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Easy® DPYD

User manual – version 2016/01

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

For *in vitro* diagnostic use

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INTENDED USE

"**Easy® DPYD**" is an *in vitro diagnostic* test for the detection by Real-Time PCR of the *DPYD* gene polymorphisms DPYD*2A (IVS14+1G>A, c.1905+1G>A, rs3918290), DPYD*13 (c.1679T>G, rs55886062), DPYD D949V (c.2846A>T, rs67376798) and DPYD IVS10 (c.1129–5923C>G, rs75017182) in human genomic DNA extracted from whole blood.

The "**Easy® DPYD**" kit is validated on the following instruments:

- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7300** - Applied Biosystems (software v.1.4.1)
- **ABI 7500** - Applied Biosystems (software v.2.0.5) in association with TaqMan Genotyper (software v.1.3)
- **Stratagene Mx3000P**, **Mx3005P** - Agilent Technologies (software v.4.10 build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

PRINCIPLE OF THE ASSAY

The "**Easy® DPYD**" kit enables the detection of the polymorphisms DPYD*2A, DPYD*13, DPYD D949V and DPYD IVS10 that cause a reduced activity of the DPD enzyme. This reduction is associated with high toxicity in patients treated with 5-florouracil. The DPYD genotyping can be used for the personalization of the fluoropyrimidines treatment.

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.

KIT CONTENTS

The kit contains sufficient reagents to carry out 24 tests for each assay for a maximum of 3 runs (5 samples and 3 controls per run).

COMP		QUAN	Color	
DPYD*2A mix (1)		3 x 20 µl	RED	Mixture of specific primers and probes targeting DPYD*2A polymorphism.
DPYD*13 mix (2)		3 x 20 µl	GREEN	Mixture of specific primers and probes targeting DPYD*13 polymorphism.
DPYD D949V mix (3)		3 x 20 µl	BLUE	Mixture of specific primers and probes targeting DPYD D949V polymorphism.
DPYD IVS10 mix (4)		3 x 20 µl	YELLOW	Mixture of specific primers and probes targeting DPYD IVS10 polymorphism.
DPYD WT pos ctrl	CONTROL +	3 x 50 µl		Positive control DNA containing a mixture of synthetic wild-type DNA sequences for DPYD polymorphisms analyzed.
DPYD MT pos ctrl	CONTROL +	3 x 50 µl		Positive control DNA containing a mixture of synthetic mutant DNA sequences for DPYD polymorphisms analyzed.
WATER	CONTROL -	1 x 1.5 ml		DNase-, RNase-free water to be use exclusively for the preparation of the PCR mix and as negative control.
Taq Premix 920		2 x 920 µl		Solution containing hot start Taq DNA polimerase, reaction buffer, Mg^{2+} and dNTP Mixture.
Dye R-I		2 x 40 µl		Inert fluorophore to be used for the amplification on ABI 7300 instrument.
Dye R-II		2 x 40 µl		Inert fluorophore to be used for the amplification on ABI 7500 instrument.
8-strip tubes & caps		1 x 5 unità		0.2 ml 8-tube strip DNase-, RNase-free to be used for the preparation of reaction mixture.

DOCUMENTS AVAILABLE ON-LINE

Easy® DPYD User Manual and Material Safety Data Sheet (MSDS) are available at www.diatechpharmacogenetics.com/en/reserved-area**.**

MATERIALS REQUIRED BUT NOT PROVIDED

Genomic DNA extraction

The "**Easy® DPYD**" kit does not contain reagents for DNA extraction.

Recommended kits:
- "**QIAamp® DNA Mini kit**" (cod. 51304, Qiagen)
- "**Genomic DNA Whole Blood Kit (Speedy installation)**" (cod. MGB400-02, RBC); to use with MagCore Automated Nucleic Acid Extractor (RBC Bioscience) automatic systems
- "**HELIX FAST BLOOD DNA**" (cod. H8010, Diatech Pharmacogenetics)
- "**Helix DNA plus®**" (cod. H8020, Diatech Pharmacogenetics) to use with "**X-tractor Gene™**" (Corbett Robotics) or "**Helix Extraction System®**" (cod. H8000, Diatech Pharmacogenetics)

ⓘ In case you employ kits which are different from those recommended, it is the user's responsibility to use standardized samples (e.g,: VEQ – EQAS quality schemes, Horizon Diagnostics samples) to verify that this does not imply a reduction of the performance of the system under analysis.

Amplification

Real-Time PCR Instruments:
- **CFX96** - Bio-Rad (software v. 3.1)
- **ABI 7300**- Applied Biosystems (software v. 1.4.1)
- **ABI 7500** - Applied Biosystems (software v.2.0.5)
- **Stratagene Mx3000P**, **Mx3005P** - Agilent Technologies (software v. 4.10 - Build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

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

Materials:
- 1,5 ml polypropylene twist-lock tubes (DNase-, RNase-, DNA-, PCR inhibitor-free)
- Micropipettes (volumes from 1 to 1.000 µl)
- Sterile filter tips DNase-, RNase-free (volumes from 1 to 1.000 µl)
- 96 well plates and foil or caps compatibles with the thermal-cycler used (check the compatibility in the user manual of the instrument) for instance:
  1. Bio-Rad CFX96: Hard Shell PCR plates 96-well WHT/CLR cod. HSP 9601; MICROSEAL B SEALS cod. MSB 1001
  2. ABI 7300/ ABI 7500: MICROAMP OPTICAL 96 WELL RNX PLATE cod. N8010560; OPTICAL ADHESIVE COVERS cod. 4360954
  3. Stratagene Mx3000P/Mx3005P: Optical caps (8x strip) cod. 401425; QPCR 96-Well Plates, Non-Skirted cod. 401333
- DNase-and RNase-free, thin-wall, PCR tubes with flat cap or 0.1 ml tubes in strip, suitable for use on Rotor-Gene, for instance:
- Qiagen, cat. no. 981103
- LTF-Labortechnik GmbH & Co, no. 102.0170
- Powder-free disposable gloves

STABILITY AND STORAGE

Store all the reagents according to the instructions on the packages, in particular:
- Store all the reagents at -35/-20°C in the original package immediately upon receipt.
- After thawing, store **Taq PreMix 920** at +2/+8°C and use it within 6 months or within the expiration date.
- Avoid thawing and re-freezing the reagents more than twice, as this could lead to poor performance.
- Protect all mixes containing probes from light to avoid degradation of the fluorescent dyes.
- If properly stored, the reagents remain stable until the expiration date displayed on the individual label.

SYMBOLS

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

PRODUCT USE LIMITATIONS

- The "**Easy® 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 "**Easy® DPYD**" kit is designed to be used with the instruments "**Rotor-Gene Q**" (Qiagen), "**Rotor-Gene™ 6000**" (Corbett Research), **CFX96** (Bio-Rad), **ABI 7300/ABI 7500** (Applied Biosystems) and Stratagene **Mx3000P/Mx3005P** (Agilent Technologies).
- The reliability of the results also depends on the procedures carried out in the pre-amplification stages, including the selection of starting biological speciments, the preservation of the samples and DNA extraction.
- Any diagnostic results generated by this procedure must be interpreted with reference to other clinical or laboratory findings.

QUALITY CONTROL

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

WARNINGS AND PRECAUTIONS

- The kit may only be used by specialist personnel, properly instructed and trained to perform *in vitro* laboratory techniques.
- Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
- Follow the laboratory safety procedures described in "Biosafety in Microbiological and Biomedical Laboratories" (Richmond, JY and McKinney, RW (eds) - 5th edition (2009) and in the NCCLS (National Committee for Clinical Laboratory Standards) Document M29-T. Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative guidelines. – Villanova, PA:NCCLS, 1989).
- Do not eat, drink or smoke in the laboratory. When handling biological samples, disposable gloves, gowns and goggles or face masks should be worn to protect against biological agents.
- ⓘ Constantly check that the gloves are free from contamination by the biological material being treated. If not, replace them immediately to avoid the possibility of cross-contamination between samples and contamination of the workplace. Wash hands thoroughly after handling samples and reagents.
- The Material Safety Data Sheet (MSDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com.
- Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
- It is recommended to ensure that the laboratory work flow proceeds in a unidirectional manner, preparing, if possible, two separate work areas for:
  - extraction of nucleic acids;

