Instructions for use

DPYD PharmaKitDx

Ref. IMG-413

CE IVD

Manufactured by:

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All Health in Code products undergo strict quality control. DPYD PharmaKitDx has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, please contact our Technical Department:

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Modifications to the Instructions for Use (IFU)		
Version 04	NOV 2022	Change of manufacturer's address: HEALTH IN CODE, S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, España.
Version 03	SEP 2022	Compatible equipment update. Sections 7.2, 8, and 10
Version 02	JUL 2022	Kit platforms update (version Draft)
Version 01	APR 2022	Document created

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01 General information

Fluoropyrimidines, such as 5-fluorouracil (5-FU) and its prodrugs, capecitabine and tegafur, are a group of cytostatic agents used for the treatment of several types of solid tumors, such as breast, colon, rectal, stomach, esophageal, pancreatic, liver, kidney, bladder, endometrial, cervical, and ovarian cancer. 5-FU is an intravenous chemotherapeutic agent, while capecitabine and tegafur are administered orally and are transformed into 5-FU by different enzymes.

Dihydropyrimidine dehydrogenase (DPD, encoded by the DPYD gene) is the main enzyme in charge of 5-FU metabolism: 80% of the administered dose is metabolized into dihydrofluorouracyl by DPD in the liver. DPD activity is subjected to high inter-individual variability in association with genetic variants in the DPYD gene. An estimated 0.01%-0.5% of the Caucasian population has a complete DPD deficiency, while 3%-8% has a partial deficiency.

Patients with DPD deficiency, particularly those with complete deficiency, have a high risk of severe adverse reactions after treatment with fluoropyrimidines, and it is estimated that approximately 10-40% of patients receiving fluoropyrimidine treatment develop some type of severe toxicity that can even be lethal, with more than 70% carrying mutations in alleles *2A, *13, *hapB3, and D949V DPYD.

The **Spanish Agency of Medicines and Medical Devices (AEMPS)**, as well as international regulatory agencies, recommend genetic testing prior to administering 5_FU, capecitabine, and tegafur. Clinical practice guidelines for the implementation of pharmacogenetic testing support the utility of *DPYD* genotyping for fluoropyrimidine treatment adjustment to prevent severe adverse reactions and even death.

References

- *Agencia Española del Medicamento y Productos Sanitarios (AEMPS). Fluorouracilo, capecitabina, tegafur y flucitosina en pacientes con déficit de dihidropirimidina deshidrogenasa. Published 11 May 2020. Accessed July 14, 2021. https://www.aemps.gob.es/informa/notasinformativas/medicamentosusohumano-3/seguridad-1/2020-seguridad-1/fluorouracilo-capecitabina-tegafur-y-flucitosina-en-pacientes-con-deficit-de-dihidropirimidina-deshidrogenasa/*
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- *Quaranta, S. & Thomas, F. (2017). Pharmacogenetics of anti-cancer drugs: State of the art and implementation – recommendations of the French National Network of Pharmacogenetics. Therapie. doi.org/10.1016/j.therap.2017.01.005*
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02 Intended use

The DPYD PharmaKitDx uses a combination of oligonucleotides and fluorescent hydrolysis probes for quantitative real-time PCR testing, validated for the simultaneous detection of polymorphisms in the DPYD gene associated with dihydropyrimidine dehydrogenase (DPD) deficiency, which entails a higher risk of toxicity by fluoropyrimidines. Specifically, this assay allows detecting the following alleles:

- DPYD*2A [NM_000110.4:c.1905+1G>A (rs3918290)].
- DPYD*13 [NM_000110.4:c.1679T>G (rs55886062)].
- DPYD*hapB3 [NM_000110.4:c.1129-5923C>G (rs75017182)].
- DPYD-D949V [NM_000110.4:c.2846A>T (rs67376798)].

