol | Meaning | Symbol | Meaning |
|---|---|---|---|
| REF | Catalogue number (product code) | CONTROL + | Positive control |
| GTIN | Global Trade Item Number | CONTROL - | Negative control |
| LOT | Batch code | | Consult the instruction for use |
| Σ | Content sufficient for <n> tests | H B | User manual (handbook) |
| IVD | For *in vitro* diagnostic use | | Use by date |
| CONT | Contents | | Temperature limits |
| COMP | Components | | Manufacturer |
| NUM | Number of aliquots | i | Important Note |
| QUAN | Quantity per aliquot | 2°C 25°C ⇨ | Storage temperature |

## PRODUCT USE LIMITATIONS

- The "**EasyPGX® ready 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 "**EasyPGX® ready NRAS**" kit has been designed and validated for the use with the real-time qPCR instrument **EasyPGX® qPCR instrument 96** (code RT800-96) and with the accessories **EasyPGX® dry block** (code RT801), **EasyPGX® centrifuge/vortex 1.5 ml** (code RT802) and **EasyPGX® centrifuge/vortex 8-well strips** (code RT803). All these items are manufactured and put on the market by Diatech Pharmacogenetics.
- Diatech Pharmacogenetics can't respond of results obtained using instruments or accessories other than those recommended in this user manual.
- 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 the DNA extraction.
- Any diagnostic results generated by this procedure must be interpreted with reference to other clinical or laboratory findings.
- The "**EasyPGX® ready NRAS**" kit is covered by the CE Mark in compliance with the European directive 98/79/EC on the in vitro diagnostic (IVD) medical devices, only in those countries that accept the user manual translated in the languages available on the website www.diatechpharmacogenetics.com/area-riservata.

