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EasyPGX® ready DPYD

User manual – version 2018/08

The "**EasyPGX® ready DPYD**" kit detects the main polymorphisms associated with the toxicity due to the treatment with fluoropyrimidines by Real-Time PCR.

For *in vitro* diagnostic use

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Index **page**

INTENDED USE

The *in vitro* diagnostic "**EasyPGX® ready DPYD**" kit is intended for the detection by Real-Time PCR of the *DPYD* gene polymorphisms in human genomic DNA extracted from whole blood.

List of detectable polymorphisms:

Allele	Reference SNP	HGVS	AA Variation
DPYD*2A	rs3918290	c.1905+1G>A	IVS14+1G>A
DPYD*13	rs55886062	c.1679T>G	p.I560S
\	rs67376798	c.2846A>T	p.D949V
\	rs75017182	c.1129–5923C>G	IVS10C>G, HapB3

The "**EasyPGX® ready DPYD**" 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

PRINCIPLE OF THE ASSAY

The "**EasyPGX® ready DPYD**" 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 whole blood.

The "**Easy® DPYD**" kit includes four assays for the detection of the polymorphisms of the *DPYD* gene by allelic discrimination.

Each assay contains primers and probes for the detection of mutant sequence (FAM) as well as the wild-type sequence (HEX).

Homozygous mutant sample generates a signal in the FAM channel, wild-type sample in HEX channel. Amplification signal for both FAM and HEX channel indicates an heterozygous sample.

1. **DPYD*2A**: the assay detects the polymorphism rs3918290, c.1905+1G>A, IVS14+1G>A
2. **DPYD*13**: the assay detects the polymorphism rs55886062, c.1679T>G, p.I560S
3. **DPYD D949V**: the assay detects the polymorphism rs67376798, c.2846A>T, p.D949V
4. **DPYD IVS10**: the assay detects the polymorphism rs75017182, c.1129–5923C>G, IVS10C>G

CLINICAL RELEVANCE

Molecular evaluation of the mutational status of the *DPYD* gene is used at the clinical level for the personalization of fluoropymidine chemotherapy.
Although fluoropyrimidine treatment is generally well tolerated, severe and sometimes lethal toxicity has been reported in some patients.

Several prospective and retrospective studies as well as meta-analysis have shown a correlation between variants of the *DPYD* gene, causing a reduction of the enzyme dihydropimidine dehydrogenase (DPD), and the risk of serious toxicity associated with fluoropyrimidine treatment. The DPD enzyme, in fact, plays a primary role in the catabolic inactivation of fluoropyrimidines and therefore in determining the levels of active drug available in the patient. Increased bioavailability of the drug can cause serious haematological, neurological and gastrointestinal toxicity.

Currently four polymorphisms of the *DPYD* gene are validated as clinical markers for the evaluation of the toxicity correlated with the chemotherapeutic treatment with fluoropyrimidine [1, 2]: c.1905+1G>A (DPYD*2A, rs3918290, IVS14+1G>A), c.1679T>G (DPYD*13, rs55886062, p.I560S), c.2846A>T (rs67376798, p.D949V) and c.1129-5923C>G (rs75017182, IVS10C>G)

Recent studies have characterized the phenotypic impact of each *DPYD* mutation on the activity of the enzyme DPD and have stratified variants based on their effect on enzyme activity. This allowed to define a score related to the enzymatic activity, on the basis of which guidelines for the customization of fluoropyrimidine dosage have been defined [2, 3, 4].

The evaluation of polymorphisms of the *DPYD* gene can therefore significantly reduce the risk of toxicity of fluoropyrimidine treatment.

References
1. *DPYD genotype-guided dose individualization to improve patient safety of fluoropyrimidine therapy: call for a drug label update*. L.M. Henricks et al. Annals of oncology 28:2945-2922, 2017.
2. *Clinical Pharmacogenetics Implementation Consortium (CPIC) Guideline for Dihydropyrimidine Dehydrogenase Genotype and Fluoropyrimidine Dosing: 2017 Update*. U. Amstutz et.al Clinical Pharmacology and therapeutics vol.0, n.0, 2017
3. *Raccomandazioni per analisi farmacogenetiche*, AIOM-SIF, 2015.
4. https://www.pharmgkb.org/gene/PA145

KIT CONTENTS

The "**EasyPGX® ready DPYD**" 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 whole blood.
The kit contains sufficient reagents to carry out 48 tests.