- amplification reaction;
- Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
- Use sterile filter tips. Avoid aerosols.
- Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
- During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by only opening the reagents within the hood.
- Change the pipette tip before each extraction of reagents and every time you move from one sample to another in any stage of the procedure.
- The precision pipettes used should have an accuracy of within 3% of the set volume.
- Periodically check the calibration status of the dispensing instruments.
- Do not use reagents after the expiration date shown on each container.
- All reagents supplied in the "**Easy® DPYD**" kit are intended to be used solely with the other reagents in the same "**Easy® DPYD**" kit. Do not substitute or mix reagents in the kit from different batches, in order to maintain optimal performance.
- Only use the **Taq PreMix 920** that is provided in the kit. Do not substitute with **Taq PreMix 920** from other kits or with similar reagents from other suppliers.
- Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
- Extraction area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products (e.g. FD 322, Dürr Dental, Germany) and UV irradiate the work surface of the biological cabinet where the pipettes should be carefully placed after decontamination.
- Amplification area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products to eliminate nucleic acids and amplicons (e.g. "DNA Cleaner" - code DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
- Avoid contamination of samples and reagents.
- Store reagents and samples separately.
- In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.
- Before use all reagents need to be thawed at room temperature, mixed by inverting 10 times and centrifuged briefly.
- All reagents contained in the kit are ready-to-use and don't need to be diluted. The reagent dilution may result in a loss of performance.
- Include in each run at least 1 negative control (**WATER**) and 2 positive control (**DPYD WT pos ctrl, DPYD MT pos ctrl**).
- In order to avoid any mixing up of samples pay particular attention to samples dispensation, placement of tubes into the instrument, editing the sample name in the software.
- Carefully read this User Manual.
- Check that the version of this User Manual corresponds to the one described on the "**Easy® DPYD**" kit box label.
- The right to contest the kit before the expiration date becomes void if the product is used in violation of GLP guidelines and the manufacturer's recommendations.
- The registered names and trademarks indicated in this document are to be considered protected by law, even when not explicitly stated.

ANALYTICAL PROCEDURE

Analytical procedure includes the following steps:
- Genomic DNA extraction;
- Amplification;
- Instrument setup;
- Data analysis and genotype determination.

DNA EXTRACTION

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

- The "**Easy® DPYD**" kit does not include the reagents for DNA extraction.
- Extract genomic DNA from whole blood.
- The quantity of biological material required for the DNA extraction depends on protocols.
- Commercial kits working with silica filters or magnetic beads are recommended. Avoid methods that use phenol.
- Perform the DNA extraction following the instructions of the extraction kit in use.
- After sampling, store blood at +2/+8°C for maximum 12 hours, otherwise at ≤-20°C divided into aliquots univocally identified.
- 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.
- Use 10-500 ng of DNA for amplification.

AMPLIFICATION

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

ⓘ Perform this step in the area dedicated to amplification mixes preparation, using dedicated materials and instruments. Before starting decontaminate pipettes, benches and wood in order to degrade any trace of DNA and possibly radiate with UV light for at least 30 minutes.

- Switch on the instrument and the software at least 20-30 minutes before starting the reaction to allow the heating of the lamps where necessary.
- Thaw all necessary reagents before use.
- Thoroughly mix the reagents in a vortex, or inverting each tube ten times, and spin them briefly before use.
- Prepare and mark an appropriate number of tubes or wells of the plate to use.
- Each run must include at least one amplification negative control (**WATER**) and two amplification positive controls (**DPYD WT pos ctrl**, **DPYD MT pos ctrl**) for each mix.

INSTRUMENT SETUP

Rotor-Gene Q, Rotor-Gene 6000

- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:
  - "DPYD*2A Green": source 470 nm – detector 510 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 1 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD*2A Yellow": source 530 nm – detector 555 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 1 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD*13 Green": source 470 nm – detector 510 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 2 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD*13 Yellow": source 530 nm – detector 555 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 2 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD D949V Green": source 470 nm – detector 510 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 3 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD D949V Yellow": source 530 nm – detector 555 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 3 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD IVS10 Green": source 470 nm – detector 510 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 4 (**Water**), "Target Sample Range" 20-30 Fl;
  - "DPYD IVS10 Yellow": source 530 nm – detector 555 nm – "Gain Optimisation" 61°C Before 1st acquisition, "Tube Position": 4 (**Water**), "Target Sample Range" 20-30 Fl;

Thermal Profile	
Hold	95°C per 2 minutes
40 cycles	95°C per 10 seconds / 61°C per 60 seconds (acquire fluorescence in channels "DPYD*2A Green", "DPYD*13 Green", "DPYD D949V Green", "DPYD IVS10 Green" and "DPYD*2A Yellow", "DPYD*13 Yellow", "DPYD D949V Yellow", "DPYD IVS10 Yellow")

- Reaction volume: 20 µl.

Stratagene Mx3000P, Mx3005P

- Select Allele Discrimination / SNP's Real-Time, then ok.
- Check for the presence of the message "Lamp – Warm-up".
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:
  - "FAM": source 492 nm – detector 516 nm – Filter gain factor x4
  - "HEX": source 535 nm – detector 555 nm – Filter gain factor x2

Thermal profile	
Hold	95°C per 2 minutes
40 cycles	95°C per 10 seconds / 61°C per 60 seconds (acquire fluorescence in channels "FAM" e "HEX" setting up END 1)

- Reaction volume: 20 µl.

CFX96

- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:

Thermal profile	
Step	
1	95°C per 2 minutes
2	95°C per 10 seconds
3	61°C per 60 seconds (Plate read – All Channels)
4	GOTO 2 39 more times

- Reaction volume: 20 µl.

ⓘ Select the option "All Channels" to acquire the signal in both FAM and HEX.

ABI 7300

- Select Create new document – In Assay: Allelic Discrimination, then Next.
- Follow the instructions reported in the user manual of the instrument to set up the following detectors with "Passive Reference: ROX":
  - "Name: *2A MT" - "Reporter Dye: FAM" - "Quencher Dye: none";
  - "Name: *2A WT" - "Reporter Dye: VIC" - "Quencher Dye: none";
  - "Name: *13 MT" - "Reporter Dye: FAM" - "Quencher Dye: none";
  - "Name: *13 WT" - "Reporter Dye: VIC" - "Quencher Dye: none";
  - "Name: D949V MT" - "Reporter Dye: FAM" - "Quencher Dye: none";
  - "Name: D949V WT" - "Reporter Dye: VIC" - "Quencher Dye: none";
  - "Name: IVS10 MT" - "Reporter Dye: FAM" - "Quencher Dye: none";
  - "Name: IVS10 WT" - "Reporter Dye: VIC" - "Quencher Dye: none".
- Create the markers DPYD*2A, DPYD*13, DPYD D949V and DPYD IVS10 with the two specific detectors.
- Set Sample Volume (µl): 20 and click Pre-Read. At the end save the run.
- Select Create new document – In Assay: Standard Curve (Absolute Quantification), then Next.
- Follow the instructions reported in the user manual of the instrument to link all detectors to each sample and to set up the following thermal profile:

Thermal profile	
Hold	95°C per 2 minutes
40 cycles	95°C per 10 seconds / 61°C per 60 seconds (fluorescence acquisition for all detectors)

- Set Sample Volume (µl): 20 and click Start. At the end save the run.
- Open Pre-Read run.
- In Instrument, click Post-Read. At the end save the run.

ABI 7500

- Select New Experiment, Genotyping, TaqMan Reagents and Standard.
- Follow the instructions reported in the user manual of the instrument to set up the following four SNP Assays with "Passive Reference: ROX":
  - "SNP Assay Name: DPYD*2A":
  "Allele 1 Name or Base(s): WT" - "Reporter: VIC" - "Quencher Dye: none"
  "Allele 2 Name or Base(s): MT" - "Reporter: FAM" - "Quencher Dye: none"
  - "SNP Assay Name: DPYD*13":
  "Allele 1 Name or Base(s): WT" - "Reporter: VIC" - "Quencher Dye: none"
  "Allele 2 Name or Base(s): MT" - "Reporter: FAM" - "Quencher Dye: none"
  - "SNP Assay Name: DPYD D949V":
  "Allele 1 Name or Base(s): WT" - "Reporter: VIC" - "Quencher Dye: none"
  "Allele 2 Name or Base(s): MT" - "Reporter: FAM" - "Quencher Dye: none"
  - "SNP Assay Name: DPYD IVS10":
  "Allele 1 Name or Base(s): WT" - "Reporter: VIC" - "Quencher Dye: none"
  "Allele 2 Name or Base(s): MT" - "Reporter: FAM" - "Quencher Dye: none"
- Each sample will be analyzed with all SNP assays.
- Follow the instructions reported in the user manual of the instrument to setup the following thermal profile:

Thermal profile	
Hold	60°C per 60 seconds (fluorescence acquisition for all SNP assays)
Hold	95°C per 2 minutes
40 cycles	95°C per 10 seconds / 61°C per 60 seconds (fluorescence acquisition for all SNP assays)
Hold	60°C per 60 seconds (fluorescence acquisition for all SNP assays)

- Reaction volume: 20 µl.

ⓘ Each sample must be amplified with four different mixes: **DPYD*2A mix (1)**, **DPYD*13 mix (2)**, **DPYD D949V mix (3)** and **DPYD IVS10 mix (4)**.