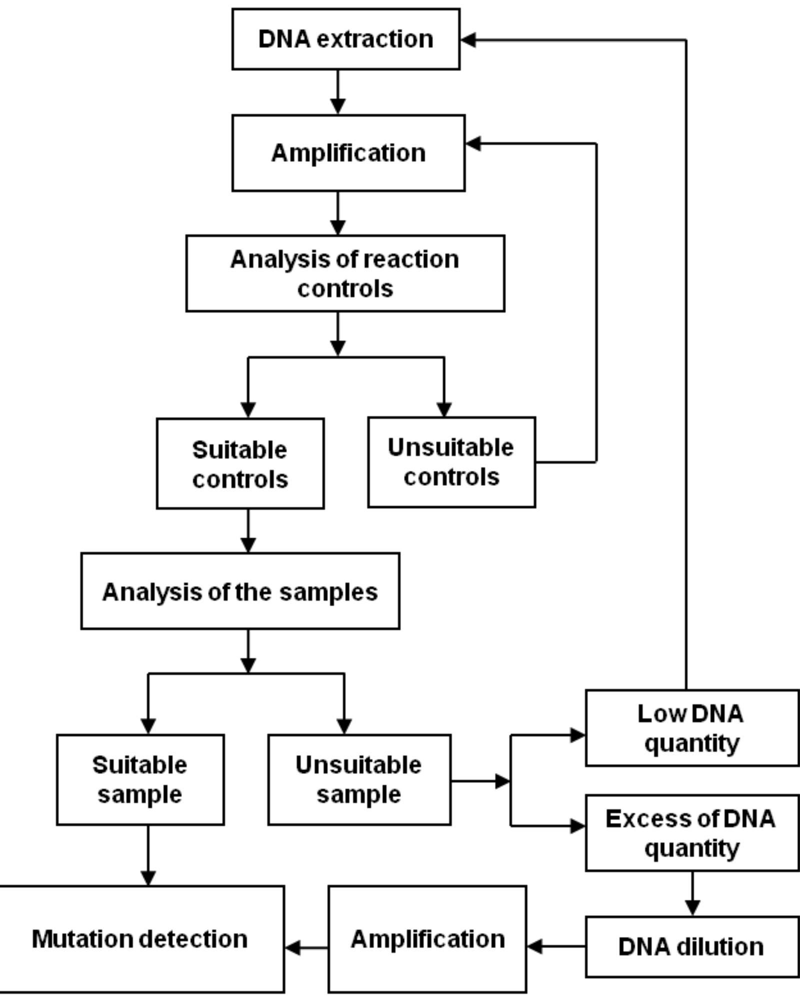
## QUALITY ASSURANCE

- The "**EasyPGX® ready NRAS**" kit has been designed, developed and validated in compliance with the Directive 98/79/EC on in vitro diagnostic (IVD) medical devices, transposed in Italy in the D.Lgs No 332/2000 and subsequent legislative changes, and in accordance with the procedures of the Company's Quality System certified for conformance to the European regulatory standards EN ISO 9001 and ISO 13485.
- The consistent quality of the "**EasyPGX® ready NRAS**" kit is guaranteed by the application of a tight process control on materials and on operative procedures for product realization and its management till the Customer. The quality of each lot is attested in the related Certificate of Analysis available upon request to the Customer Service (support@diatechpharmacogenetics.com).

## WARNINGS AND PRECAUTIONS

1. The kit may only be used by specialist personnel, properly instructed and trained to perform *in vitro* laboratory techniques.
2. Carefully read this User Manual.
3. Check that the version of the User Manual in use corresponds to the one described on the "**EasyPGX® ready NRAS**" kit box label.
4. Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
5. Follow the laboratory safety procedures described in "Biosafety in Microbiological and Biomedical Laboratories" (Richmond, JY and McKinney, RW (eds) - 5th edition (2009) and in the NCCLS (National Committee for Clinical Laboratory Standards) Document M29-T. Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative guidelines. – Villanova, PA:NCCLS, 1989).
6. Do not eat, drink or smoke in the laboratory. When handling biological samples, disposable gloves, gowns and goggles or face masks should be worn to protect against biological agents.
7. Constantly check that the gloves are free from contamination by the biological material being treated. If not, replace them immediately to avoid the possibility of cross-contamination between samples and contamination of the workplace. Wash hands thoroughly after handling samples and reagents.
8. The Safety Data Sheet (SDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com, or can be requested to the the Diatech Pharmacogenetics technical support support@diatechpharmacogenetics.com.
9. Perform the procedure in accordance with *Good Laboratory Practice (GLP)* general guidelines.
10. It is recommended to ensure that the laboratory work flow proceeds in a unidirectional manner, setting up two separate working areas for:
    - extraction of nucleic acids
    - amplification reaction
11. Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
12. Use sterile filter tips. Avoid aerosols.
13. Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
14. During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by opening the reagents only within the hood.
15. Change the pipette tip before each pick up of reagents and every time you move from one sample to another in any stage of the procedure.
16. The precision pipettes used should have an accuracy of within 3% of the set volume.
17. Periodically check the calibration status of the dispensing instruments.
18. Do not use reagents after the expiration date shown on each container.
19. All reagents supplied in the "**EasyPGX® ready NRAS**" kit are intended to be used solely with the other reagents included in the same kit. Do not substitute or mix reagents from different batches, in order to maintain optimal performance.
20. Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
21. 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.
22. 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.
23. Avoid contamination of samples and reagents.
24. Store reagents and samples separately.
25. In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.
26. Before use all reagents need to be mixed by inverting 10 times and centrifuged briefly.
27. 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.
28. Include in each run at least 1 negative control (**WATER**) and 1 positive control (**EasyPGX NRAS pos ctrl**).
29. In order to avoid any mixing up of samples pay particular attention to samples dispensation, placement of strips into the instrument, editing the sample name in the software.
30. 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.
31. 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 good quality of the extracted DNA strictly depends on the conditions of paraffin-embedded tissue. In particular, 4-10% of formalin fixation and a fixation period not longer than 14-24 hours are recommended.

*For more information refer to "Tumor Samples: Preliminary Phase Protocol" available upon request to Diatech Pharmacogenetics technical support (email: support@diatechpharmacogenetics.com)*

ⓘ When cutting sections from paraffin-embedded blocks, the microtome's blade and tweezers should be cleaned between each samples to avoid cross contamination of DNA.
ⓘ All assays in the "**EasyPGX® ready NRAS**" kit amplify short DNA sequences. However heavily fragmented DNA can generate no amplification product.
ⓘ The "**EasyPGX® ready NRAS**" kit includes the reagents for DNA extraction from FFPE samples.