Destination **EXTRACTION AREA** Storage temperature **+2/+25°C**		
EasyPGX® Blood Extraction reagents		
COMP	QUAN	
EasyPGX Buffer	1 x 6 ml	Buffer for DNA extraction from whole blood
EasyPGX Enzyme	1 x 600 µl	Enzyme for DNA extraction from whole blood
PBS	2 x 30 ml	Phosphate Buffered Saline for the washing of the cellular pellet
Destination **AMPLIFICATION AREA** Storage temperature **+2/+25°C**		
COMP	QUAN	
EasyPGX DPYD strips	2 x 12 strips	CLEAR 8-well strips: 8 dry complete mixtures containing specific primers and probes targeting the following *DPYD* polymorphisms: **Position 1** and **5: DPYD*2A** **Position 2** and **6: DPYD*13** **Position 3** and **7: DPYD D949V** **Position 4** and **8**: **DPYD IVS10**
8-strip flat optical caps	1 x 25 strips	0.2 ml 8-tube cap strips DNase-, RNase-free to be used to recap the **EasyPGX DPYD strips**
EasyPGX® Pos & Neg Controls		
COMP	QUAN	
EasyPGX DPYD WT pos ctrl CONTROL +	4 tubes	DNA positive control in a dry format containing a mixture of synthetic wild-type DNA sequences for the analyzed DPYD polymorphisms. Every aliquot must be resuspended with 400 µl of **WATER** before the use.
EasyPGX DPYD MT pos ctrl CONTROL +	4 tubes	DNA positive control in a dry format containing a mixture of synthetic mutant DNA sequences for the analyzed DPYD polymorphisms. Every aliquot must be resuspended with 400 µl of **WATER** before the use.
WATER CONTROL –	8 x 1.5 ml	DNase-, RNase-free water, 2 aliquot to be used exclusively to resuspend the dry positive controls, 1 aliquot to be used exclusively as negative control in the PCR reaction and 5 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 DPYD**" - User Manual.
- Template for **EasyPGX® qPCR instrument 96**: "*RT026_template*".
- 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 DPYD**" kit contains reagents for DNA extraction from from whole blood.

Required accessories:

- **EasyPGX® dry block** (code RT801, Diatech Pharmacogenetics)
- **EasyPGX® centrifuge/vortex 1.5 ml** (code RT802, Diatech Pharmacogenetics)
- Benchtop centrifuge (maximum speed required 10000xg)

Other recommended options for DNA extraction and purification:

- "**QIAamp® DNA Mini kit**" (code 51304, Qiagen)
- "**Genomic DNA Whole Blood Kit (Speedy installation)**" (cod. MGB400-02, RBC); to be used with MagCore Automated Nucleic Acid Extractor systems (RBC Bioscience)

ⓘ 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 instruments:

- **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.

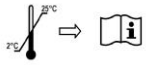
EasyPGX® Blood Extraction reagents

- 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 DPYD 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 DPYD WT pos ctrl** and **EasyPGX DPYD MT 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

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

PRODUCT USE LIMITATIONS

- The "**EasyPGX® ready DPYD**" 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 DPYD**" kit has been designed and validated for the use with the real-time qPCR instruments **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 DPYD**" 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.

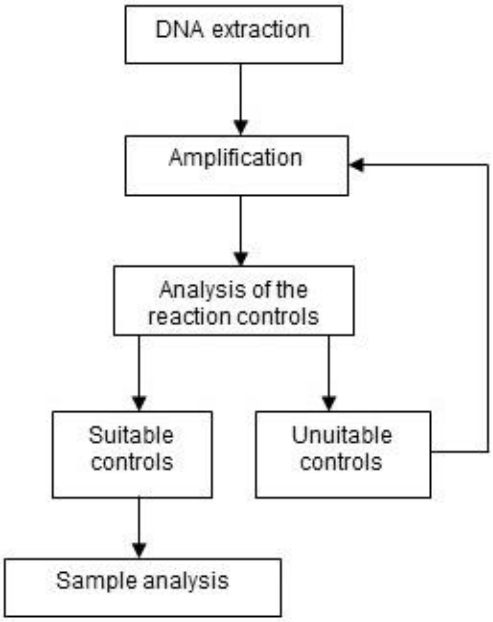
QUALITY ASSURANCE

- The "**EasyPGX® ready DPYD**" 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 DPYD**" 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 DPYD**" 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 DPYD**" 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 2 positive controls (**EasyPGX DPYD WT pos ctrl, EasyPGX DPYD MT 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 "**EasyPGX® ready DPYD**" kit includes the reagents for DNA extraction from whole blood.