This genetic test allows detecting the presence or absence of these genotypes in four multiplexed real-time PCR reactions, which include the simultaneous amplification of the reference genotype and the alternative genotype.

The DPYD PharmaKitDx kit studies the germline genotype; therefore, the optimal sample type for this analysis is genomic DNA.

The results of this assay allow clinicians to identify patients with partial or total deficiency of the DPYD gene, who have a higher susceptibility to toxicity by fluoropyrimidines, and to administer personalized doses based on their genotype. The four variants determined by the *DPYD* kit compose the group recommended by the AEMPS within the context of pre-treatment screening for DPD deficiency.

The DPYD PharmaKitDx kit is solely intended for *in vitro* diagnosis and is aimed at professionals in molecular biology.

03 Technical characteristics

The DPYD PharmaKitDx has been validated in the following platforms:

- *7500 FAST Real-time PCR cycler* (Applied Biosystems)
- *StepOne Plus Real-time PCR system (Applied Biosystems)*

Validation has taken place via the analysis of reference DNA samples from the *Coriell Institute* and of patient samples that have been previously genotyped with other technologies. In said validation, the specific detection of the variants present in the selected genes (see above) was verified, as was the analytical sensitivity, repeatability, and reproducibility of the technique.

Analytical specifications:

- Sample type: DNA from peripheral blood.
- Recommended amount of DNA: 50 ng
- Limit of detection: 1 ng DNA

The DPYD PharmaKitDx kit is compatible with real-time PCR platforms with FAM and VIC (HEX) fluorescence channels.

Health in Code S.L. is certified under the **UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems - Requirements for regulatory purposes** standard by the SPANISH AGENCY OF MEDICINES AND MEDICAL DEVICES for the Design, development, and production of medical devices for *in vitro* diagnostic use:

- Genetic testing kits
- Software for the bioinformatics analysis of genetic data

04 Safety warnings and precautions

- Strictly follow the instructions of this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with abundant water.
- Safety data-sheets (MSDS) of all hazardous components contained in this kit are available on request.
- This product requires the handling of samples and materials of human origin. You should consider all materials of human origin as potentially infectious and handle them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory verify compliance with the technical specifications of the manufacturer when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for the malfunction of the assay when the reagents included in the kit are replaced with other reagents not supplied by Health in Code.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code but are considered by the user equivalent to those provided in the kit.

05 Content and storage conditions of the kit

This kit contains sufficient reagents to carry out 48 real-time PCR reactions with each specific master mix:

- DPYD*2A *Master Mix:* specific oligonucleotides, FAM- and VIC-labeled probes, and PCR-grade water for the detection of the DPYD*2A variant.
- DPYD*13 *Master Mix:* specific oligonucleotides, FAM- and VIC-labeled probes, and PCR-grade water for the detection of the DPYD*13 A>C variant.
- DPYD*hapB3 *Master Mix:* specific oligonucleotides, FAM- and VIC-labeled probes, and PCR-grade water for the detection of the IVS10 G>C variant, included in *habB3.
- DPYD D949 *Master Mix:* specific oligonucleotides, FAM- and VIC-labeled probes, and PCR-grade water for the detection of D949V T>A variant.
- *General Master Mix:* PCR master mix with nucleotides, MgCl2, DNA polymerase and buffer required to perform real-time PCR.
- *Positive Control:* synthetic positive control for all study variants.

Reagents	Color indicator	Quantity	Conservation
DPYD*2A *Master Mix*	Black cap	2 x 180 µL	-20°C
DPYD*13 *Master Mix*	Purple cap	2 x 180 µL	-20°C
DPYD*hapB3 *Master Mix*	Yellow cap	2 x 180 µL	-20°C
DPYD D949 *Master Mix*	Red cap	2 x 180 µL	-20°C
General Master Mix	White cap	4 x 600 µL	4°C
Positive Control	Black cap	120 µL	-20°C

Table 1. Components of the DPYD PharmaKitDx kit

06 Equipment, reagents, and materials not included in the kit

Equipment:

- Real-time PCR thermal cycler able to detect FAM and VIC fluorophores
- 10 µL, 20 µL, and 200 µL micropipettes
- Vortex mixer
- Centrifuge

Reagents:

- Nucleic acid extraction kit
- Nuclease-free water

Materials:

- Optical 96-well plates or 0.2 ml optical tubes
- Optical consumables compatible with the real-time PCR thermal cycler
- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 ml tubes
- Latex gloves
- Necessary material for nucleic acid extraction

Related kits

For the genotyping analysis of pharmacogenetics-related targets, Health in Code also offers Imegen®-Gilbert Plus **(ref. IMG-288)** and Action PharmaKitDx **(ref. IMG-401)** kits. These have been validated to perform the test via fragment analysis (capillary electrophoresis) and next-generation sequencing, respectively

**Imegen is a trademark registered in Spain, which belongs to the Health in Code Group*

07 Assay protocol

07.1 | Preparation of amplification reactions

To estimate the amount of reagents needed, the number of samples and controls to be analyzed simultaneously must be taken into account. To perform the calculations, we recommend to either perform one extra reaction or to add an extra 10% of each reagent.

To carry out the qualitative analysis, we recommend preparing one amplification reaction per sample and including the positive control, as well as a negative PCR control, to rule out contamination of the reagents.

The recommended protocol for the preparation of the amplification reactions is shown below:

01 Thaw all kit reagents and DNA samples. Vortex each reagent and keep cold.

02 Prepare the PCR mix in a 1.5mL tube using the following reagents per sample:

Reagent	Volume per reaction
DPYD Specific Master Mix	7.5 µL
General Master Mix	12.5 µL

03 Vortex and spin the PCR mixes and dispense 20 µL into the corresponding wells of the optical consumables.

04 Add 5 µL of the samples, diluted to 10 ng/µL, and 5 µl of the positive control or nuclease-free water (negative control) to the corresponding wells.

05 Place the tubes or plates into the real-time PCR thermal cycler and configure settings for the amplification program as indicated in the next section.

07.2 | Settings for the real-time PCR program

Fluorophores of hydrolysis probes:

System	Reporter	Allele	Quencher
DPYD*2A	FAM	G	MGB
c.1905+1G>A	VIC	A	
DPYD*13	FAM	T	
c.1679T>G	VIC	G	

DPYD*hapB3	FAM	C	MGB
c.1129-5923C>G	VIC	G	
DPYD D949V	FAM	A	
c.2846A>T	VIC	T	

Table 2. Information about hydrolysis probes

Optimal PCR program:

7500 Fast Real-Time PCR System (Applied Biosystems)

- Type of experiment: Quantitation
- Ramp rate: Standard
- ROX™ baseline reference: included
- Fluorophores of TaqMan® probes
- Set a cycle threshold (Ct) at 0.1 for the analysis of results.

Stage	No. of cycles	Temperature	Time
Enzymatic activation	1	95°C	10 minutes
PCR Denaturation, annealing, and extension	40	95°C	15 seconds
		60°C	30 seconds (1)

Table 3. Optimal PCR program for 7500 FAST (Thermo Scientific)

(1) If other thermal cycler models are available, please see chapter 10: Limitations
(2) Fluorescence acquisition

StepOne Plus Real-time PCR System (Applied Biosystems)

- Type of experiment: Quantitation
- Ramp rate: Standard
- ROX™ baseline reference: included
- Fluorophores of TaqMan® probes
- Set a cycle threshold (Ct) at 0.2 for the analysis of results.

Stage	No. of cycles	Temperature	Time
Enzymatic activation	1	95°C	10 minutes
PCR Denaturation, annealing, and extension	40	95°C	15 seconds
		60°C	30 seconds (1)

Table 4. Optimal PCR program for StepOne Plus (Thermo Scientific).