### *Use of EasyPGX® Extraction reagents*

- Prepare a 1 x 10 µm (or 2 x 5 µm) thick paraffin-embedded tissue section of a surface area up to 250mm² and add it into a 1.5 ml twist-lock tube (not provided) using a sterilized tweezer. Alternatively, transfer an equal amount of FFPE sample from an histological slide into a 1.5 ml microcentrifuge tube (not provided) using a sterilized lancet.
- Set the **EasyPGX® dry block** at 56°C and wait the instrument to reach the temperature.
- Gently mix by invertion the **EasyPGX Dep solution** and add 100 µl into the 1.5 ml twist-lock tube (not provided).
- Gently mix by invertion the **EasyPGX buffer** and add 100 µl into the 1.5 ml twist-lock tube (not provided).
- Add 10 µl of **EasyPGX Enzyme** into the 1.5 ml twist-lock tube (not provided).
- Mix thoroughly by vortexing for 10 seconds and inverting the microcentrifuge tube 2-3 times, then centrifuge for 10 seconds using the **EasyPGX® centrifuge/vortex 1.5 ml**.
- Verify that the material is completely included into the emulsion and incubate at 56°C for 1 hour at 1400 rpm in the **EasyPGX® dry block**.
- Remove the 1.5 ml twist-lock tube, set the **EasyPGX® dry block** at 95°C and wait the instrument to reach the temperature.
- Mix 10 seconds by vortexing and incubate the 1.5 ml twist-lock tube at 95°C for 10 minutes (without shaking).

- Remove the 1.5 ml twist-lock tube and centrifuge it for 10 seconds.
- Transfer the lower phase into a new 1.5 ml twist-lock tube (not provided) paying attention to not withdraw the paraffin of the supernatant and the tissue debris.
- Use **2 µl** to do a 1:100 dilution with the provided **WATER** (2 µl extracted DNA + 220 µl **WATER**, the excess of water doesn't affect the final dilution) that will be use as the template for the PCR reaction or store the extracted DNA at ≤-20°C, divided into aliquots in order to maintain the experimental conditions constant in case of repetition.

***Use of the other recommended kits (see "Materials Required but Not Provided")***

- 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 or for pre-processing of plasma samples.
- 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 ≤-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 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, the suitability of the extracted DNA should be based on the **NRAS ctrl mix (position 8)** considering that the optimal amount of 10-20 ng/reaction corresponds to Cq (Quantification Cycle) values FAM ~ 25.

## INSTRUMENT SETUP

***EasyPGX® qPCR instrument 96***

- Follow the instructions indicated in the instrument user manual to import the correct template with the following plate setup and thermal profile:

**Plate Setup**

- All the 96 positions selected, Well type: "Unknown" and Add Dyes: "FAM" and "HEX". Click Sync Plate.

**Thermal Profile**

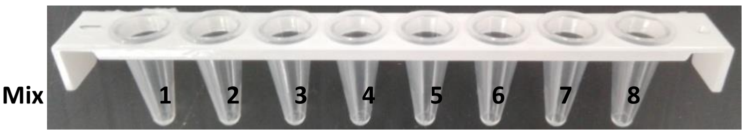
| Step | Temperature/Time |
|---|---|
| Hot Start (1 Cycle) | 95°C for 5 minutes |
| Amplification (40 Cycles) | 95°C for 15 seconds |
| | 56°C for 15 seconds |
| | 60°C for 45 seconds (Data Collection) |

## AMPLIFICATION AND MUTATION DETECTION

ⓘ Perform this step in the area dedicated to PCR 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.

ⓘ Each sample must be amplified with all the 8 different dry mixes contained in one **EasyPGX NRAS strip**: **NRAS G12x-G13x (position 1)**, **NRAS A59x-Q61H (position 2)**, **NRAS Q61K (position 3)**, **NRAS Q61R (position 4)**, **NRAS Q61L (position 5)**, **NRAS K117x (position 6)**, **NRAS A146x (position 7)**, **NRAS ctrl (position 8)**.

