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Easy® UGT1A1

User manual – version 2017/06

The "**Easy® UGT1A1**" kit detects by Real-Time PCR the polymorphism UGT1A1*1 (TA)6, UGT1A1*28 (TA)7, UGT1A1*36 (TA)5 and UGT1A1*37 (TA)8 of the UGT1A1 gene promoter associated with the toxicity due to the treatment with irinotecan.

For *in vitro* diagnostic use

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Changes made since the previous version 2016/09

- Update of section “Materials required but not provided”. – Amplification, Materials
- Validation of the kit on **ABI 7500 Fast**.

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

INTENDED USE

The "**Easy® UGT1A1**" kit detects by Real-Time PCR the polymorphism UGT1A1*1 (TA)6, UGT1A1*28 (TA)7, UGT1A1*36 (TA)5 and UGT1A1*37 (TA)8 of the *UGT1A1* gene promoter in human genomic DNA extracted from whole blood.

The "**Easy® UGT1A1**" 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, 7500 Fast** - 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® UGT1A1**" kit detects, by allelic discrimination, the polymorphism UGT1A1*1, UGT1A1*28, UGT1A1*36 and UGT1A1*37 that can be used for the personalization of the irinotecan treatment.

The assay contains two probes specific for the sequences UGT1A1*28 and UGT1A1*37 (HEX), and two probes specific for the sequences UGT1A1*1 and UGT1A1*36 (FAM).
Homozygous mutant sample generates a signal in the FAM channel, wild-type sample in HEX channel. Amplification signal for both FAM and HEX channels indicate an heterozygous sample.
Samples with UGT1A1*28/*28, UGT1A1*37/*37 or UGT1A1*28/*37 genotype generates a signal in the HEX channel otherwise samples with UGT1A1*1/*1, UGT1A1*36/*36 or UGT1A1*1/*36 genotype generates a signal in the FAM channel.
Amplification signal for both FAM and HEX channel indicates an heterozygous sample with UGT1A1*1/*28, UGT1A1*1/*37, UGT1A1*36/*28 or UGT1A1*36/*37 genotype.

CLINICAL BACKGROUND

The irinotecan can cause severe gastrointestinal and haematological toxicity resulting from the deficiency of its metabolism by glucuronidation catalyzed by the enzyme UGT1A1[1]. Molecular analysis of the UGT1A1 gene promoter (wild-type allele *1 [$(TA)_6$TAA] and mutant allele *28 [$(TA)_7$TAA] rs8175347) is recommended as in patients homozygous for the UGT1A1*28 allele [$(TA)_{7/7}$TAA], there is a significant reduction of UGT enzyme activity[2,3] that makes necessary to reduce at least by 30% the starting dosage of irinotecan in order to avoid the risk of severe toxicity[4-7]. The frequency of the UGT1A1*28 allele is about 40% in the European population[8] while variants with 5 or 8 TA repeats occur at much lower frequencies, primarily in individuals of African descent.
The promoter variant lacking one TA repeat [UGT1A1*36 (TA)5] leads to an increased transcriptional activity respect to the wild-type variant and it is present in Africans with a frequency up to 10%. The promoter variant with an additional TA insertion leads to 8 TA repeats [UGT1A1*37 (TA)8] resulting in a further reduction of glucuronidation activity respect to wild-type variant and it is present in Africans with a frequency up to 7%[9]. About the dosage personalization of the irinotecan chemotherapy, the rare allele *36 (proficient) can be interpreted as an *1 allele and allele *37 (deficient) as an allele *28[10].
Thus, knowledge of the UGT1A1 polymorphism status could help guide the selection of appropriate starting dosages, reducing the risk of severe toxicity and improving the chances that therapy could be maintained.

References

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3. Iyer L, Das S, Janisch L, et al. UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. Pharmacogenomics J. 2002;2:43–47.
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6. Innocenti F, Undevia SD, Iyer L, et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol. 2004;22:1382-1388.
7. Raccomandazioni per Analisi Farmacogenetiche UGT ed Irinotecan (Gruppo di lavoro AIOM-SIF)
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9. Horsfall L, Zeitlyn D, Tarekegn A, et al. Prevalence of Clinically Relevant UGT1A Alleles and Haplotypes in African Populations. Annals of human genetics 2011; 75:236–246.
10. https://www.pharmgkb.org/guideline/PA166127626.