Use of EasyPGX® Extraction reagents

- Set the **EasyPGX® dry block** at 56°C and wait the instrument to reach the temperature.
- Transfer 200 µl of whole blood into a 1.5 ml microcentrifuge tube (not provided).
- Centifuge for 5 minutes at 10000xg. Discard the supernatant.
- Add 1 ml of **PBS** and mix 10 seconds by vortexing.
- Centifuge for 5 minutes at 10000xg. Discard the supernatant.
- Gently mix by invertion the **EasyPGX buffer** and add 100 µl into the 1.5 ml twist-lock tube with the cellular pellet on the bottom.
- Add 10 µl of **EasyPGX® Enzyme** into the same 1.5 ml twist-lock tube.
- Mix thoroughly by vortexing for 10 seconds, then centrifuge for 10 seconds using the **EasyPGX® centrifuge/vortex 1.5 ml**.
- 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 supernatan into a new 1.5 ml twist-lock tube (not provided) paying attention to not withdraw the cellular debris on the bottom.
- Use **20 µl** to prepare a 1:10 dilution with the provided **WATER** (20 µl extracted DNA + 180 µl **WATER**) 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 blood required for the DNA extraction depends on protocols.
- 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 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.
- The recommend amount of DNA for each amplification reaction is 10-500 ng.

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.

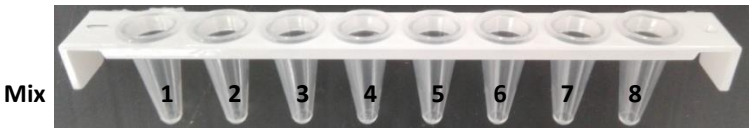
Posizione	Well Type	Dye	Target Name
Line A and E	Unknown	FAM	*2A MUT
		HEX	*2A WT
Line B and F	Unknown	FAM	*13 MUT
		HEX	*13 WT
Line C and G	Unknown	FAM	D949V MUT
		HEX	D949V WT
Line D and H	Unknown	FAM	IVS10 MUT
		HEX	IVS10 WT

Thermal Profile

Step	Temperature/Time
Hot Start (1 Cycle)	95°C for 5 minutes
Amplification (40 Cycles)	95°C for 15 seconds
	61°C for 1 minute (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 4 different dry mixes contained in the **EasyPGX DPYD strips** (from mix 1 to mix 4 or from mix 5 to mix 8): **DPYD*2A (position 1** and **5)**, **DPYD*13 (position 2** and **6)**, **DPYD D949V (position 3** and **7)**, **DPYD IVS10 (position 4** and **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 21 clinical samples and 3 controls (**EasyPGX DPYD WT pos ctrl**, **EasyPGX DPYD MT pos ctrl** and **WATER**) in each run.

BEFORE TO START:

- Centrifuge for 10 seconds the needed number of **EasyPGX DPYD strips** using the **EasyPGX® centrifuge/vortex 8-well strips** considering that each run must include at least one amplification negative control (**WATER**) and two amplification positive controls (**EasyPGX DPYD WT pos ctrl** and **EasyPGX DPYD MT pos ctrl**).
- Verify that the dry cakes are on the bottom of each well of the **EasyPGX DPYD strips**.
- Centrifuge for 10 seconds the **EasyPGX DPYD WT pos ctrl** and **EasyPGX DPYD MT pos ctrl** and resuspend them by adding 400 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	DNA3	DNA5	DNA7	DNA9	DNA11	DNA13	DNA15	DNA17	DNA19	DNA21	POS MT
B	DNA1	DNA3	DNA5	DNA7	DNA9	DNA1	DNA13	DNA15	DNA17	DNA19	DNA21	POS MT
C	DNA1	DNA3	DNA5	DNA7	DNA9	DNA11	DNA13	DNA15	DNA17	DNA19	DNA21	POS MT
D	DNA1	DNA3	DNA5	DNA7	DNA9	DNA11	DNA13	DNA15	DNA17	DNA19	DNA21	POS MT
E	DNA2	DNA4	DNA6	DNA8	DNA10	DNA12	DNA14	DNA16	DNA18	DNA20	POS WT	WATER
F	DNA2	DNA4	DNA6	DNA8	DNA10	DNA12	DNA14	DNA16	DNA18	DNA20	POS WT	WATER
G	DNA2	DNA4	DNA6	DNA8	DNA10	DNA12	DNA14	DNA16	DNA18	DNA20	POS WT	WATER
H	DNA2	DNA4	DNA6	DNA8	DNA10	DNA12	DNA14	DNA16	DNA18	DNA20	POS WT	WATER