(1) If other thermal cycler models are available, please see chapter 10: Limitations
(2) Fluorescence acquisition

08 Analysis of results

You should follow the instructions below to ensure an adequate analysis of results:

- Verify that there is no amplification signal in the negative PCR control, neither in the FAM nor in the VIC channels.
- Confirm the presence of an amplification signal for the positive control, both in the FAM channel and in the VIC channel.
- To analyze the samples, a specific software of the real-time PCR thermal cycler employed must be used, assessing amplification curves.

The possible results obtained using the DPYD PharmaKitDx kit are shown below:

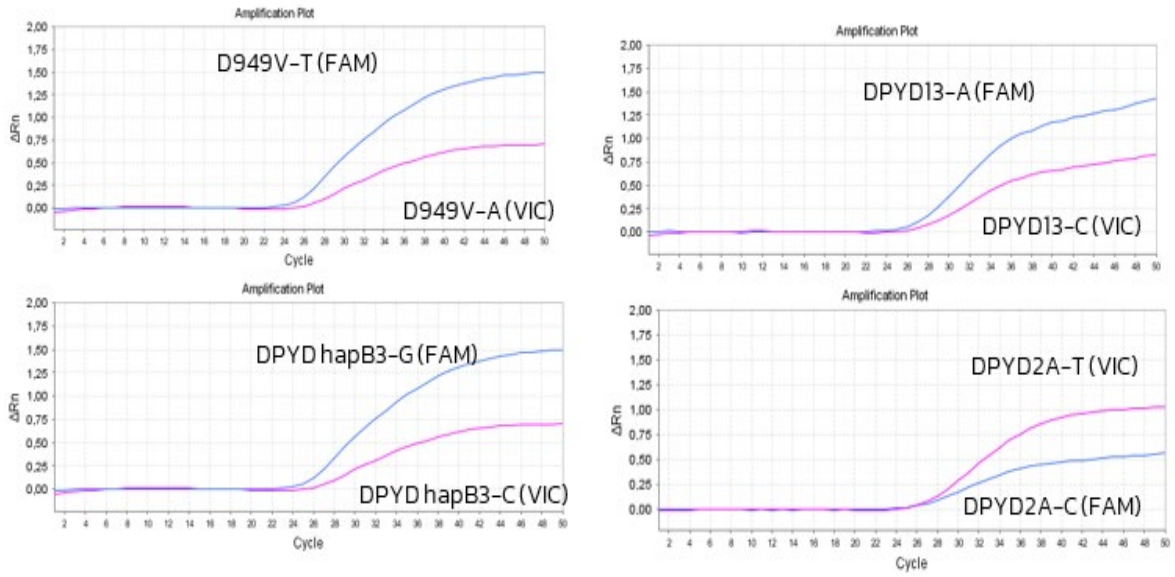

Figure 1. Results obtained from a wild-type sample (reference genotype) for each variant of interest. Amplification is only detected in the FAM channel.