KIT CONTENTS

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

COMP		QUAN	Color	
UGT1A1 mix		3 x 20 µl	RED	Mixture of specific primers and probes targeting the UGT1A1 gene polymorphism.
UGT1A1 WT pos ctrl	CONTROL +	3 x 20 µl		Positive control DNA containing synthetic DNA with wild-type UGT1A1*1/*1 genotype.
UGT1A1 MT pos ctrl	CONTROL +	3 x 20 µl		Positive control DNA containing synthetic DNA with mutant UGT1A1*28/*28 genotype.
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 310		1 x 310 µl		Solution containing hot start Taq DNA polymerase, reaction buffer, Mg^{2+} and dNTP mixture.
Dye R		1 x 18 µl		Inert fluorophore to be used for the amplification on ABI 7300 instrument.
Dye R II		1 x 18 µl		Inert fluorophore to be used for the amplification on ABI 7500 and 7500 Fast instrument.

DOCUMENTS AVAILABLE ON-LINE

Easy® UGT1A1 User Manual and Safety Data Sheet (SDS) are available at www.diatechpharmacogenetics.com/en/reserved-area

For any clarification contact the Diatech Pharmacogenetics technical support (support@diatechpharmacogenetics.com)

MATERIALS REQUIRED BUT NOT PROVIDED

Genomic DNA extraction

The "**Easy® UGT1A1**" 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 test 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 (in alternative):
- **CFX96** - Bio-Rad (software v. 3.1)
- **ABI 7300**- Applied Biosystems (software v. 1.4.1)
- **ABI 7500, 7500 Fast** - 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. **ABI 7500 Fast: MICROAMP FAST OPTICAL 96 WELL RNX PLATE** cod. 4346907; **OPTICAL ADHESIVE COVERS** cod. 4360954
    4. **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:
    - **PCR tubes 0.1 (1000)** – cod. DIA-PL1, Diatech Pharmacogenetics
    - **Strip tubes and caps, 0.1 ml (250)** – cod. 981103, Qiagen
- 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 310** 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 +	Positive control
GTIN	Global Trade Item Number	CONTROL -	Negative control
LOT	Lot number		Consult the instruction
Σ	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		

PRODUCT USE LIMITATIONS

- The "**Easy® UGT1A1**" 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® UGT1A1**" 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/ABI 7500 Fast** (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.
- **"Easy® UGT1A1"** is a CE-IVD marked product under the European Directive 98/79/EC on IVD medical devices only into those countries wich are accepting the handbook translated into the languages listed at www.diatechpharmacogenetics.com/area-riservata.
- Diatech Pharmacogenetics does not respond to the quality of the result obtained using accessories other than those recommended in this manual.

QUALITY CONTROL

- The **"Easy® UGT1A1"** Kit was designed, developed and validated in accordance with the Directive 98/79/EC on in vitro diagnostic medical devices, transposed at national level with the D.Lgs n. 332/2000 and the subsequent legislative changes, and in accordance with the company system of full quality assurance certified in accordance to the European standards EN ISO 9001 and ISO 13485
- The consistent product quality of the **"Easy® UGT1A1"** kit is guaranteed by the application of a tight quality control process on operating procedures for the realization of the product and subsequent managment to the customer. The quality of each lot is attested in the Certficate of Analysis available upon request to the Customer Service (support@diatechphamaracogenetics.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 this User Manual corresponds to the one described on the "**Easy® UGT1A1**" 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 Material Safety Data Sheet (MSDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com.
9. Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
10. It is recommended to ensure that the laboratory workflow proceeds in a unidirectional manner, preparing, if possible, two separate work areas for:
    a. extraction of nucleic acids;
    b. 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 only opening the reagents within the hood.
15. 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.
16. The precision pipettes used should have an accuracy 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 "**Easy® UGT1A1**" kit are intended to be used solely with the other reagents in the same "**Easy® UGT1A1**" kit. Do not substitute or mix reagents in the kit from different batches, in order to maintain optimal performance.
20. Only use the **Taq PreMix 310** that is provided in the kit. Do not substitute with **Taq PreMix 310** from other kits or with similar reagents from other suppliers.
21. Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
22. 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.
23. 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.
24. Avoid contamination of samples and reagents.
25. Store reagents and samples separately.
26. In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.
27. Before use all reagents need to be thawed at room temperature, mixed by inverting 10 times and centrifuged briefly.
28. 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.
29. Include in each run at least 1 negative control (**WATER**) and 2 positive control (**UGT1A1 WT pos ctrl, UGT1A1 MT pos ctrl**).
30. 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.
31. 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.
32. 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® UGT1A1**" kit does not include the reagents for DNA extraction.
- Extract genomic DNA from EDTA-anticoagulated whole blood. Avoid the use of heparin as anticoagulant because it may inhibit the amplification reaction.
- 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 25-500 ng of DNA for the amplification reaction.