	X
1	DPYD*2A mix (pos1)
2	DPYD*13 mix (pos2)
3	DPYD D949V mix (pos3)
4	DPYD IVS10 mix (pos4)
5	DPYD*2A mix (pos5)
6	DPYD*13 mix (pos6)
7	DPYD D949V mix (pos7)
8	DPYD IVS10 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 DPYD WT pos ctrl**	CONTROL +
positive control	25 µl of the resuspended **EasyPGX DPYD MT 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 3.0.0 or above (code RT800-SW, Diatech Pharmacogenetics) or manually using the following procedure.

ⓘ Analyze first the negative control **WATER** and the positive controls **EasyPGX DPYD WT pos ctrl**, **EasyPGX DPYD MT 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.

- At the end of the run click Plate Setup, and enter the sample names.
- Click Graphical Displays and in the box Threshold Fluorescence set the following threshold values for FAM and HEX channel:

Assay	Threshold FAM (MUT)	Threshold HEX (WT)
DPYD*2A	350	350
DPYD*13	250	200
DPYD D949V	750	1300
DPYD IVS10	300	200

- 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).
- Click File and Save.

1. Analysis of reaction controls

	Assay	Cq FAM (MUT)	Cq HEX (WT)	Results
WATER	**DPYD*2A**	Cq > 35	Cq > 35	Proceed with analysis of the samples.
	DPYD*13	Cq > 35	Cq > 34	
	DPYD D949V	Cq > 35	Cq > 36	
	DPYD IVS10	Cq > 34	Cq > 35	
	DPYD*2A	Cq ≤ 35	Cq ≤ 35	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
	DPYD*13	Cq ≤ 35	Cq ≤ 34	
	DPYD D949V	Cq ≤ 35	Cq ≤ 36	
	DPYD IVS10	Cq ≤ 34	Cq ≤ 35	

- Calculate, for each assay, the **ΔCq FAM-HEX** values as reported below, taking care that the Cq values (for both channels FAM-MUT and HEX-WT of the same assay) belong to the same positive control (consider No Cq as 40):

ΔCq FAM-HEX = Cq **FAM (MUT**) – Cq **HEX (WT)**

- Compare **Cq FAM**, **Cq HEX** and **ΔCq FAM-HEX** values of the controls with those reported in the following table:

	Assay	Cq FAM (MUT)	Cq HEX (WT)	ΔCq FAM-HEX	Results
EasyPGX DPYD WT pos ctrl	**DPYD*2A**	Any value	23 ≤ Cq ≤ 28	ΔCq > 0	Proceed with analysis of the samples.
	DPYD*13		18 ≤ Cq ≤ 24	ΔCq > 3	
	DPYD D949V		23 ≤ Cq ≤ 28	ΔCq > 1	
	DPYD IVS10		19 ≤ Cq ≤ 25	ΔCq > 4	
	DPYD*2A		Cq < 23	ΔCq > 0	Probably excess of DNA. Proceed with analysis of the samples.
	DPYD*13		Cq < 18	ΔCq > 3	
	DPYD D949V		Cq < 23	ΔCq > 1	
	DPYD IVS10		Cq < 19	ΔCq > 4	
	DPYD*2A	All the other conditions			Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	DPYD*13				
	DPYD D949V				
	DPYD IVS10				
EasyPGX DPYD MT pos ctrl	**DPYD*2A**	23 ≤ Cq ≤ 33	Any value	ΔCq < -5	Proceed with analysis of the samples.
	DPYD*13	19 ≤ Cq ≤ 32		ΔCq < -2	
	DPYD D949V	23 ≤ Cq ≤ 34		ΔCq < -5	
	DPYD IVS10	23 ≤ Cq ≤ 33		ΔCq < -3	
	DPYD*2A	Cq < 23		ΔCq < -5	Probably excess of DNA. Proceed with analysis of the samples.
	DPYD*13	Cq < 19		ΔCq < -2	
	DPYD D949V	Cq < 23		ΔCq < -5	
	DPYD IVS10	Cq < 23		ΔCq < -3	
	DPYD*2A	All the other conditions			Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).
	DPYD*13				
	DPYD D949V				
	DPYD IVS10				