ⓘ The number imprinted on the top and the small hole on the bottom of each strip indicate respectively the mix position 1 and the mix position 8.




ⓘ The kit content is optimized to analyze 10 clinical samples and 2 controls (**EasyPGX NRAS pos ctrl** and **WATER**) in each run.

BEFORE TO START:

- Centrifuge for 10 seconds the needed number of **EasyPGX NRAS strips** using the **EasyPGX® centrifuge/vortex 8-well strips** considering that each run must include at least one amplification negative control (**WATER**) and one amplification positive control (**EasyPGX NRAS pos ctrl**).
- Verify that the dry cakes are on the bottom of each well of the **EasyPGX NRAS strips**.
- Centrifuge for 10 seconds the **EasyPGX NRAS pos ctrl** and resuspend it by adding 700 µl of the provided **WATER**. Vortex carefully for 10 seconds and then centrifuge for 10 seconds (perform this step in the area dedicated to DNA isolation and dilution, using dedicated materials and instruments). To achieve a complete resuspension of the dry cake, after adding **WATER**, store the liquid positive control at room temperature for 30 minutes before use.
- Identify uniquely each strip. Please pay attention because in each package more than one strip may have the same number imprinted on the top.
- Setup the sample grid:

**EasyPGX® qPCR instrument 96 (sample grid 96)**

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

| | X |
|---|---|
| 1 | G12x-G13x mix (pos1) |
| 2 | A59x-Q61H mix (pos2) |
| 3 | Q61K mix (pos3) |
| 4 | Q61R mix (pos4) |
| 5 | Q61L mix (pos5) |
| 6 | K117x mix (pos6) |
| 7 | A146x mix (pos7) |
| 8 | ctrl mix (pos8) |

**X = Number imprinted on the top of the strip**

- Gently remove the seals from the strips paying attention to not get out the dry cakes.
- Add to the respective strip:

| | | |
|---|---|---|
| negative control | 25 µl **WATER** | CONTROL - |
| sample | 25 µl DNA | |
| positive control | 25 µl of the resuspended **EasyPGX NRAS pos ctrl** | CONTROL + |

- Close carefully all the strips using the **8-strip flat optical caps**. Verify that all caps are correctly closed.
- Centrifuge the strips for 10 seconds.
- Check that the thermal profile is setted up correctly and start the run.

ⓘ It is strongly recommended to use as negative control the **WATER** provided with the kit and in particular those aliquots to be used exclusively as negative control in the PCR reaction.

ⓘ Before starting the run, check the right 8-well strip orientation on the instrument block: the number imprinted on the strip must be on the top

ⓘ Before starting the run, if the instrument plate is not completely full, balance the plate with 8-well strips on columns 1 and 12.

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

## DATA ANALYSIS

*General reccomandations regarding both instruments*

ⓘ Data analysis can be performed automatically using the **EasyPGX® analysis software** version 1.1.0 or above (code RT800-SW, Diatech Pharmacogenetics) or manually using the following procedure.

ⓘ Analyze first the negative control **WATER** and the positive control **EasyPGX 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.

ⓘ It is necessary to verify that the Cq 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.

ⓘ Only the samples that are suitable for quality/quantity of DNA (**NRAS ctrl mix (position 8)**) can be analyzed for the presence of mutations.

- At the end of the run click Plate Setup, and enter the sample names.
- Click Graphical Displays and in the box Threshold Fluorescence set threshold values 50 for both FAM and HEX channel.
- Click on the Lock icon (in this way the selected values cannot be modified). Set default values for all the other parameters.
- In the box Result Table, click Column Options, Select All and Ok to have the results in both channels with their respective Cq (ΔR) and ΔR last values.
- Click File and Save.