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 (**UGT1A1 WT pos ctrl**, **UGT1A1 MT pos ctrl**).

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:
  - "UGT1A1 Green": source 470 nm – detector 510 nm – "Gain Optimisation" 60°C Before 1st acquisition, "Tube Position": 1 (**Water**), "Target Sample Range" 10-20 Fl;
  - "UGT1A1 Yellow": source 530 nm – detector 555 nm – "Gain Optimisation" 60°C Before 1st acquisition, "Tube Position": 1 (**Water**), "Target Sample Range" 30-40 Fl;

Thermal Profile	
Hold	95°C for 2 minutes
40 cycles	95°C for 15 seconds / 62°C for 15 seconds / 60°C for 45 seconds (acquire fluorescence in channels "UGT1A1 Green" and "UGT1A1 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 x1

Thermal Profile	
Hold	95°C for 2 minutes
40 cycles	95°C for 15 seconds / 62°C for 15 seconds / 60°C for 45 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 for 2 minutes
2	95°C for 15 seconds
3	63°C for 15 seconds
4	60°C for 45 seconds (Plate read – All Channels)
5	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: UGT1A1 WT" - "Reporter Dye: FAM" - "Quencher Dye: none";
  - "Name: UGT1A1 MT" - "Reporter Dye: VIC" - "Quencher Dye: none";
- Create the UGT1A1 marker with the two 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 for 2 minutes
40 cycles	95°C for 15 seconds / 63°C for 15 seconds / 60°C for 45 seconds (fluorescence acquisition for both detectors)

- Set Sample Volume (µl): 20 and click Start. At the end save the run.
- Open Pre-Read run.
- In Instrument, click File, Save As…, name the run as post-read and then click Post-Read. At the end save the run.

ABI 7500, ABI 7500 Fast

- Select New Experiment, Genotyping, TaqMan Reagents and Standard.
- Follow the instructions reported in the user manual of the instrument to set up the SNP Assay with "Passive Reference: ROX":
  - "SNP Assay Name: UGT1A1":
    "Allele 1 Name or Base(s): WT" - "Reporter: FAM" - "Quencher Dye: none"
    "Allele 2 Name or Base(s): MT" - "Reporter: VIC" - "Quencher Dye: none"
- Follow the instructions reported in the user manual of the instrument to setup the following thermal profile:

Thermal profile	
Hold	60°C for 60 seconds (fluorescence acquisition)
Hold	95°C for 2 minutes
40 cycles	95°C for 15 seconds / 63°C for 15 seconds / 60°C for 45 seconds (fluorescence acquisition)
Hold	60°C for 60 seconds (fluorescence acquisition)

- Reaction volume: 20 µl.

ⓘ The kit content is optimized to analyze five clinical samples and three controls (**UGT1A1 WT pos ctrl, UGT1A1 WT pos ctrl** e **WATER**) in each run.

Mx3000P/3005P, ABI 7300, ABI 7500, ABI 7500 Fast, CFX96 (Easy® UGT1A1 sample grid A)

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

UGT1A1 mix

Rotor-Gene (Easy® UGT1A1 sample grid B)

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

- Prepare, for each sample and control, an amplification mixture (**Amp-Mix**), as indicated in the following scheme, where N is the number of samples and controls to be tested.

Rotor-Gene, Stratagene, CFX96		
Amp-Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
Taq Premix 310	10	
WATER CONTROL -	3	
UGT1A1 mix	2	
Total volume	15	

ABI 7300		
Amp-Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
Taq Premix 310	10	
Dye R	0.4	
WATER CONTROL -	2.6	
UGT1A1 mix	2	
Total volume	15	

ABI 7500, ABI 7500 Fast		
Amp-Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
Taq Premix 310	10	
Dye R II	0.2	
WATER CONTROL -	2.8	
UGT1A1 mix	2	
Total volume	15	

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

negative control	5 µl **WATER**	CONTROL -
sample	5 µl DNA	
positive control	5 µl **UGT1A1 WT pos ctrl**	CONTROL +
positive control	5 µl **UGT1A1 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