2. Analysis of the samples

- Calculate, for each assay, the **ΔCq FAM-HEX** values as reported below, taking care that the Cq values (for both channels FAM-MUT and HEX-WT of the same assay) belong to the same sample (consider No Cq as 40):

ΔCq FAM-HEX = Cq **FAM (MUT**) – Cq **HEX (WT)**

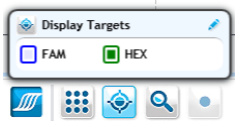
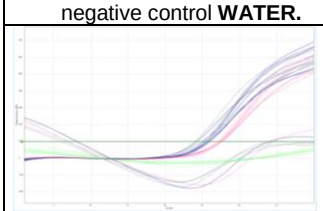
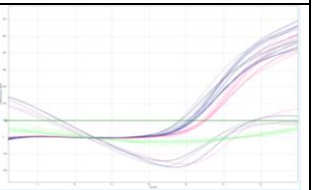
- Compare **Cq FAM**, **Cq HEX** and **ΔCq FAM-HEX** values of the controls with those reported in the following table:

	Assay	Cq FAM (MUT)	Cq HEX (WT)	∆Cq FAM-HEX	Results
Campione	DPYD*2A	Cq ≥ 20	22 ≤ Cq ≤ 35	∆Cq > 0	**WILD-TYPE**
		20 ≤ Cq ≤ 32	Cq ≥ 22	∆Cq < -5	**MUTANT**
		20 ≤ Cq ≤ 32	22 ≤ Cq ≤ 35	-5 ≤ ∆Cq ≤ 0	**HETEROZYGOUS**
		Cq < 20	\	\	Excess of DNA. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM. 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.
		\	Cq < 22	\	
		Cq > 32	Cq > 35	\	Suboptimal amount of starting DNA or PCR inhibition. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM: ▪ 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**. Consider that the dilution reduces the presence of inhibitor, but decreases also the concentration of the target DNA.
	DPYD*13	Cq ≥ 18	17 ≤ Cq ≤ 32	∆Cq > 3	**WILD-TYPE**
		18 ≤ Cq ≤ 32	Cq ≥ 17	∆Cq < -2	**MUTANT**
		18 ≤ Cq ≤ 32	17 ≤ Cq ≤ 32	-2 ≤ ∆Cq ≤ 3	**HETEROZYGOUS**
		Cq < 18	\	\	Excess of DNA. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM. 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.
		\	Cq < 17	\	
		Cq > 32	Cq > 32	\	Suboptimal amount of starting DNA or PCR inhibition. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM: ▪ 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**. Consider that the dilution reduces the presence of inhibitor, but decreases also the concentration of the target DNA.
\ Any value					

	Assay	Cq FAM (MUT)	Cq HEX (WT)	ΔCq FAM-HEX	Results
Campione	DPYD D949V	Cq ≥ 20	22 ≤ Cq ≤ 36	ΔCq > 1	**WILD-TYPE**
		20 ≤ Cq ≤ 34	Cq ≥ 22	ΔCq < -5	**MUTANT**
		20 ≤ Cq ≤ 34	22 ≤ Cq ≤ 36	-5 ≤ ΔCq ≤ 1	**HETEROZYGOUS**
		Cq < 20	\	\	Excess of DNA. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM. 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.
		\	Cq < 22	\	
		Cq > 34	Cq > 36	\	Suboptimal amount of starting DNA or PCR inhibition. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM: ▪ 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**. Consider that the dilution reduces the presence of inhibitor, but decreases also the concentration of the target DNA.
	DPYD IVS10	Cq ≥ 20	17 ≤ Cq ≤ 30	ΔCq > 4	**WILD-TYPE**
		20 ≤ Cq ≤ 31	Cq ≥ 17	ΔCq < -3	**MUTANT**
		20 ≤ Cq ≤ 31	17 ≤ Cq ≤ 30	-3 ≤ ΔCq ≤ 4	**HETEROZYGOUS**
		Cq < 20	\	\	Excess of DNA. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM. 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.
		\	Cq < 17	\	
		Cq > 31	Cq > 30	\	Suboptimal amount of starting DNA or PCR inhibition. YOU CAN NOT CONTINUE WITH THE ANALYSIS OF POLYMORPHISM: ▪ 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**. Consider that the dilution reduces the presence of inhibitor, but decreases also the concentration of the target DNA.
\ Qualsiasi valore					