1. Analysis of reaction controls

| | **Cq FAM** | **ΔR last FAM** | | | | | | | | **Cq HEX** | **ΔR last HEX** | **Results** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **All mixes** | **G12x-G13x mix** | **A59x-Q61H mix** | **Q61K mix** | **Q61R mix** | **Q61L mix** | **K117x mix** | **A146x mix** | **ctrl mix** | **All mixes** | | |
| **WATER** | > 32 | \ | \ | \ | \ | \ | \ | \ | \ | > 30 | \ | Proceed with analysis of the samples. |
| | ≤ 32 | < 100 | < 400 | < 300 | < 200 | < 100 | < 350 | < 350 | < 300 | ≤ 30 | < 250 | |
| | ≤ 32 | ≥ 100 | ≥ 400 | ≥ 300 | ≥ 200 | ≥ 100 | ≥ 350 | ≥ 350 | ≥ 300 | ≤ 30 | ≥ 250 | Possible contamination: it is not possible to analyze the samples (see Troubleshooting). |
| **EasyPGX NRAS pos ctrl** | 16 ≤ Cq ≤ 29 | ≥ 100 | ≥ 400 | ≥ 300 | ≥ 200 | ≥ 100 | ≥ 350 | ≥ 350 | ≥ 300 | 18 ≤ Cq ≤ 27 | ≥ 250 | Proceed with analysis of the samples. |
| | Cq < 16 | ≥ 100 | ≥ 400 | ≥ 300 | ≥ 200 | ≥ 100 | ≥ 350 | ≥ 350 | ≥ 300 | Cq < 18 | ≥ 250 | Probably excess of DNA. Proceed with analysis of the samples. |
| | 16 ≤ Cq ≤ 29 | < 100 | < 400 | < 300 | < 200 | < 100 | < 350 | < 350 | < 300 | 18 ≤ Cq ≤ 27 | < 250 | Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting). |
| | Cq > 29 | \ | \ | \ | \ | \ | \ | \ | \ | Cq > 27 | \ | |

\ Any value

2. Analysis of **NRAS ctrl mix (position 8)** for the suitability of the DNA quality/quantity

| NRAS ctrl mix | Cq FAM | ∆R last FAM | Cq HEX[1] | ∆R last HEX[1] | Results |
|---|---|---|---|---|---|
| **DNA samples** | 21 ≤ Cq ≤ 30 | ≥ 300 | 19 ≤ Cq ≤ 28 | ≥ 250 | Suitable sample. Proceed with the mutation analysis. |
| | Cq < 21 | ≥ 300 | Cq < 19 | ≥ 250 | Excess of DNA.<br>YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION.<br>Samples must be diluted with **WATER** so that Cq fall in the ranges indicated above. Consider that the dilution 1:2 of the DNA increases the Cq of 1 unit. |
| | 21 ≤ Cq ≤ 30 | < 300 | 19 ≤ Cq ≤ 28 | < 250 | Suboptimal amount of starting DNA or PCR inhibition.<br>YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION:<br>▪ Proceed with a new DNA extraction to obtain an higher concentration of template and/or a DNA of higher quality.<br>▪ If the presence of inhibitors is suspected, dilute the sample with **WATER**. Consider that the dilution reduces the presence of inhibitor, but decreases also the concentration of the target DNA. |
| | Cq > 30 | \ | Cq > 28 | \ | |

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

3. Analysis of the mutation assays (both the ΔCq and the ΔR last criteria must be satisfied)

ⓘ Only samples that are suitable for DNA quality/quantity 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 (on both FAM and HEX channel) for the mutation and the equivalent for the control assay belongs to the same sample:

ΔCq **Internal Control** = Cq **HEX** (mutation mix) – Cq **HEX** (ctrl mix)

ΔCq **Mutation** = Cq **FAM** (mutation mix) – Cq **FAM** (ctrl mix)