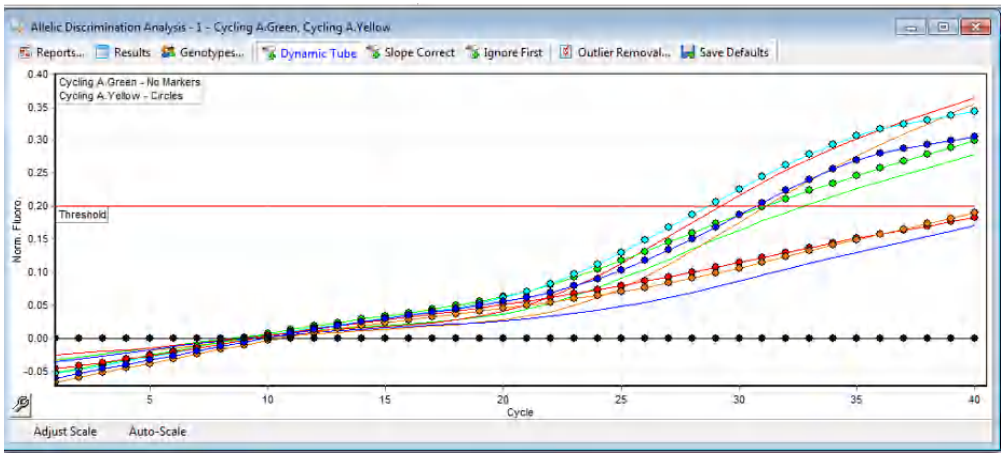
Rotor-Gene

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

Allelic Discrimination Analysis

- Name all the samples.
- Select Analysis, Other, Allelic Discrimination.
- Select the channels Cycling A.Green and Cycling A.Yellow (clicking CTRL) and click Show.
- Select Dynamic Tube.
- Click Outlier Removal, in the "NTC Threshold" box set the value to "30" and click OK.
- Click on Genotypes and link the specific channel to each genotype:
  - Wild-Type: "Cycling A.Green";
  - Heterozygous: "Cycling A.Green", "Cycling A.Yellow";
  - Mutant: "Cycling A.Yellow".
- 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 **UGT1A1 WT pos ctrl** is identified as "wild-type" and **UGT1A1 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