ⓘ The kit content is optimized to analyze five clinical samples and three controls (**DPYD WT pos ctrl, DPYD WT pos ctrl** e **WATER**) in each run.

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

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

1	DPYD*12 mix (1)
2	DPYD*13 mix (2)
3	DPYD D949V mix (3)
4	DPYD IVS10 mix (4)

Rotor-Gene (Easy® DPYD sample grid B)

WATER		WT POS CTRL		MT POS CTRL		DNA1		DNA2		DNA3		DNA4		DNA5			
1	•	9	•	17	•	25	•	33	•	41	•	49	•	57	•	65	•
2	•	10	•	18	•	26	•	34	•	42	•	50	•	58	•	66	•
3	•	11	•	19	•	27	•	35	•	43	•	51	•	59	•	67	•
4	•	12	•	20	•	28	•	36	•	44	•	52	•	60	•	68	•
5	•	13	•	21	•	29	•	37	•	45	•	53	•	61	•	69	•
6	•	14	•	22	•	30	•	38	•	46	•	54	•	62	•	70	•
7	•	15	•	23	•	31	•	39	•	47	•	55	•	63	•	71	•
8	•	16	•	24	•	32	•	40	•	48	•	56	•	64	•	72	•

▪ Prepare, for each sample and control, 4 different amplification mixtures (**Amp-Mix**), one for each assay, as indicated in the following scheme, where N is the numer of samples and controls to be tested for each assay.

ⓘ If you are working with a 96 well plate, it is possible to preprare the **Amp-Mix** in the strip provided with the kit, so you can dispense the all **Amp-Mix** with a multichannel pipette.

Rotor-Gene, Stratagene, CFX96			
Amp-Mix		**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
Taq Premix 920		10	
WATER	CONTROL -	3	
DPYD*2A mix (1) **DPYD*13 mix (2)** **DPYD D949V mix (3)** **DPYD IVS10 mix (4)**	or or or or	2	
Total Volume		15	

ABI 7300			
Amp-Mix		**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
Taq Premix 920		10	
Dye R-I		0.4	
WATER	CONTROL -	2.6	
DPYD*2A mix (1) **DPYD*13 mix (2)** **DPYD D949V mix (3)** **DPYD IVS10 mix (4)**	or or or or	2	
Total volume		15	

ABI 7500		
Amp-Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
Taq Premix 920	10	
Dye R-II	0.2	
WATER CONTROL -	2.8	
DPYD*2A mix (1) or **DPYD*13 mix (2)** or **DPYD D949V mix (3)** or **DPYD IVS10 mix (4)** or	2	
Total volume	15	

- Mix all **Amp-Mix** thoroughly by repeated pipetting or rapid vortexing, then centrifuge briefly.
- Pipette 15 µl of each **Amp-Mix** in all the tubes/wells previously marked.
- Add to the respective tubes/wells for each of the four assays:

negative control	5 µl **WATER**	CONTROL -
sample	5 µl DNA	
positive control	5 µl **DPYD WT pos ctrl**	CONTROL +
positive control	5 µl **DPYD MT pos ctrl**	CONTROL +

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

ⓘ Before starting the run, please pay attention to the plate orientation (well A1 on the upper left position) or to the Rotor-Gene 0.1ml strips of tubes orientation (mark the first tube of each strip).

DATA ANALYSIS AND GENOTYPE DETERMINATION

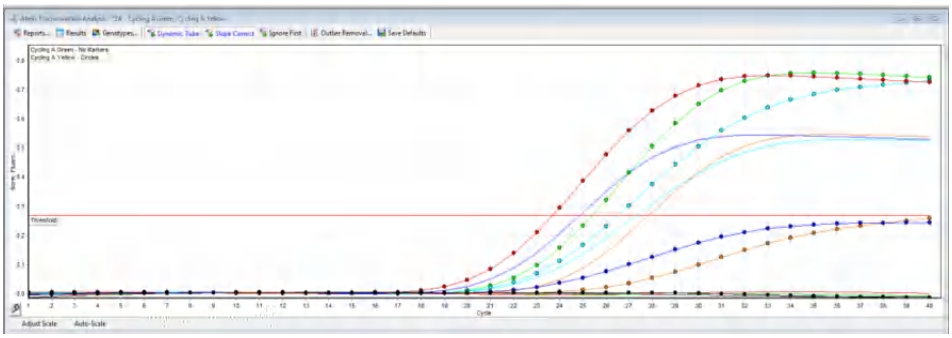
Rotor-Gene

- This section describes how to perform the “Allelic Discrimination Analysis” using Rotor-Gene software version software 1.7.87.

Allelic Discrimination Analysis

ⓘ Analyze each assay separately, selecting only the respective samples.
- Create four different samples pages, one for each assay.
- Select Analysis, Other, Allelic Discrimination.
- Select the channels Cycling A.Green and Cycling A.Yellow of the respective assay (clicking CTRL) and click Show. Select assay sample page.
- Select Dynamic Tube and Slope Correct.
- Click on Genotypes and link the specific channel to each genotype:
  - Wild-Type: “Cycling A.Yellow”;
  - Heterozygous: “Cycling A.Green”, “Cycling A.Yellow”;
  - Mutant: “Cycling A.Green”.
- In Edit Samples, select only amplification positive and negative controls.
- Manually set the Discrimination Threshold, so that:
  1) the threshold has the minimum possible value;
  2) negative control **WATER** is identified as “No Reaction”;
  3) positive control **DPYD WT pos ctrl** is identified as “wild-type” and **DPYD MT pos ctrl** is identified as “Mutant”.
- In Edit Samples, select the samples.
- Sample genotype is shown in the “Allelic Discrimination Analysis” and “Allelic Discrimination Results” windows:

Allelic Discrimination Analysis



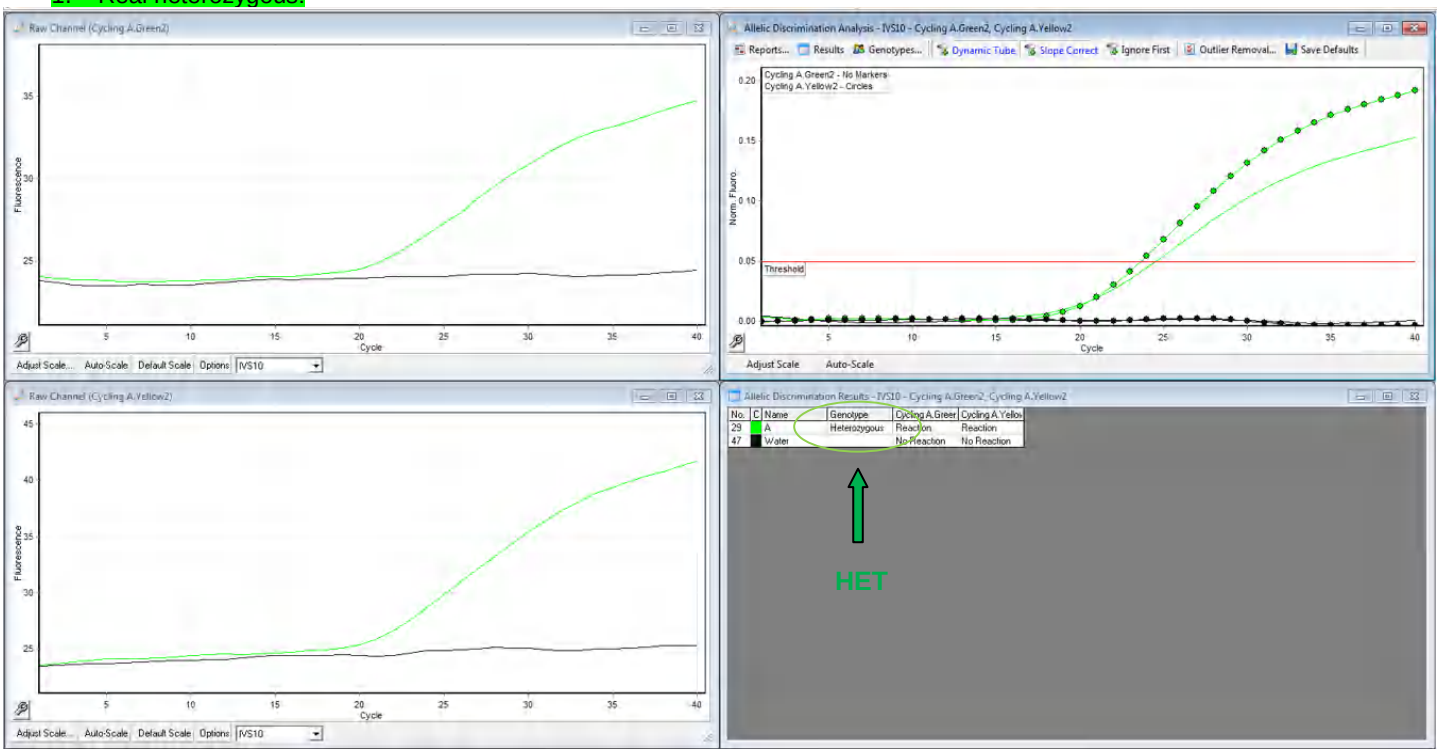
Allelic Discrimination Results

Allelic Discrimination Results - *2A - Cycling A.Green, Cycling A.Yellow

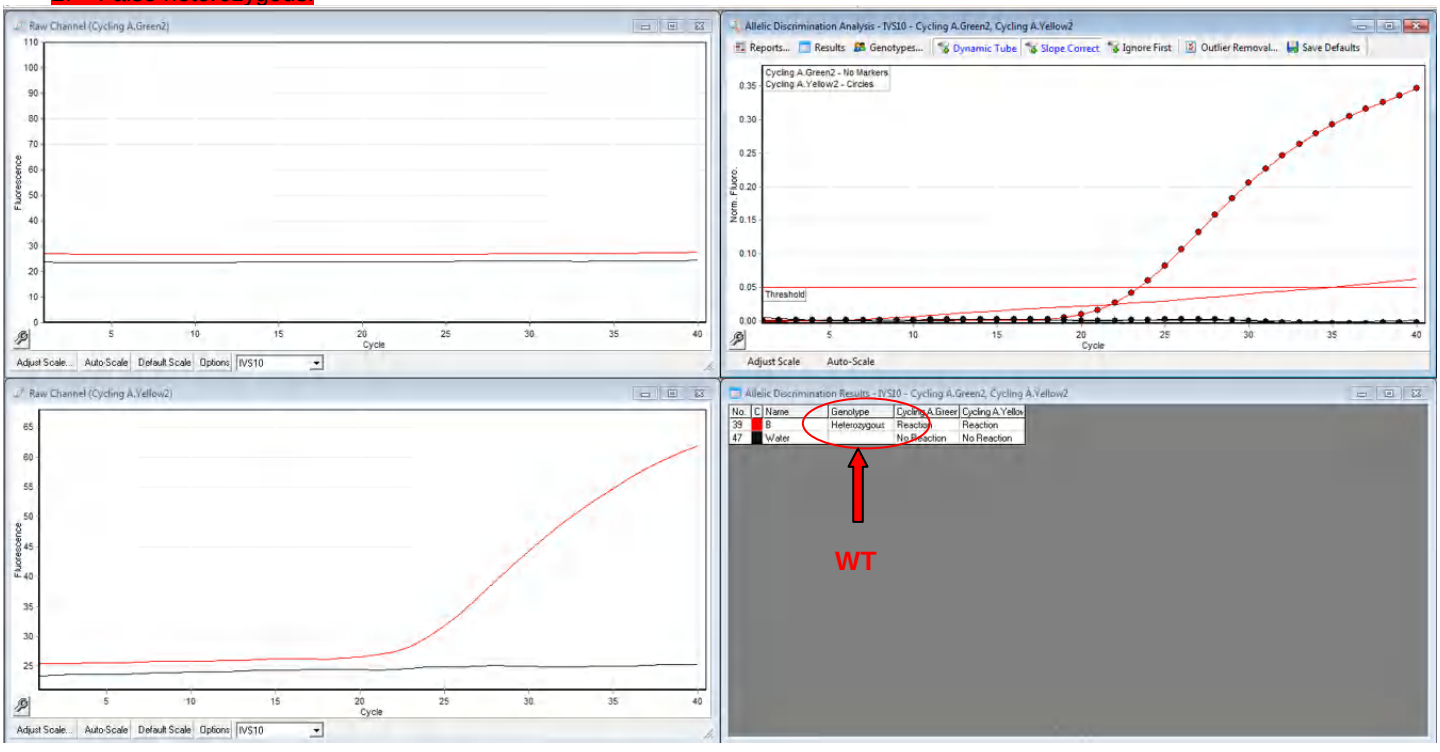
No.	C	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1		A	Wild Type	No Reaction	Reaction
5		B	Heterozygous	Reaction	Reaction
9		DPYD MT pos ctrl	Mutant	Reaction	No Reaction
10		C	Mutant	Reaction	No Reaction
22		DPYD WT pos ctrl	Wild Type	No Reaction	Reaction
24		Water		No Reaction	No Reaction

ⓘ In case of “Heterozygous” result, check for the presence of a real sigmoidal curve for both channels “Green” and “Yellow”. Refer to the graphics below:

1. Real heterozygous:



2. False heterozygous:



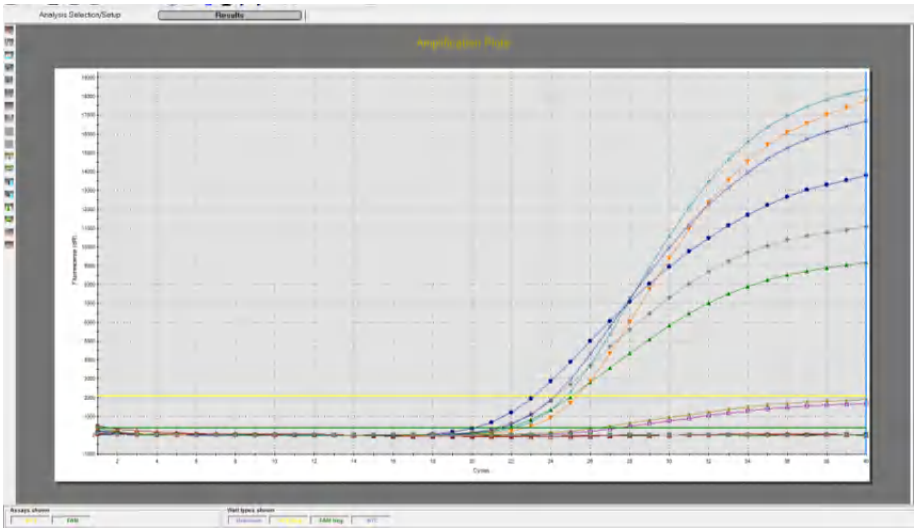
ⓘ Sometimes to have the correct genotype it is necessary to exclude the last 5 amplification cycles from the analysis. In “Raw Channel” “Cycling A.Green” and “Cycling A.Yellow” - “Options” select Crop end cycles and Remove data after cycle: 35. Proceed with “Allelic Discrimination Analysis” for channels “Cycling A(to 35).Green” and “Cycling A(to 35).Yellow”.

Stratagene Mx3000P, Mx3005P

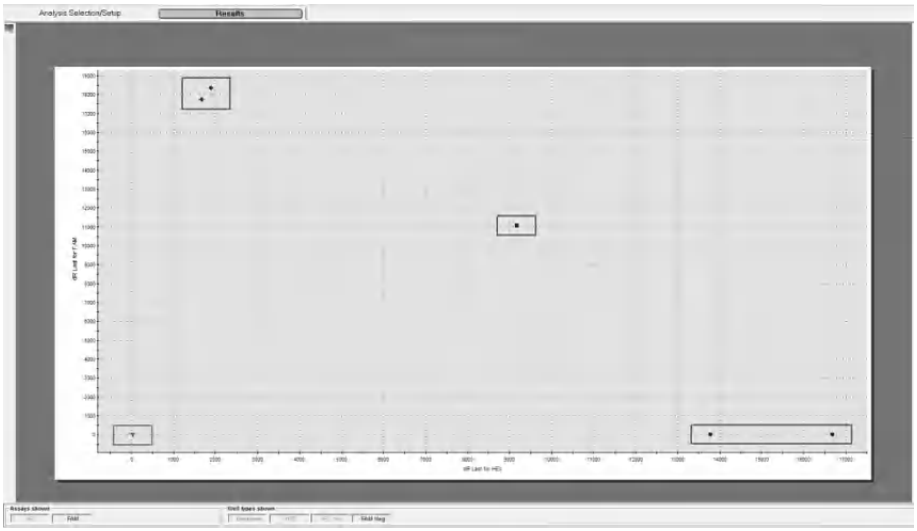
ⓘ Analyze each assay separately, selecting only the respective samples.