| Assay | ΔCq Internal Control (HEX) | ΔR last Internal Control (HEX) | ΔCq Mutation (FAM) | ΔR last Mutation (FAM) | Results |
|---|---|---|---|---|---|
| G12x-G13x | ± 3 | ≥ 250 | ≤ 6 | ≥ 100 | G12x-G13x positive |
| A59x-Q61H | | | ≤ 7.5 | ≥ 400 | A59x-Q61H positive |
| Q61K | | | ≤ 8 | ≥ 300 | Q61K positive |
| Q61R | | | ≤ 8 | ≥ 200 | Q61R positive |
| Q61L | | | ≤ 10 | ≥ 100 | Q61L positive |
| K117x | | | ≤ 9 | ≥ 350 | K117x positive |
| A146x | | | ≤ 9 | ≥ 350 | A146x positive |
| G12x-G13x | ± 3 | ≥ 250 | > 6 | \ | Wild type sample or beneath the LOD[1] |
| A59x-Q61H | | | > 7.5 | \ | |
| Q61K | | | > 8 | \ | |
| Q61R | | | > 8 | \ | |
| Q61L | | | > 10 | \ | |
| K117x | | | > 9 | \ | |
| A146x | | | > 9 | \ | |
| G12x-G13x | ± 3 | ≥ 250 | ≤ 6 | < 100 | |
| A59x-Q61H | | | ≤ 7.5 | < 400 | |
| Q61K | | | ≤ 8 | < 300 | |
| Q61R | | | ≤ 8 | < 200 | |
| Q61L | | | ≤ 10 | < 100 | |
| K117x | | | ≤ 9 | < 350 | |
| A146x | | | ≤ 9 | < 350 | |
| G12x-G13x | < -3 or > +3 | \ | \ | \ | Failed: not sufficient template/PCR inhibition/mistake during samples dispensation |
| A59x-Q61H | | | \ | \ | |
| Q61K | | | \ | \ | |
| Q61R | | | \ | \ | |
| Q61L | | | \ | \ | |
| K117x | | | \ | \ | |
| A146x | | | \ | \ | |
| G12x-G13x | \ | < 250 | \ | \ | |
| A59x-Q61H | | | \ | \ | |
| Q61K | | | \ | \ | |
| Q61R | | | \ | \ | |
| Q61L | | | \ | \ | |
| K117x | | | \ | \ | |
| A146x | | | \ | \ | |

1. LOD = Limit Of Detection
\ Any value

ⓘ To confirm the mutation, check that normalized fluorescence at cycle 40 derives from a real amplification reaction (sigmoidal fluorescence curve) and not from an artifact (linear fluorescence curve).

ⓘ If multiple assays show a ΔCq 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 ΔCq.

ⓘ If a sample fails for one or more assays, to confirm the results, it should be retested with all the mixes.