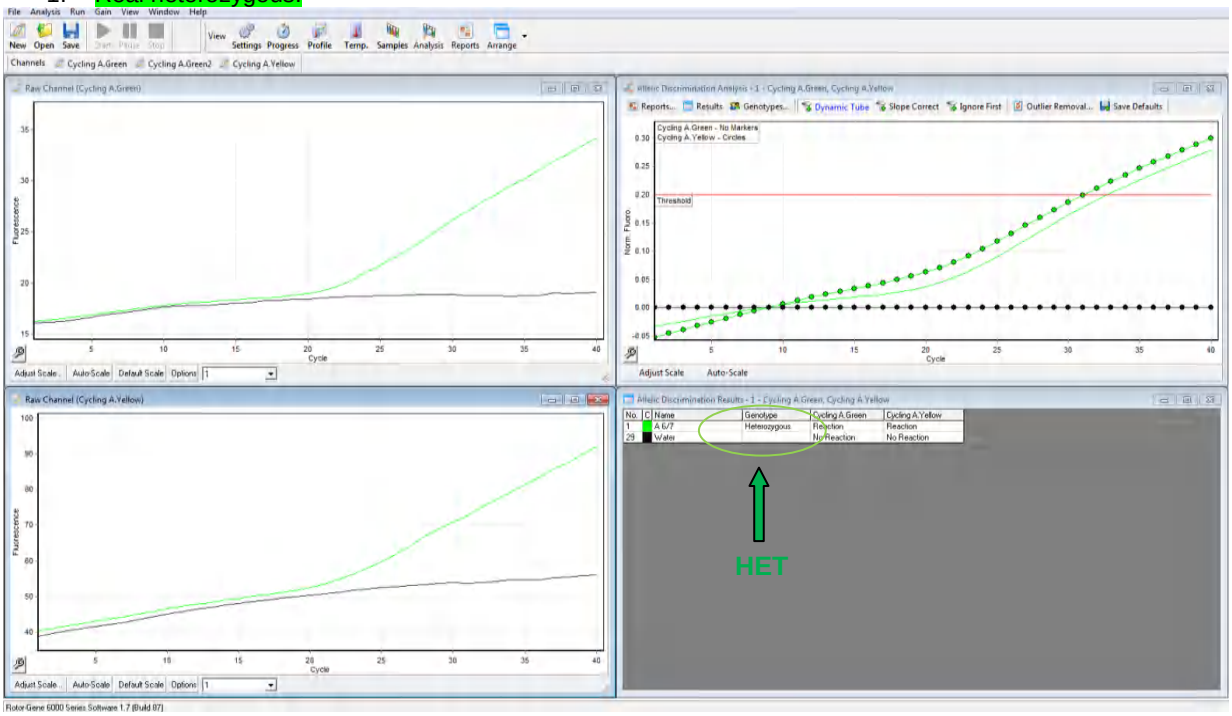
Allelic Discrimination Results

Allelic Discrimination Results - 1 - Cycling A.Green, Cycling A.Yellow

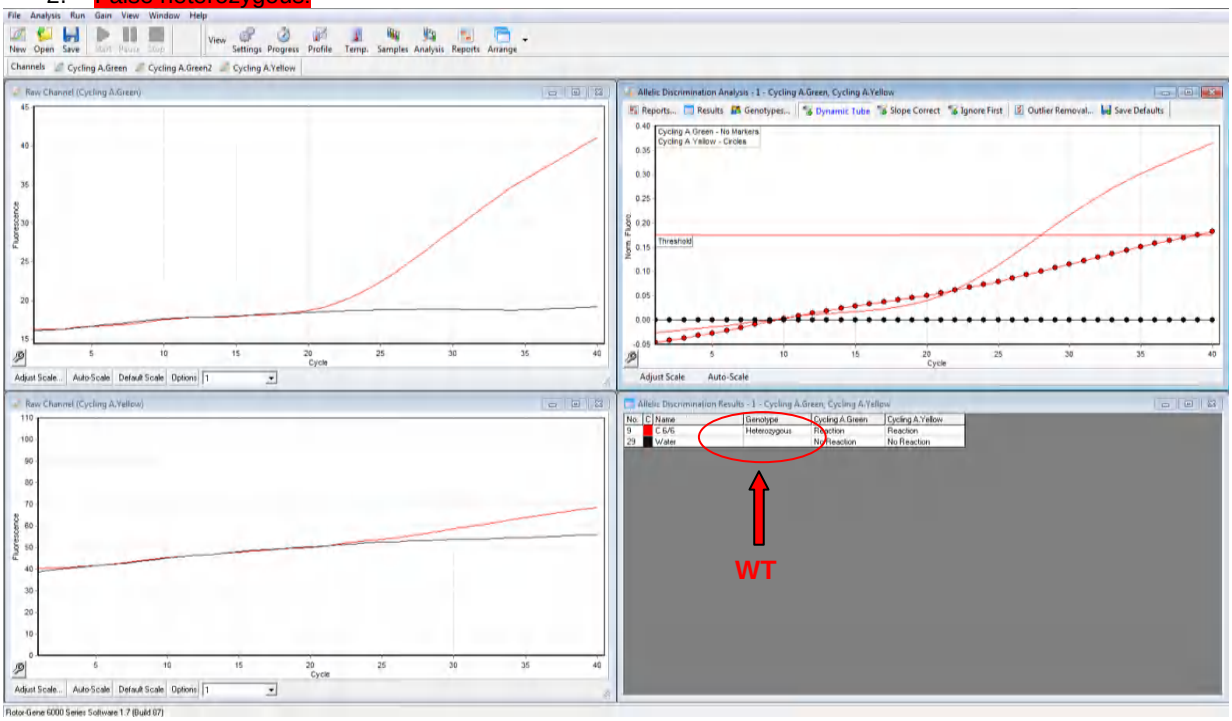
No.	C	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1		A 6/7	Heterozygous	Reaction	Reaction
5		B 7/7	Mutant	No Reaction	Reaction
9		C 6/6	Wild Type	Reaction	No Reaction
13		UGT1A1 WT pos ctrl	Wild Type	Reaction	No Reaction
15		UGT1A1 MT pos ctrl	Mutant	No Reaction	Reaction
29		Water		No Reaction	No 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:

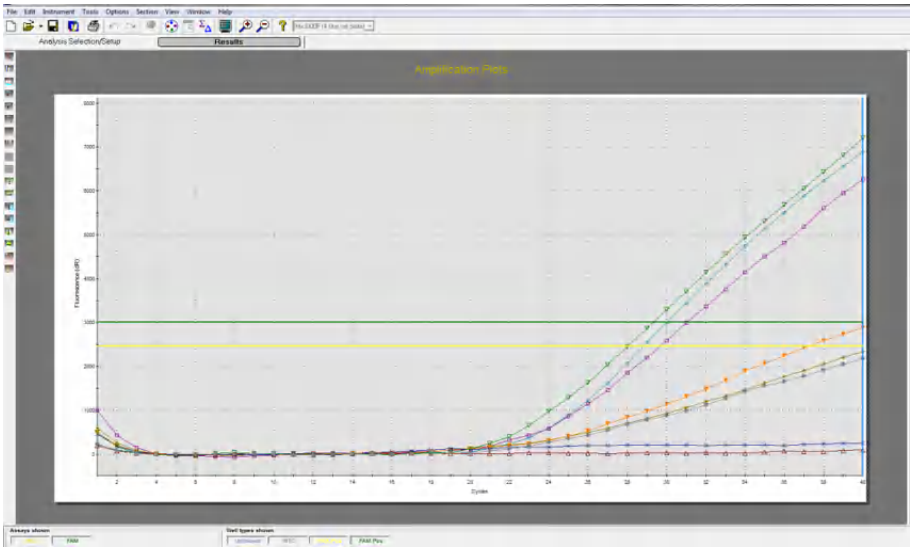


ⓘ Sometimes to have the correct genotype it is necessary to exclude the last 5 amplification cycles from the analysis. In “Raw Channel” “Cycling A.Green” and “Cycling A.Yellow” - “Options” select Crop end cycles and Remove data after cycle: 35. Proceed with “Allelic Discrimination Analysis” for channels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

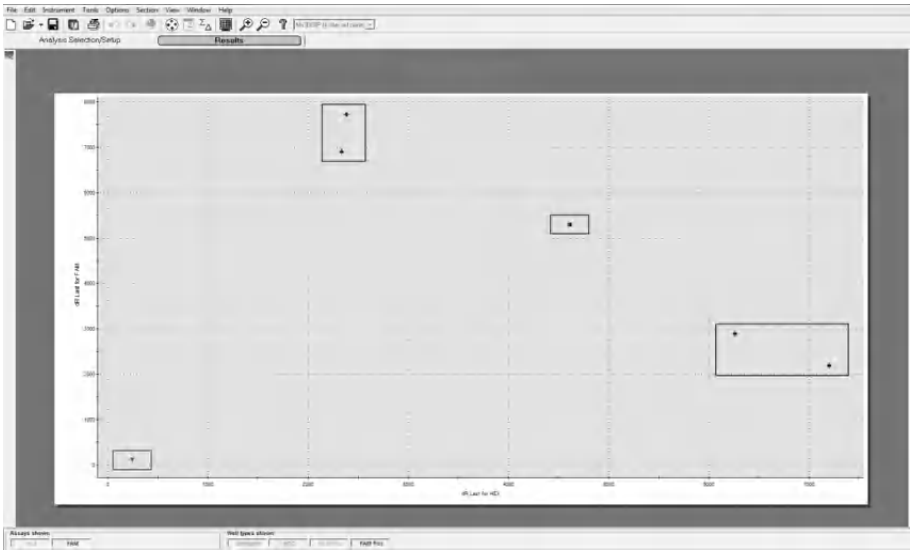
Stratagene Mx3000P, Mx3005P

- 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 Positive Control” for **UGT1A1 WT pos ctrl**, “HEX Positive Control” for **UGT1A1 MT pos ctrl** and “NTC” for **WATER**.
- In Analysis, Analysis Selection/Setup, in the Algorithm enhancements box select only “Moving average” and in the plate scheme select only **UGT1A1 WT pos ctrl**, **UGT1A1 MT pos ctrl** and **WATER**.
- In Analysis, Results, Area to analyze, Amplification plot, manually set the Fluorescence Threshold, moving the line in the Amplification Plots grah, both for FAM and Hex channels, in such a way that:
  1) the threshold has the minimum possible value;
  2) negative control **WATER** is identified as “No Ct” for both channels;
  3) positive control **UGT1A1 WT pos ctrl** (FAM positive control) shows a Ct only for the FAM channel;
  4) positive control **UGT1A1 MT pos ctrl** (HEX positive control) shows a Ct only for the HEX 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: MT, Allele B: WT and Both: HET.
- In Area to Analyze, Text Report, the sample genotype is reported in the “Genotype (dR)” column.
- Click Save.