- At the end of the run, click Set-up, Plate Setup, select the wells to be analyzed and load samples names.
- Select “Unknown” for samples, “FAM Negative Control” for **DPYD WT pos ctrl**, “HEX Negative Control” for **DPYD MT pos ctrl** and “NTC” for **WATER**.
- In Analysis, Analysis Selection/Setup, select only **DPYD WT pos ctrl**, **DPYD MT pos ctrl** and **WATER**.
- In Analysis, Results, Area to analyze, Amplification plot, manually set the Fluorescence Threshold, so that:
  1) the threshold has the minimum possible value;
  2) negative control **WATER** is identified as “No Ct” for both channels;
  3) positive control **DPYD WT pos ctrl** (FAM negative control) shows a Ct only for the HEX channel;
  4) positive control **DPYD MT pos ctrl** (HEX negative control) shows a Ct only for the FAM channel.
- In the section Analysis, Results, click on the lock icon in the box Threshold Fluorescence (in this way the selected values cannot be modified).
- In Area to Analyze, Dual color scatter plot, click Rename Alleles/Genotypes and set Allele A: WT, Allele B: MT and Both: HET.
- In Area to Analyze, Text Report, the sample genotype is reported in the “Genotype (dR)” column.
- Click Save.

Amplification Plots



Dual color scatter plot



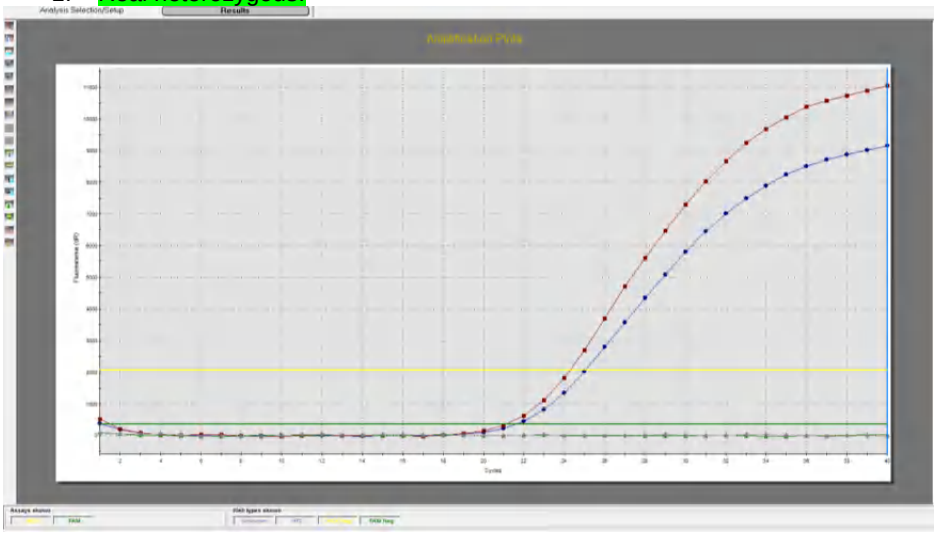
Text report

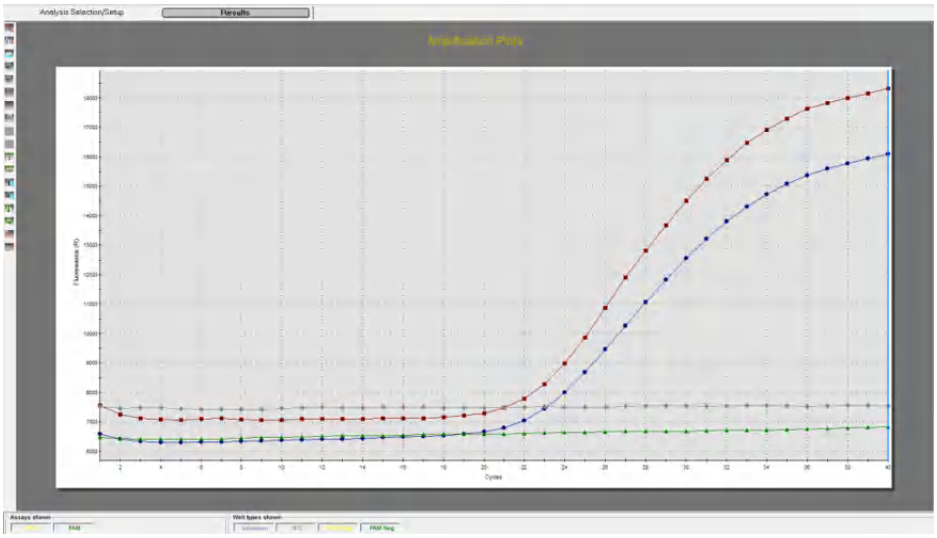
Analysis Selection/Setup | **Results**

Well	Well Name	Dye	Well Type	Threshold (dR)	Ct (dR)	Final Call (dR)	Genotype (dR)
A1	A	HEX	Unknown	2063.538	23.17	+	WT
A1	A	FAM	Unknown	367.017	No Ct	-	WT
A5	B	HEX	Unknown	2063.538	25.05	+	HET
A5	B	FAM	Unknown	367.017	21.18	+	HET
A9	DPYD MT pos ctrl	HEX	HEX Negative Control	2063.538	No Ct	-	MT
A9	DPYD MT pos ctrl	FAM	HEX Negative Control	367.017	22.13	+	MT
A10	C	HEX	Unknown	2063.538	No Ct	-	MT
A10	C	FAM	Unknown	367.017	22.71	+	MT
B10	DPYD WT pos ctrl	HEX	FAM Negative Control	2063.538	24.23	+	WT
B10	DPYD WT pos ctrl	FAM	FAM Negative Control	367.017	No Ct	-	WT
B12	Water	HEX	NTC	2063.538	No Ct	-	None
B12	Water	FAM	NTC	367.017	No Ct	-	None

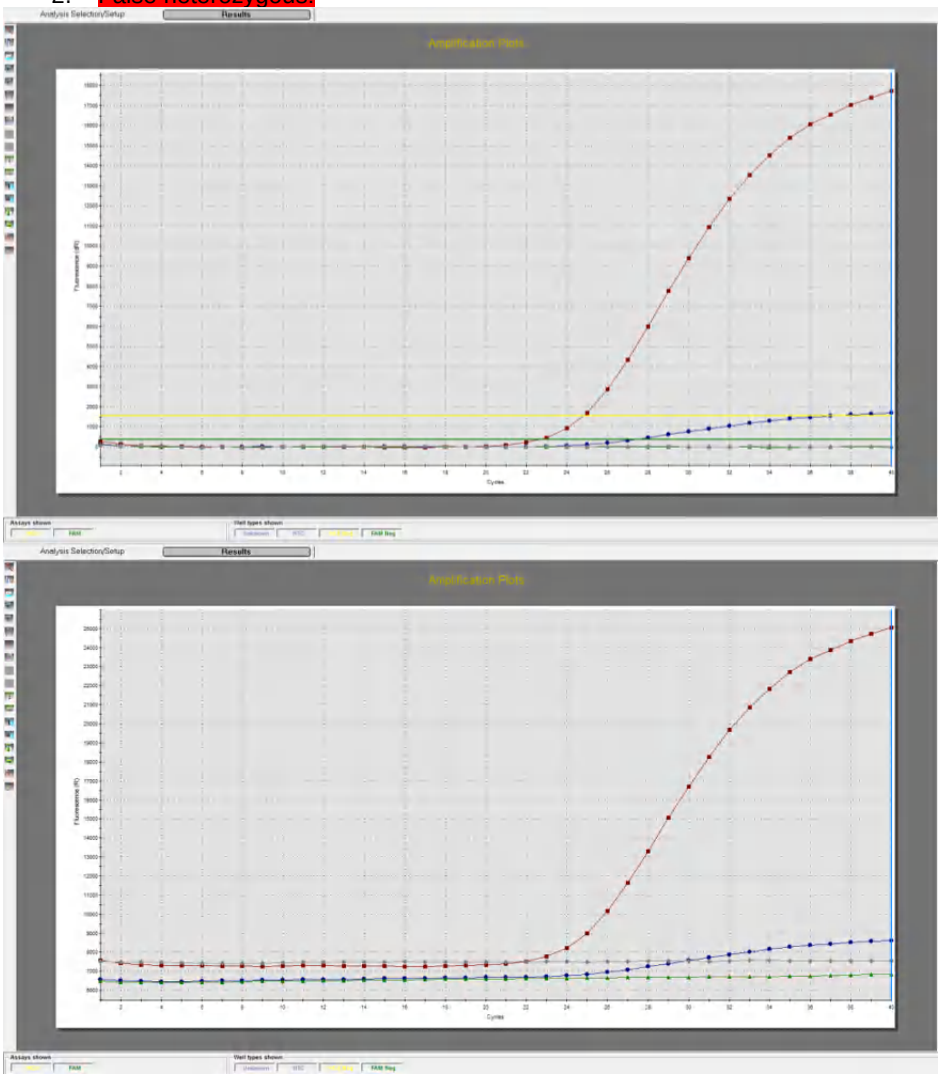
ⓘ In case of “HET” result, check for the presence of a real sigmoidal curve for both channels “FAM” and “HEX”. Refer to the graphics below:

1. Real heterozygous:





2. False heterozygous:

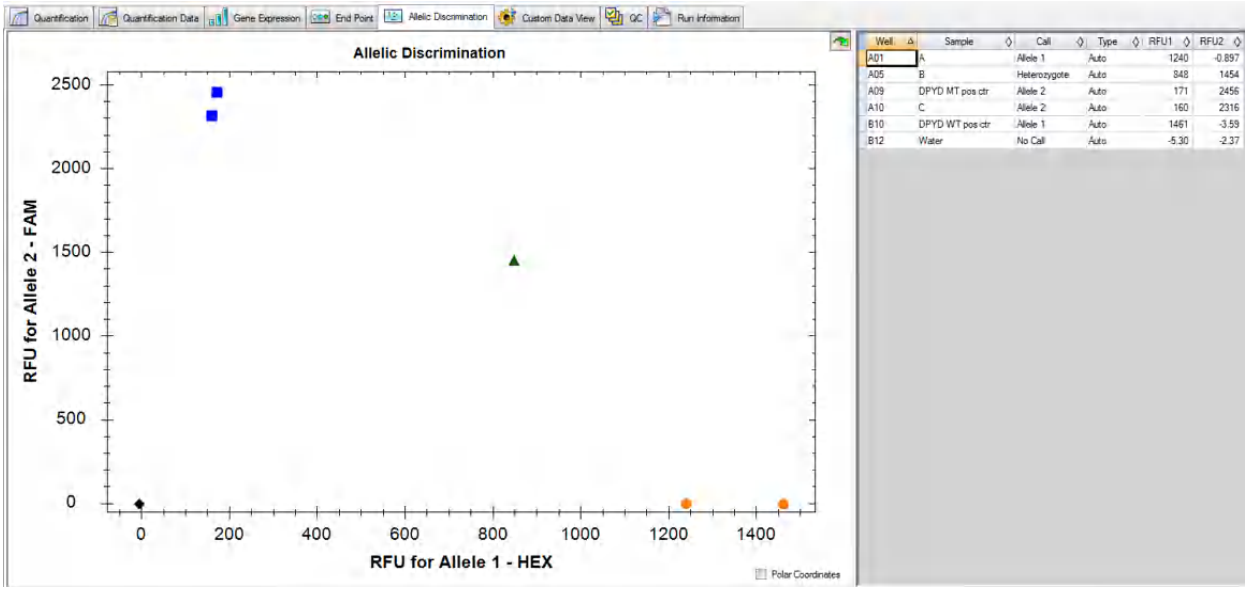


ⓘ Sometimes to have the correct genotype it is necessary to exclude the last 5 amplification cycles from the analysis. In Amplification Plots window drag and drop the blue line up to cycle 35.

CFX96

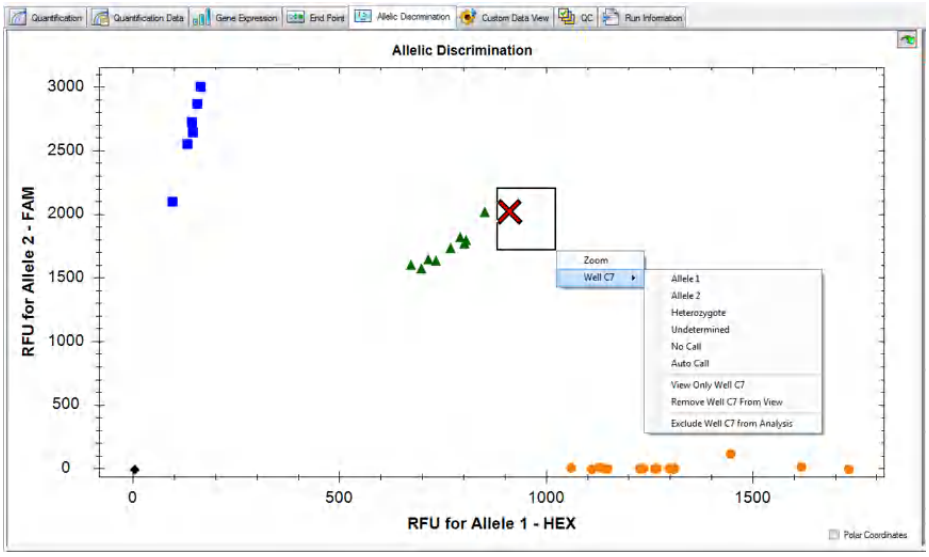
ⓘ Analyze each assay separately, selecting only the respective samples.

- At the end of the run, click Plate Setup – View/Edit Plate, select FAM and HEX fluorophores, select the wells to be analyzed and load samples names.
- Select "Unknown" for samples **DPYD WT pos ctrl** and **DPYD MT pos ctrl**.
- Select "NTC" for negative control **WATER**.
- In Allelic Discrimination, Settings select the default analysis criteria:
  - Cq determination mode: Single Threshold
  - Baseline settings: Baseline Subtracted Curve fit e Apply Fluorescence Drift Correction
  - Analysis Mode: Fluorophore
- In Selected Fluorophores, set X: HEX and Y: FAM, Select cycle: 40 and select View call map.
- In the "Call" column check that:
  1) negative control **WATER** is identified as "No Call";
  2) positive control **DPYD WT pos ctrl** and **DPYD MT pos ctrl** are identified respectively as "Allele 1" and "Allele 2".
- Sample genotype is reported in the "Call" column.
- click File and Save.



ⓘ In Quantification tab check that heterozygous samples have a comparable fluorescence level at cycle 40 for both FAM and HEX channels.

ⓘ If an "Undetermined" sample shows a graphic pattern similar to a wild-type sample (X axis – Allele 1), mutant sample (Y axis – Allele 2) or heterozygous sample (diagonal axis), to assign the genotype right click on the graph and select the correct genotype. The graph below shows an heterozygous sample identified as "Undetermined":



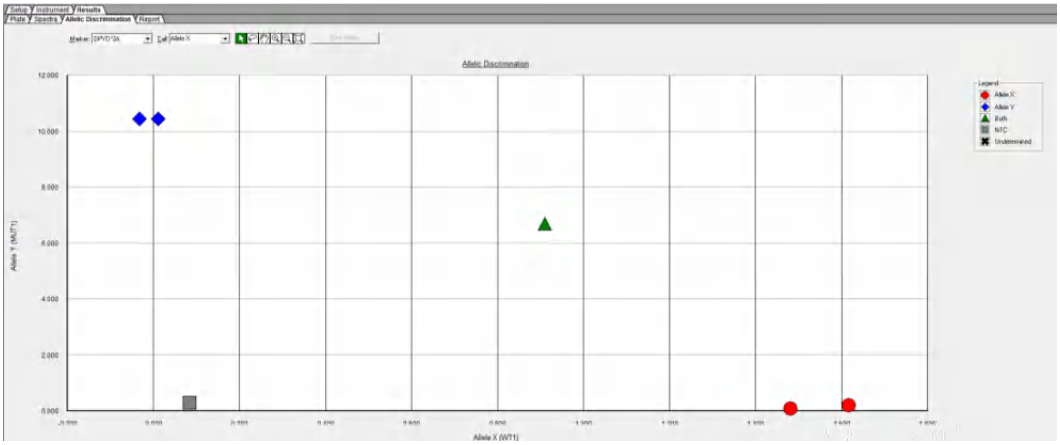
ABI 7300

ⓘ Analyze each assay separately, selecting only the respective samples.

- At the end of the run, click Setup – Plate, select the wells to be analyzed and load samples names.
- Select "Unknown" for samples, **DPYD WT pos ctrl** and **DPYD MT pos ctrl** and "NTC" for **WATER**.
- In Results – Allelic Discrimination, click Analysis, Analysis Settings, deselect the options Analyze post-read data only and Keep Manual Calls from previous Analysis, in the drop-down menu select Marker: "assay name", Automatic Allele Calling and set Quality Value: 90%.
- Click Apply, Ok & Reanalyze then Yes.
- In Results – Report, in the column "Call" check that:

1) negative control **WATER** is identified as "NTC"
2) positive controls **DPYD WT pos ctrl** and **DPYD MT pos ctrl** are identified respectively as "assay name WT" and "assay name MT".