## TROUBLESHOOTING

| Problem | Possible reason | Recommendation |
|---|---|---|
| Low or absent amplification signal in the channel “FAM” and/or in the channel “HEX” for both **EasyPGX NRAS 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 setting of the thermal profile. | ▪ Check the temperature profile and repeat amplification with the settings described in this manual. |
| | Incorrect resuspension/storage of the **EasyPGX NRAS pos ctrl**. | ▪ Add 700 µl of the provided **WATER** to a new aliquot. Vortex and centrifuge for 10 seconds.<br>▪ Store the resuspended **EasyPGX NRAS pos ctrl** at -35/-20°C and avoid thawing and refreezing more than four times. |
| | Reagents improperly stored or expired. | ▪ Protect the 8-well strips from light in their original package with desiccant sachet.<br>▪ Store the 8-well strips at +2/+25°C.<br>▪ Once a **EasyPGX NRAS PCR strips** package is opened, store it at +2/+8°C and use the contained strips within 2 months and within the expiration date.<br>▪ Once the dry mixes are resuspended, use immediately the 8-well strips in the PCR reaction.<br>▪ Do not use expyred reagents. |
| Cq values for "FAM" and "HEX" channels ouside the acceptability range for ctrl assay. **EasyPGX NRAS pos ctrl** is within the expected values. | Excess of DNA | ▪ Samples must be diluted with **WATER** so that Cq fall in the ranges indicated in the section analysis of the ctrl mix. Consider that the dilution 1:2 of the DNA increases the Cq of 1 unit. |
| | Insufficient amount of starting DNA and / or presence of PCR inhibitors. | ▪ When using **EasyPGX® Extraction reagents** contained in the kit:<br>1) if it is assumed that the amount of starting DNA is insufficient repeat the amplification diluting the sample 1:50 with **WATER** or repeat the DNA extraction;<br>2) if you suspect the presence of inhibitors, repeat the amplification diluting the sample 1:200 with **WATER**.<br>▪ When using other recommended reagents not included in the kit for DNA extraction and purification:<br>1) check the quantity and quality of the extracted DNA e.g with the spectrophotometer and, if not appropriate, repeat the extraction faithfully following the instructions of the extraction kit;<br>2) 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;<br>3) if it is assumed that the amount of starting DNA is insufficient, repeat the DNA extraction by reducing the volume of elution or the diluition factor;<br>4) if you suspect the presence of inhibitors, repeat the amplification diluting the sample 1:5 or 1:10 with **WATER**. |
| | Incorrect or no dispensation of the samples. | ▪ Repeat the amplification dispensing the correct volume of DNA and including positive and negative controls. |
| Amplification signal weak or absent in "FAM" and "HEX" only for the positive control **EasyPGX NRAS pos ctrl**. | Degradation of the **EasyPGX NRAS pos ctrl**. | ▪ Repeat the amplification resuspending and testing a new aliquot of **EasyPGX NRAS pos ctrl**. |
| | Incorrect or failure dispensation of the **EasyPGX NRAS pos ctrl**. | ▪ Repeat the amplification by pipetting the appropriate volume of **EasyPGX NRAS pos ctrl**. |
| For **EasyPGX® qPCR instrument 96** “HEX” signal is not correcty normalized in one or more than one well. | Wrong normalization of fluorescence in “HEX” channel. | ▪ In Plate Setup select all samples except negative control **WATER**.<br>▪ In Analysis - Graphical Displays deselect “FAM” channel by clicking Display Targets.<br>▪ In Analysis – Graphical Displays section Amplification Plots – Baseline Correction, click Adjust – Select All, set Start Cycle: 3, End Cycle:15 then click Apply - OK.<br>▪ Check that the “HEX” fluorescence is correctly normalized, select “FAM” channel and repeat the analysis including also negative control **WATER.** |

| | | |
|---|---|---|
| | | Wrongly normalized fluorescence / Correctly normalized fluorescence |
| The positive control **EasyPGX NRAS pos ctrl** shows no amplification signal in the channel "FAM" for the assays of mutations or the signal is detectable only for some mixes; while one or more samples show a signal amplification in all assays for mutations. | Wrong 8-well strip identification. | ▪ Repeat the amplification after marking unambiguously the reaction strips for samples and controls. |
| | Incorrect dispensing of the samples. | ▪ Repeat the amplification paying attention to the dispensation of the DNA and the **EasyPGX NRAS pos ctrl** in the 8-well reaction strips. |
| | Incorrect samples names set-up in the software. | ▪ Check samples names set-up. |
| One sample shows HEX Cq values different from each other for the assays. | DNA dispensation error. | ▪ Repeat the amplification paying attention to the dispensation of the DNA in the 8-well reaction strips. |
| The negative control **WATER**, shows an amplification signal in both "FAM" and "HEX" channel. | Contamination. | ▪ The results shall be rejected and samples must be reamplified using new reagents.<br>▪ Prepare the PCR reaction in a dedicated area. Carefully decontaminate benches, pipettes and instruments. |
| Fluorescence intensity variable. | Cutaneous fat on the tubes. | ▪ Wear gloves. |

If the problems persist despite the implementation of the given recommendations and for any further questions or problems, please contact the Diatech Pharmacogenetics technical support:

- e-mail support@diatechpharmacogenetics.com
- telephone +39 0731 213243
- fax +39 0731 213239

## PERFORMANCE VALIDATION

Performance validation has been performed using all the reagents included in the "**EasyPGX® ready NRAS**" kit.
The experiments have been performed according to the instructions reported in this user manual on the following instruments and accessories:

- **EasyPGX® qPCR instrument 96** - Diatech Pharmacogenetics (96-well plate)
- **EasyPGX® dry block** - Diatech Pharmacogenetics
- **EasyPGX® centrifuge/vortex 1.5 ml** - Diatech Pharmacogenetics
- **EasyPGX® centrifuge/vortex 8-well strips** - Diatech Pharmacogenetics