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

TROUBLESHOOTING

Problem	Possible reason	Recommendation
Low or absent amplification signal in the channel “FAM” and/or in the channel “HEX” for both **EasyPGX DPYD MT pos ctrl** and/or **EasyPGX DPYD WT 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 DPYD MT pos ctrl** and/or **EasyPGX DPYD WT pos ctrl**	▪ Add 400 µl of the provided **WATER** to a new aliquot. Vortex and centrifuge for 10 seconds. ▪ Store the resuspended **EasyPGX DPYD MT pos ctrl** e/o **EasyPGX DPYD WT 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. ▪ Store the 8-well strips at +2/+25°C. ▪ Once a **EasyPGX DPYD strips** package is opened, store it at +2/+8°C and use the contained strips within 2 months and within the expiration date. ▪ Once the dry mixes are resuspended, use immediately the 8-well strips in the PCR reaction. ▪ Do not use expyred reagents.
Cq values for "FAM" and "HEX" channels ouside the acceptability range for ctrl assay. **EasyPGX DPYD MT pos ctrl** and **EasyPGX DPYD WT pos ctrl** are 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®** **Blood Extraction reagents** contained in the kit: 1) if it is assumed that the amount of starting DNA is insufficient repeat the amplification diluting the sample 1:5 with **WATER** or repeat the DNA extraction; 2) if you suspect the presence of inhibitors, repeat the amplification diluting the sample 1:50 with **WATER**. ▪ When using other recommended reagents not included in the kit for DNA extraction and purification: 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; 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; 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; 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.
“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**. ▪ In Analysis - Graphical Displays deselect “FAM” channel by clicking Display Targets.  ▪ In Analysis – Graphical Displays section Amplification Plots – Baseline Correction, click Adjust – Select All, set Start Cycle: 3, End Cycle:15 then click Apply - OK. ▪ Check that the “HEX” fluorescence is correctly normalized, select “FAM” channel and repeat the analysis including also negative control **WATER.**
		 Wrongly normalized fluorescence
		 Wrongly normalized fluorescence

Amplification signal weak or absent in "FAM" and "HEX" only for **EasyPGX DPYD MT pos ctrl** and **EasyPGX DPYD WT pos ctrl**	Degradation of the **EasyPGX DPYD MT pos ctrl** and **EasyPGX DPYD WT pos ctrl**.	▪ Repeat the amplification resuspending and testing a new aliquot of **EasyPGX DPYD MT pos ctrl** and **EasyPGX DPYD WT pos ctrl**.
	Incorrect or failure dispensation of the **EasyPGX DPYD MT pos ctrl** and **EasyPGX DPYD WT pos ctrl** .	▪ Repeat the amplification by pipetting the appropriate volume of **EasyPGX DPYD MT pos ctrl** and **EasyPGX DPYD WT pos ctrl** .
The positive control **EasyPGX DPYD MT pos ctrl** shows no amplification signal in the channel "FAM" or the signal is detectable only for some mixes; while one or more samples show a signal amplification in all assays.	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 DPYD MT pos** in the 8-well reaction strips.
	Incorrect samples names set-up in the software.	▪ Check samples names set-up.
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. ▪ 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 DPYD**" 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 sensitivity and specificity

In order to evaluate the clinical sensitivity and specificity of the kit, DNA samples isolated from whole blood have been tested. Samples were suitable in terms of starting DNA amount and and have been already genotyped through Pyrosequencing technology ("**FLUOROPYRIMIDINES response®**", cod. UP024 Diatech Pharmacogenetics), or by MALDI-TOF Mass Spectrometry using MassArray® platform ("**Myriapod® ADMET**" cod. SQ040 Diatech Pharmacogenetics), Real-Time PCR (**Easy® DPYD** cod. RT006 - Diatech Pharmacogenetics), direct sequencing, High Resolution Melt Analysis or samples with known genotype from Coriell Institute.
If no homozygous mutant samples were available, clinical sensitivity for these genotypes has been evaluated using synthetic oligos.