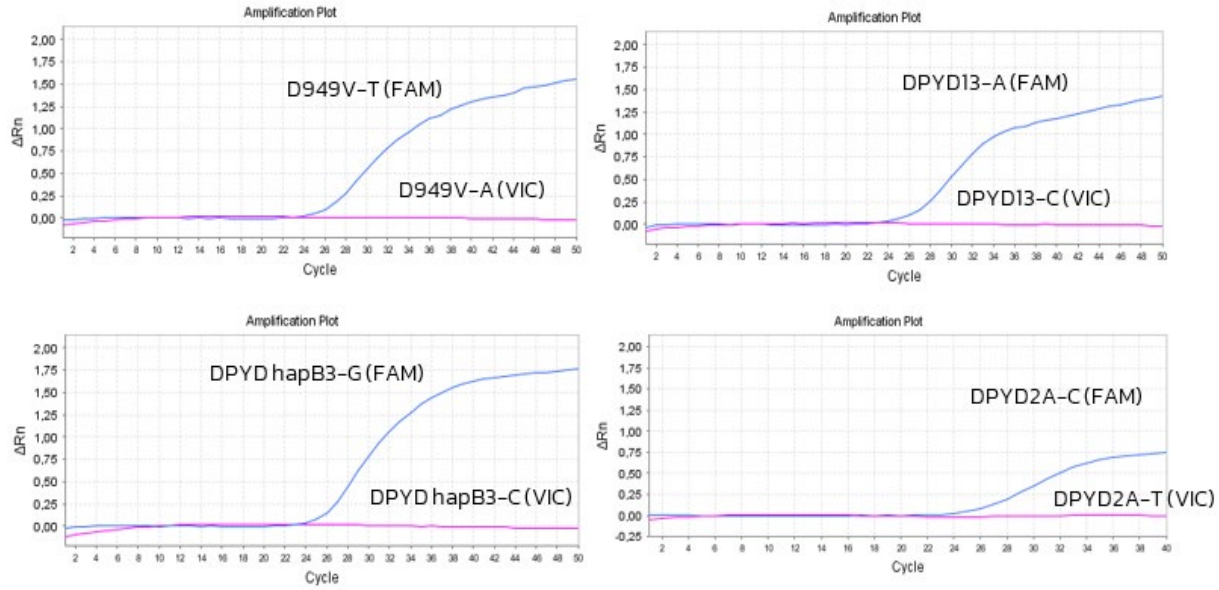

Figure 2. Expected result from a heterozygous sample/kit's positive control for each variant of interest. Signals are detected in both fluorescence channels (FAM and VIC), with higher intensity in the FAM channel.

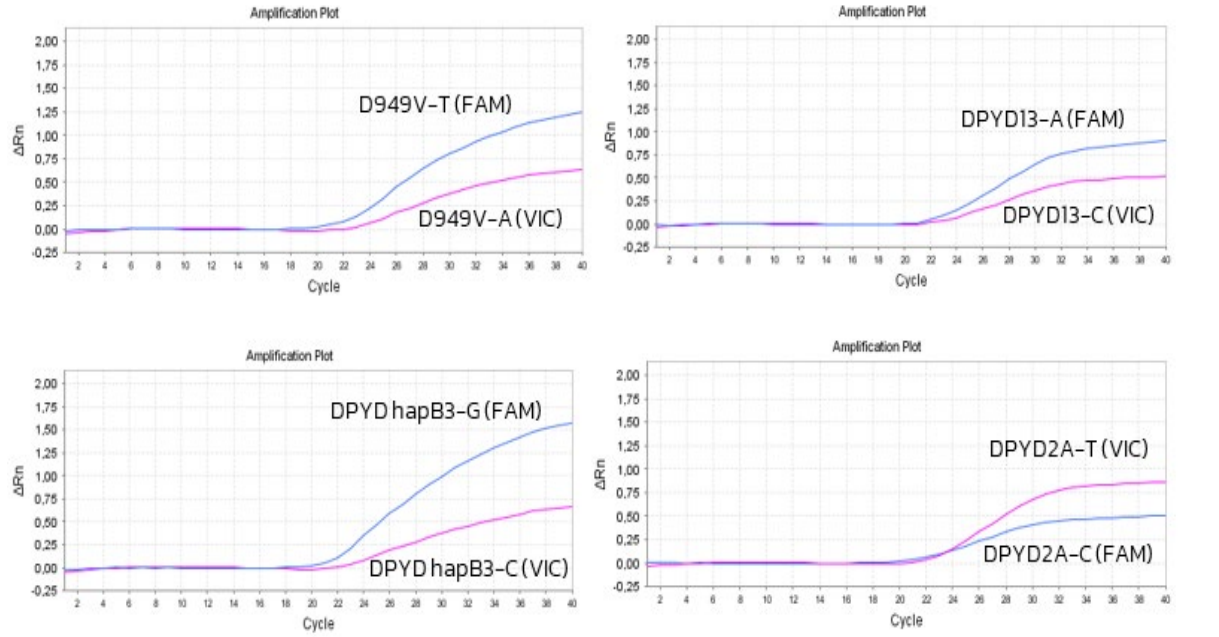

Figure 3. Expected result from the kit's positive control for each variant of interest. Signals are detected in both fluorescence channels (FAM and VIC).