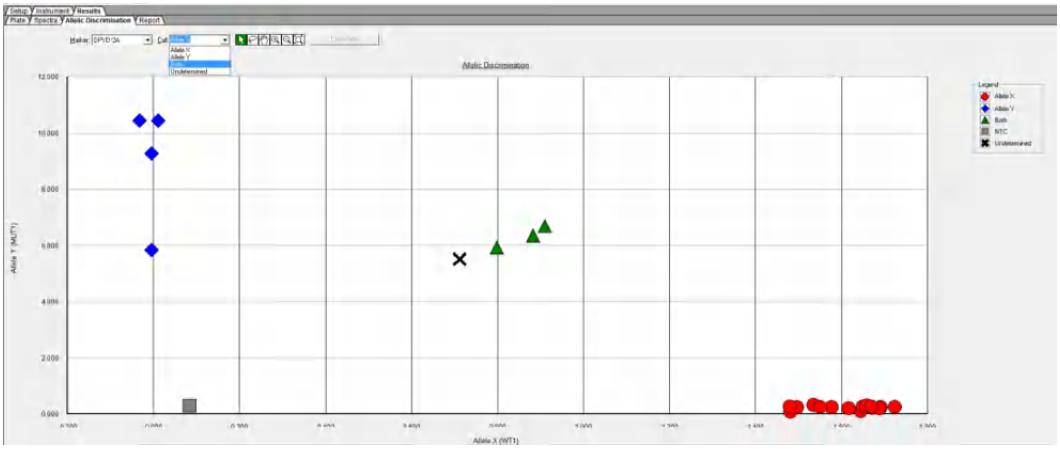
- Samples genotype is reported in the "Call" column.
- Click Save.



Setup | Instrument | Results
Plate | Spectra | Allelic Discrimination | Report

Well	Sample Name	Marker	Task	Call	Quality(%)	Method
A1	A	DPYD*2A	Unknown	WT1	99.37	Auto Call
A5	B	DPYD*2A	Unknown	Both	99.85	Auto Call
A9	DPYD MT pos ctrl	DPYD*2A	Unknown	MUT1	98.53	Auto Call
A10	C	DPYD*2A	Unknown	MUT1	98.53	Auto Call
B10	DPYD WT pos ctrl	DPYD*2A	Unknown	WT1	100.00	Auto Call
B12	Water	DPYD*2A	NTC	NTC		

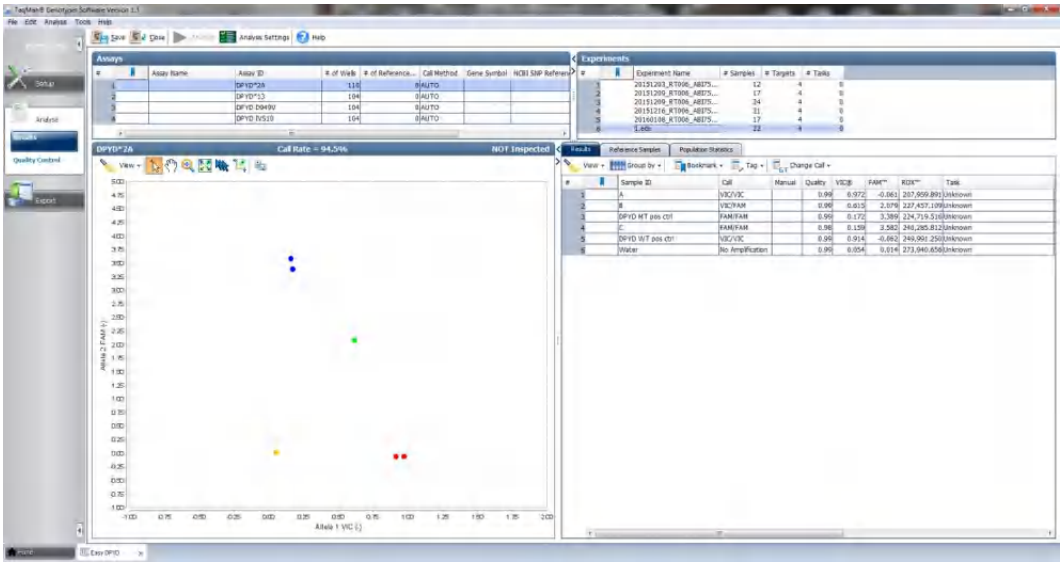
ⓘ If an "Undetermined" sample shows a graphic pattern similar to a wild-type sample (X axis), mutant sample (Y axis) or heterozygous sample (diagonal axis), assign the correct genotype in the "Call" drop-down menu. The graph below shows an heterozygous sample identified as "Undetermined":



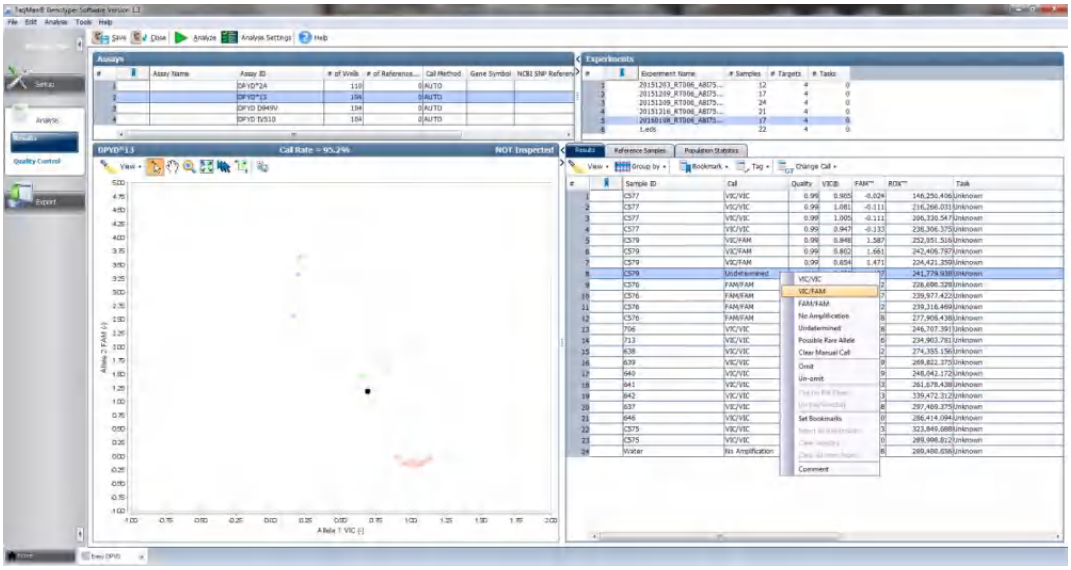
ABI 7500

ⓘ Analyze each assay separately, selecting only the respective samples.

- At the end of the run, in Setup, Plate Set-up, set samples names.
- Select “Unknown” for samples, “Positive Control Allele 1/Allele 1” for **DPYD WT pos ctrl**, “Positive Control Allele 2/Allele 2” for **DPYD MT pos ctrl** and “Negative Control” for **WATER**.
- In Analysis click Analyze.
- Repeat the steps above for each assay.
- Click Save.
- Open TaqMan® Genotyper Software v1.3 (available on the Themo Fisher Scientific website) and click Create Study.
- In Setup, Properties set Study Name: Easy DPYD, Instrument Type: 7500 Fast Real-Time PCR System and Experiment type: Endpoint+pre-PCR read.
- In Setup, Experiments, click Import and select the run file.
- In Analysis, Results, in Assays select the Assay ID to be analyzed.
- In Analysis, Results, in “Call” column check that:
  1) negative control **WATER** is identified as “No Amplification”;
  2) positive controls **DPYD WT pos ctrl** and **DPYD MT pos ctrl** are identified respectively as “VIC/VIC” and “FAM/FAM”.
- Samples genotype is reported in the “Call” column.



ⓘ If an “Undetermined” sample shows a graphic pattern similar to a wild-type sample (X axis – Allele 1), mutant sample (Y axis – Allele 2) or heterozygous sample (diagonal axis), to assign the genotype right click on the call and select the correct genotype. The graph below shows an heterozygous sample identified as “Undetermined”:



TROUBLESHOOTING

Problem	Possible reason	Recommendation
Low or absent amplification signal for one or more assays in the channel "VIC/Yellow/HEX" for both **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.
	No dispensation of the specific DPYD mix into Amp-Mix.	▪ Samples show no signal also for the FAM/Green channel. ▪ Repeat amplification.
Low or absent amplification signal for one or more assays in the channel "FAM/Green" for both **DPYD MT 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.
	No dispensation of the specific DPYD mix into Amp-Mix.	▪ Samples show no signal also for the VIC/HEX/Yellow channel. ▪ Repeat amplification.
Amplification signal weak or absent in "Green / FAM" and "Yellow / HEX/VIC" for one or more than one sample.	Insufficient amount of starting DNA and / or presence of PCR inhibitors.	▪ Check the quantity and quality of the extracted DNA and, if appropriate, repeate the extraction faithfully following the instructions of the extraction kit. ▪ If the extraction protocol involves the use of washing buffers containing ethanol, it is advisable to carry out a further centrifugation prior to final elution to remove any possible trace of alcohol. ▪ If it is assumed that the amount of starting DNA is insufficient, repeat the reaction amplifying > 5ul DNA (max volume 8 ul), reducing the corresponding volume of **WATER** in the **Amp-Mix**. Otherwise repeat the DNA extraction by reducing the volume of elution and repeat the amplification using at least 2ng/ul of DNA. ▪ If you suspect the presence of inhibitors, repeat the amplification diluting sample 1:5 or 1:10. ▪ In each run test also amplification positive and negative controls.
	Incorrect or no dispensation of the samples.	▪ Repeat the amplification dispensing the correct volume of DNA and including positive and negative controls. ▪ In each run test also amplification positive and negative controls.
You can not perform the analysis on Rotor-Gene in Cycling A. Green and/or Cycling A. Yellow because of a fluorescence intensity too high or out of scale for one or more than one assay.	Rotor-Gene gain too high.	▪ Perform the calibration in order to set the initial fluorescence between 20-30FI.
Low or absent amplification signal.	Degradation of the fluorophores due to improper storage of the DPYD mix.	▪ Store DPYD mix protected from light. ▪ Store DPYD mix at -35/-20°C and avoid thawing and re-freezing the reagents more than twice. ▪ Do not store DPYD mix at +2/+8°C for more than 5 hours.
	Incorrect manipulation of the reagents.	▪ Mix reagents by vortexing or inverting the tubes ten times and briefly spin before use. ▪ Keep reagents on ice or refrigerated blocks during the preparation of the Amp-Mix.
	Incorrect set-up of the amplification thermal profile.	▪ Check the thermal profile and fluorescence acquisition channels and repeat amplification with the settings described in this manual.
Incorrect genotypization of **DPYD WT pos ctrl** and/or **DPYD MT pos ctrl**. One or more than one sample cannot be genotyped.	Aberrant fluorescent signals on Rotor-Gene or Mx3000/3005P	▪ Exclude the last 5 cycles of amplification from the analysis. ▪ Increase the threshold value in order to exclude unspecific signals.
	Heterozygous sample with high difference between "FAM" / "Green" and "VIC" / "Yellow" / "HEX" signals.	
	Wrong tubes identification.	▪ Repeat the amplification after marking unambiguously the reaction tubes for samples and controls.
	Incorrect dispensing of the samples.	▪ Repeat the amplification paying attention to the dispensation of the DNA and the controls in reaction tubes/wells.
	Incorrect samples names set-up in the software.	▪ Check samples names set-up.

The negative control **WATER**, shows an amplification signal in both “FAM/Green” and “VIC/HEX/Yellow” channels.	Contamination.	▪ The results should be rejected and samples must be reamplified using new reagents. ▪ Prepare **Amp Mix** in a dedicated area. Carefully decontaminate benches, pipettes and instruments.
	Unspecific or aberrant fluorescent signal on Rotor-Gene o Mx3000/3005P	▪ Exclude the last 5 cycles of amplification from the analysis. ▪ Increase the threshold value in order to exclude unspecific signals.
Fluorescence intensity variable.	Cutaneous fat on the reaction tube.	▪ Wear gloves.
If the problems persist despite the implementation of the recommendations given and for any other questions or problems, please contact technical support of Diatech Pharmacogenetics: ▪ e-mail support@diatechpharmacogenetics.com ▪ telephone +39 0731 213243 ▪ fax +39 0731 213239		

PERFORMANCE VALIDATION

The performance validation has been performed using all the reagents supplied with the kit.

The experiments have been performed according to the instructions reported in this user manual on the following real-time instruments:

- **CFX96** - Bio-Rad (software v. 3.1)
- **ABI 7300**- Applied Biosystems (software v. 1.4.1)
- **ABI 7500** - Applied Biosystems (con software v. 2.0.5) in association with TaqMan Genotyper (software v.1.3)
- **Stratagene Mx3000P**, **Mx3005P** - Agilent Technologies (software v. 4.10 - Build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

Clinical specificity

In order to evaluate the kit specificity DNA samples isolated from whole blood have been tested. Samples were suitable in terms of starting DNA amount and have been already genotyped through pyrosequencing technology (kit "**FLUOROPYRIMIDINES response®**", cod. UP024 Diatech Pharmacogenetics), Mass Spectrometry using MassArray® platform ("**Myriapod® ADMET**" cod. SQ040 Diatech Pharmacogenetics), direct sequencing or High Resolution Melt Analysis. Also samples with known genotype from Coriell Institute have been analyzed.
No homozygous mutant samples were available, so clinical specificity for these genotypes has been evaluated using synthetic oligos.

	Rotor-Gene		**Stratagene Mx3000P, Mx30005P**		**CFX96**		**ABI 7300**		**ABI 7500**	
	N° samples tested	**N° samples correctly genotyped**	**N° samples tested**	**N° samples correctly genotyped**	**N° samples tested**	**N° samples correctly genotyped**	**N° samples tested**	**N° samples correctly genotyped**	**N° samples tested**	**N° samples correctly genotyped**
DPYD*2A het	6	6/6	6	6/6	6	6/6	6	6/6	7	7/7
DPYD*13 het	1	1/1	1	1/1	1	1/1	1	1/1	1	1/1
DPYD D949V het	7	7/7	8	8/8	8	8/8	6	6/6	8	8/8
DPYD IVS10 het	2	2/2	2	2/2	2	2/2	1	1/1	2	2/2
DPYD wild-type	29	29/29	24	24/24	25	25/25	24	24/24	30	30/30
Totale	45	45/45	41	41/41	42	42/42	38	38/38	48	48/48

Analytical sensitivity

The sensitivity limit of the kit was evaluated as the minimum amount of DNA necessary to correctly genotype 95% of the samples.
The analytical sensitivity of the kit was evaluated by testing for each assay, serial dilutions 100-20-2 ng/ul of two DNA samples (homozygous wild-type and heterozygous from Coriell Institute). For homozygous mutant samples serial dilution of synthetic oligo were tested.
Wild-type and mutant samples have been tested in duplicates at 100ng/ul, while heterozygous samples have been tested in duplicates at 2ng/ul.
Four independent sessions have been performed on each instrument (Rotor-Gene, Mx3000P/Mx3005P, ABI 7300, ABI 7500 e CFX96).
Given the above criteria, the sensitivity limit was 2 ng/μl.

Repeatability and Reproducibility

Reproducibility (*inter-assay* variability) and repeatability (*intra-assay* variability) have been evaluated testing four replicates of samples with known genotype in four independent sessions. Results are reproducible and repeatable in terms of correct genotypization for all assays.

Sample	**Genotype**	**N° replicates**	**N° sessions**	**Results**
NA18633	Wild-type	16	4	64/64 wild-type for all assays
NA20812	DPYD*2A het	4	4	16/16 heterozygous for DPYD*2A assay
M4620	DPYD*2A mut	4	4	16/16 mutant for DPYD*2A assay
HG00332	DPYD*13 het	4	4	16/16 heterozygous for DPYD*13 assay
M4628	DPYD*13 mut	4	4	16/16 mutant for DPYD*13 assay
NA20515	DPYD D949V het	4	4	16/16 heterozygous for DPYD D949V assay
M4623	DPYD D949V mut	4	4	16/16 mutant for DPYD D949V assay
NA20531	DPYD IVS10 het	4	4	16/16 heterozygous for DPYD IVS10 assay
M4622	DPYD IVS10 mut	4	4	16/16 mutant for DPYD IVS10 assay

Robustness

Lot to lot consistency
Different batches of **Taq PreMix 920** have been tested with the same DNA samples. Results from the different lots are comparable.
Two different batches of primers and probes have been tested with the same DNA samples. Results from the different lots are comparable.

RT006 sample grid A – Easy® DPYD

DATE		RUN NAME	

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

DPYD*2A mix (1)

DPYD*13 mix (2)

DPYD D949V mix (3)

DPYD IVS10 mix (4)

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

RT006 sample grid B – Easy® DPYD

DATE		RUN NAME	

	WATER	WT POS CTRL	MT POS CTRL						
DPYD*2A mix (1)	1 ●	9 ●	17 ●	25 ●	33 ●	41 ●	49 ●	57 ●	65 ●
DPYD*13 mix (2)	2 ●	10 ●	18 ●	26 ●	34 ●	42 ●	50 ●	58 ●	66 ●
DPYD D949V mix (3)	3 ●	11 ●	19 ●	27 ●	35 ●	43 ●	51 ●	59 ●	67 ●
DPYD IVS10 mix (4)	4 ●	12 ●	20 ●	28 ●	36 ●	44 ●	52 ●	60 ●	68 ●
	5 ●	13 ●	21 ●	29 ●	37 ●	45 ●	53 ●	61 ●	69 ●
	6 ●	14 ●	22 ●	30 ●	38 ●	46 ●	54 ●	62 ●	70 ●
	7 ●	15 ●	23 ●	31 ●	39 ●	47 ●	55 ●	63 ●	71 ●
	8 ●	16 ●	24 ●	32 ●	40 ●	48 ●	56 ●	64 ●	72 ●

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

OPERATOR NOTES

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