### Clinical specificity

In order to evaluate the kit sensitivity and specificity, DNA samples isolated from FFPE tumor tissue and from plasma have been tested. Samples were suitable in terms of starting DNA amount and for the presence of mutations detected by the kit, and have been already genotyped through Pyrosequencing technology ("**Anti-EGFR MoAb response® (NRAS status)",** code UP038 - Diatech Pharmacogenetics), or by MALDI-TOF Mass Spectrometry using MassArray® platform ("**Myriapod® Colon Status**", code SQ010 and "**Myriapod® Lung Status**" code SQ011 - Diatech Pharmacogenetics), or with **Easy® NRAS** (code RT004 - Diatech Pharmacogenetics) or with direct sequencing. If no FFPE or plasma samples were available, Horizon Diagnostics standards, cell lines or plasmids have been tested.

| | **EasyPGX® qPCR instrument 96** | | |
|---|---|---|---|
| Assay | N° mutant samples tested | N° wild-type samples tested | N° samples correctly genotyped |
| NRAS G12x-G13x | 5 | 21 | 26/26 |
| NRAS A59x-Q61H | 1 | 25 | 26/26 |
| NRAS Q61K | 3 | 23 | 26/26 |
| NRAS Q61R | 3 | 23 | 26/26 |
| NRAS Q61L | 1 | 25 | 26/26 |
| NRAS K117x | 3* | 26 | 29/29 |
| NRAS A146x | 2* | 26 | 28/28 |
| * FFPE or plasma samples not available: Horizon Diagnostics standards, 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 at least 95% of tests.
To determine the LOD, samples with different percentage of mutation and with a medium input DNA concentration (12.5 ng/reaction) have been tested.
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 "**EasyPGX® ready NRAS**" kit, considering all the instruments tested is:

| **Assay** | **LOD $C_{95}$ (at medium DNA input concentration)** |
|---|---|
| G12x-G13x | 7.0% |
| A59x-Q61H | 1.0% |
| Q61K | 0.5% |
| Q61R | 0.5% |
| Q61L | 0.5% |
| K117x | 2.0% |
| A146x | 5.0% |

### Reproducibility

System reproducibility (*inter-assay* variability) has been evaluated analyzing the data deriving from three independent runs with Horizon Diagnostics standards (12.5 ng/reaction). Results are 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 duplicates of Horizon Diagnostics standards (12.5 ng/reaction) from three independent runs. Results are repeatable in terms of genotyping for all assays and samples analyzed.

**Robustness**

Freeze-drying cycles

Two different batches of **EasyPGX NRAS strips** have been prepared. The freeze-dried strips have been tested with the same Horizon Diagnostics standards (12.5 ng/reaction). Results from different batches are comparable in terms of genotyping.

Lot to lot consistency

Two different batches of primers and probes have been tested with the same Horizon Diagnostics standards (12.5 ng/reaction). Results from the different batches are comparable in terms of genotyping.

Three different batches of master mixes have been tested with the same Horizon Diagnostics standards (12.5 ng/reaction). Results from the different batches are comparable in terms of genotyping.

Two different batches of **EasyPGX Dep solution, EasyPGX buffer** and three different batches of **EasyPGX Enzyme** have been tested with the same Horizon Diagnostics FFPE standards (1 x 15-20um section). Results from the different batches are comparable in terms of quality/quantity of extracted DNA.

# EasyPGX® ready NRAS - sample grid 96

**Appendix A – sample grid 96**

| DATE | | RUN NAME | |
|---|---|---|---|

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

| X |
|---|
| G12x-G13x mix (pos1) |
| A59x-Q61H mix (pos2) |
| Q61K mix (pos3) |
| Q61R mix (pos4) |
| Q61L mix (pos5) |
| K117x mix (pos6) |
| A146x mix (pos7) |
| ctrl mix (pos8) |

| INSTRUMENT (s/n) | | | | USER MANUAL version | |
|---|---|---|---|---|---|
| PRODUCT CODE | RT024 | LOT | | EXPIRY DATE | |
| NOTES | | | | | |
| OPERATOR | | | | SIGN | |

**OPERATOR NOTES**

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