08.1 | Pharmacogenetic testing

According to clinical guidelines, result interpretation is performed by assigning phenotypes based on genotype results. Three pharmacogenetic phenotypes are established for DPD according to their metabolizing capacity.

- Normal metabolizer (NM),
- Intermediate metabolizer (IM), and
- Slow metabolizer (SM)

These groups are established based on the different DPYD alleles carried by the individual, which are assigned an activity score (AS) that represents the enzymatic activity of the DPD protein. Variants can be classified as:

- Normal DPYD*1 function for the wild-type allele (AS=1),
- Reduced function (AS=0.5)
- Complete loss of function (AS=0)

The following activity score (AS) is assigned to the different DPYD alleles:

System	Reporter	Allele	DPD enzymatic function	Activity Score (AS)
DPYD*2A c.1905+1G>A	FAM	G	Normal function (wild type)	1
	VIC	A	Complete loss	0
DPYD*13 c.1679T>G	FAM	T	Normal function (wild type)	1
	VIC	G	Complete loss	0
DPYD*hapB3 c.1129-5923C>G	FAM	C	Normal function (wild type)	1
	VIC	G	Reduced function	0.5
DPYD D949V c.2846A>T	FAM	A	Normal function (wild type)	1
	VIC	T	Reduced function	0.5

Table 5. Activity score for the different DPYD alleles

The individual's global activity score (GA) is calculated by adding the individual activity scores (AS) of the two DPYD alleles. This allows classifying individuals into the following phenotypic categories:

Pharmacogenetic phenotypes for DPD	DPD genotype	Global activity (GA)
Normal metabolizer	Homozygote with 2 normal-function alleles	2
Intermediate metabolizer	Heterozygote with 1 normal-function allele and 1 reduced-function allele	1.5
	Heterozygote with 1 normal-function allele and 1 complete-loss allele or homozygous with 2 reduced-function allele	1
Slow metabolizer	Heterozygote with 1 complete-loss allele and 1 reduced-function allele	0.5
	Homozygote with 2 complete-loss alleles	0

Table 6. Classification of pharmacogenetic phenotypes for DPD.

The table below represents the possible results that could be obtained from this study:

Pharmacogenetic phenotypes for DPD	DPYD Alleles					DPYD genotype	Global activity
	WT*1	*2A	*13	*hapB3	D949V		
Normal metabolizer	+					*1/*1	2
Intermediate metabolizer	+			+		*1/*hapB3	1.5
	+				+	*1/D949V	1.5
	+	+				*1/*2A	1
	+		+			*1/*13	1
				+	+	*hapB3/D949V	1
				+		*hapB3/*hapB3	1
					+	D949V/D949V	1
Slow metabolizer		+		+		*2A/*hapB3	0.5
		+			+	*2A/D949V	0.5
			+	+		*13/*hapB3	0.5
			+		+	*13/D949V	0.5
		+	+			*2A/*13	0
		+				*2A/*2A	0
			+			*13/*13	0

Table 7. Classification of the potential results obtained with the DPYD PharmaKitDx kit.