Assay	N° wild-type samples tested	N° heterozygous samples tested	N° mutant samples tested	N° samples correctly genotyped
DPYD*2A	49	5	1	55/55
DPYD*13	52	1	1*	54/54
DPYD D949V	45	9	1*	55/55
DPYD IVS10	52	1	1	54/54

*Synthetic oligos

Analytical sensitivity

The analytical sensitivity limit of the kit was evaluated as the minimum amount of DNA necessary to correctly genotype 95% of the samples.
It was evaluated by testing for each assay, serial dilutions of homozygous wild-type, heterozygous and homozygous mutant DNA samples from Coriell Institute with the following concentrations: 500 – 100 - 10 ng/reaction. For homozygous mutant samples not available from Coriell Institute (DPYD*13 and D949V), serial dilutions of synthetic oligos were tested.
Four independent sessions have been performed with samples that have been tested in duplicates.
The analytical sensitivity limit was 10 ng/reaction.

Repeatability and Reproducibility

The reproducibility of the system (*inter-assay* variability) was evaluated by testing samples with a known genotype in four independent sessions. The results are reproducible, in terms of genotyping, for all the assays and samples analyzed.
The repeatability of the system (*intra-assay* variability) was evaluated by testing two replicates at each of the above concentrations of samples at known genotype in four independent sessions. The results are repeatable, in terms of genotyping, for all the assays and samples analyzed.

Campione	Genotipo	N° Replicates	N° Sessions	Risultati
NA06994	Wild-type	6	4	24/24 wild-type for all assays
NA20812/NA19921	DPYD*2A het	6	4	24/24 heterozygous for the specific assay
HG02684	DPYD*2A mut	6	4	24/24 mutant for the specific assay
HG00332	DPYD*13 het	6	4	24/24 heterozygous for the specific assay
M4628*	DPYD*13 mut	6	4	24/24 mutant for the specific assay
NA20515/NA20797	DPYD D949V het	6	4	24/24 heterozygous for the specific assay
M4623*	DPYD D949V mut	6	4	24/24 mutant for the specific assay
NA20531	DPYD IVS10 het	6	4	24/24 heterozygous for the specific assay
NA21119	DPYD IVS10 mut	6	4	24/24 mutant for the specific assay

Robustness

Freeze-drying cycles

Overall two different batches of **EasyPGX DPYD strips** have been prepared using two different freeze-dryer instruments. The freeze-dried strips have been tested with the same standard samples from Coriell Institute. 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 standard samples from Coriell Institute. Results from the different batches are comparable in terms of genotyping.

Two different batches of master mix have been tested with the same standard samples from Coriell Institute. Results from the different batches are comparable in terms of genotyping.

Two different batches of **EasyPGX buffer** and of **EasyPGX Enzyme** have been tested with the same whole blood samples. Results from the different batches are comparable in terms of quality/quantity of extracted DNA.

EasyPGX® ready DPYD - sample grid 96

Appendix A – sample grid 96

DATE		RUN NAME	

	1	2	3	4	5	6	7	8	9	10	11	12	X
Sample ID	…	…	…	…	…	…	…	…	…	…	…	…	
A													DPYD*2A mix (pos1)
B													DPYD*13 mix (pos2)
C													DPYD D949V mix (pos3)
D													DPYD IVS10 mix (pos4)
Sample ID	…	…	…	…	…	…	…	…	…	…	…	…	
E													DPYD*2A mix (pos5)
F													DPYD*13 mix (pos6)
G													DPYD D949V mix (pos7)
H													DPYD IVS10 mix (pos8)

INSTRUMENT (s/n)				USER MANUAL version	
PRODUCT CODE	RT026	LOT		EXPIRY DATE	
NOTES					

OPERATOR NOTES

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