(+) Allele detected by the genotyping assay

08.2 | Clinical recommendations for phenotypes for DPD

Based on the different phenotypes and according to the CPIC and SEFF clinical guidelines, the following dosage recommendations aim to minimize the risk of severe toxicity in patients with DPD enzyme deficiency:

Pharmacogenetic phenotypes for DPD	Implications	Dosage recommendation
Normal metabolizer	Normal DPD activity and normal risk of fluoropyrimidine toxicity	According to the product specifications sheet
Intermediate metabolizer	Decreased DPD activity (between 30% and 70%) and increased risk of severe or even lethal toxicity when treated with fluoropyrimidines	Reduce initial dosage by 50%. Subsequently adjust the dosage based on toxicity or pharmacogenetics
Slow metabolizer	Complete DPD deficiency and increased risk of severe or even lethal toxicity when treated with fluoropyrimidines	Treatment with fluoropyrimidines is contraindicated. Alternative drugs must be sought

Table 8. Clinical recommendations for pharmacogenetic phenotypes for DPD.

- **Intermediate metabolizers (IM).** 50% of the recommended initial dosage should be administered followed by dosage escalation:
  - To maximize the efficacy of the drug, 5-FU doses can be progressively increased in patients who have not experienced toxicity in the first 2 cycles or in whom plasma concentrations are sub-therapeutic.
  - To minimize toxicity, the dose of 5-FU should be reduced when the patient does not tolerate the initial dose. In the case of topical administration, systemic absorption is very low and, therefore, toxicity is not expected to occur in IM patients.

- **Slow metabolizers (SM).** The use of fluorouracil, capecitabine, and/or tegafur is contraindicated:
  - CPIC guidelines indicate that patients with GA=0.5 who cannot be treated with alternative drugs could receive a reduced dose of 5-FU, <25% of the normal dose, undergoing early monitoring of plasma 5-FU concentration with the purpose of interrupting the treatment if drug levels are too high.
  - Clinical guidelines contraindicate the use of fluoropyrimidines in patients with GA=0. Treatment decisions must be made on a case-by-case basis and exclusively based on clinical criteria, assessing risks and benefits.

09 Troubleshooting

The table below represents the expected test results that could be obtained from the analysis of the different controls and one sample in one run, along with their interpretation:

Control	Result		Cause
	FAM	VIC	
Positive control	+	+	Expected result
	–	–	Failure of PCR amplification [1]
Sample	+	+	Expected result
	+	–	
	–	+	
	–	–	Failure of sample amplification [2]
Negative PCR control	–	–	Expected result
	+	+	PCR contamination with human DNA [3]
	+	–	
	–	+	

Table 9. Interpretation of possible results.

(1) Failure of PCR amplification: Make sure the amplification program and fluorescence detection settings are correct. An amplification error may be due to a technical issue during PCR program setup.

(2) Failure of sample amplification: Verify that sample quantification meets the recommendations; if so, the specified result may be due to a highly degraded sample.

(3) PCR contamination by human DNA: PCR contamination may be due to mishandling of the sample, the use of contaminated reagents or environmental contamination. Thoroughly clean the laboratory where the PCR was prepared, as well as the equipment and material used. If necessary, use fresh aliquots of the PCR reagents. Prepare the PCR reaction containing the positive control last, in order to avoid cross-contamination. It is recommended that the assay be repeated in this case. Using the touchscreen for run setup presents usability issues.

10 Limitations

10.1 | Analytical

DPYD*2A, DPYD*13, DPYD*hapB3, and DPYD-D949V variants are the most relevant ones for the study of DPD deficiency in the population. However, other variants have also been detected at very low frequencies in association with altered DPD activity. Overall, *DPYD* variants *2A, *13, *hapB3, and D949V only partly explain toxicity caused by fluoropyrimidines.

10.2 | Equipment

DPYD PharmaKitDx has been validated using the following amplification platform:

- 7500 FAST Real-time PCR cycler
- StepOne Plus Real-time PCR system

10.3 | Reagents

DPYD PharmaKitDx has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Required equipment and materials not included in the kit).

10.4 | Product stability

Optimal performance of this product is achieved when the specified recommended storage conditions are applied, within the optimal product expiration date associated with each production batch.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

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