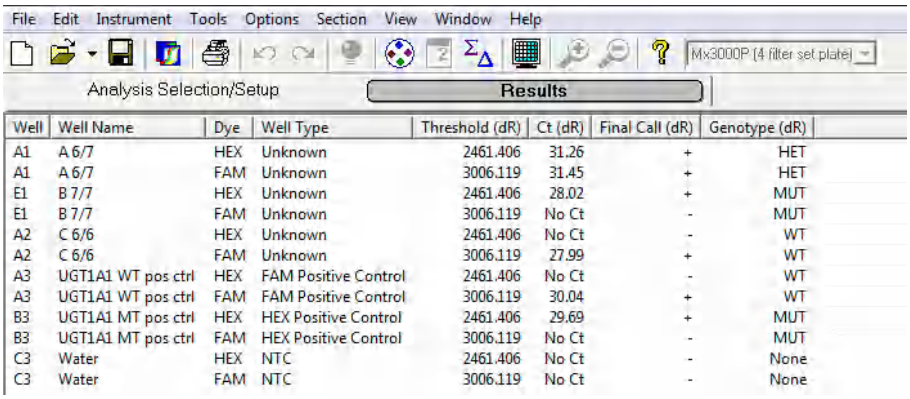
Amplification Plots



Dual scatter plot



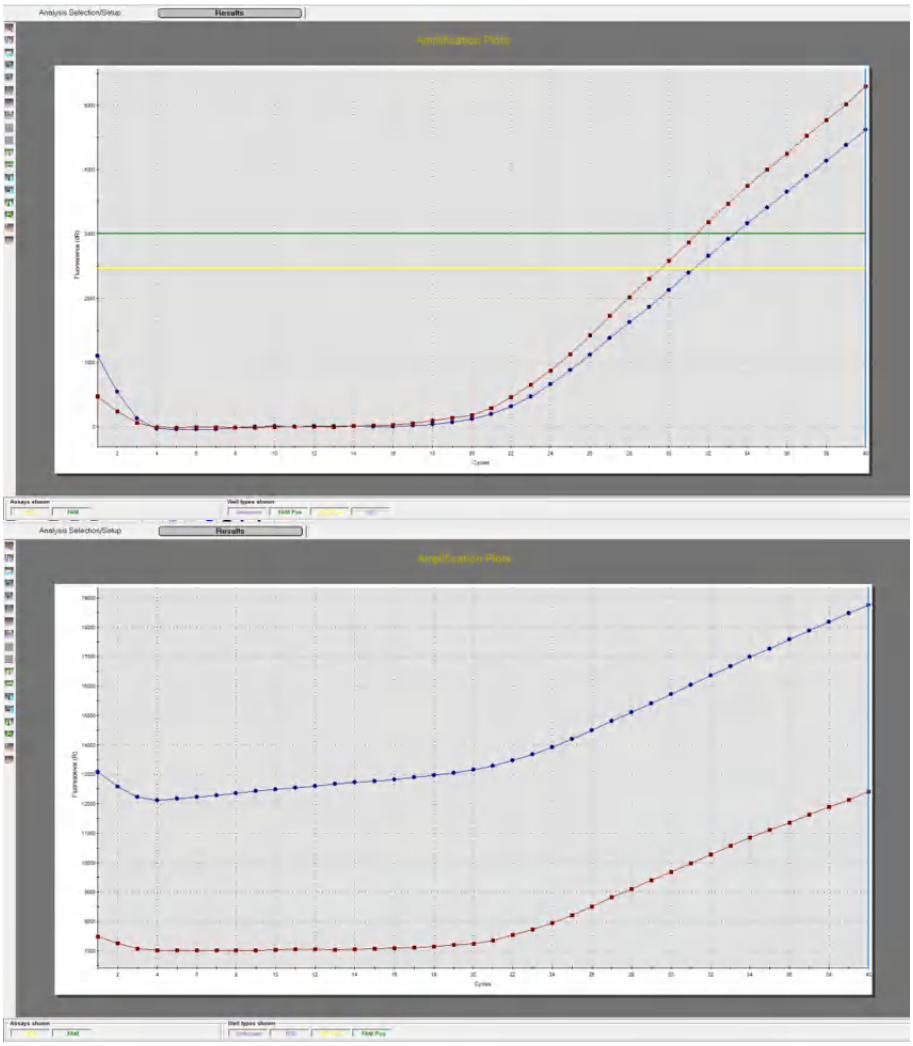
Text report



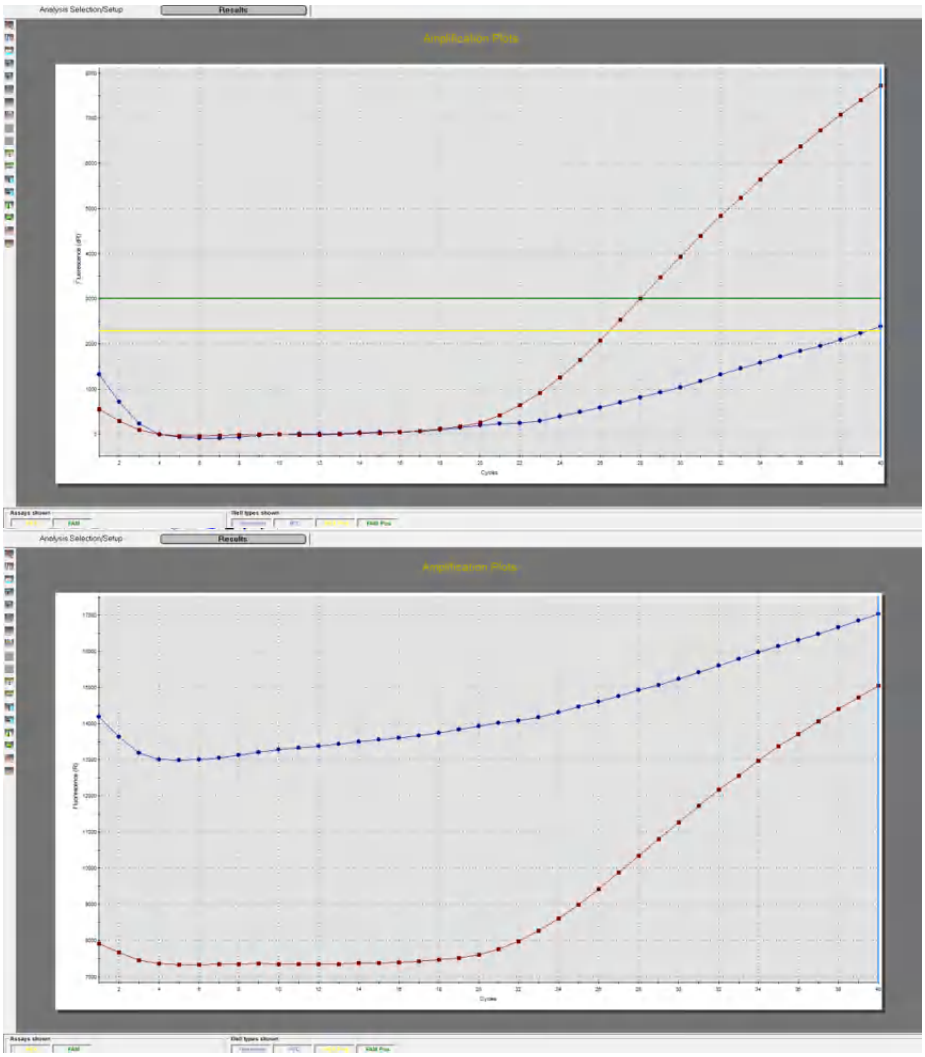
Well	Well Name	Dye	Well Type	Threshold (dR)	Ct (dR)	Final Call (dR)	Genotype (dR)
A1	A 6/7	HEX	Unknown	2461.406	31.26	+	HET
A1	A 6/7	FAM	Unknown	3006.119	31.45	+	HET
E1	B 7/7	HEX	Unknown	2461.406	28.02	+	MUT
E1	B 7/7	FAM	Unknown	3006.119	No Ct	-	MUT
A2	C 6/6	HEX	Unknown	2461.406	No Ct	-	WT
A2	C 6/6	FAM	Unknown	3006.119	27.99	+	WT
A3	UGT1A1 WT pos ctrl	HEX	FAM Positive Control	2461.406	No Ct	-	WT
A3	UGT1A1 WT pos ctrl	FAM	FAM Positive Control	3006.119	30.04	+	WT
B3	UGT1A1 MT pos ctrl	HEX	HEX Positive Control	2461.406	29.69	+	MUT
B3	UGT1A1 MT pos ctrl	FAM	HEX Positive Control	3006.119	No Ct	-	MUT
C3	Water	HEX	NTC	2461.406	No Ct	-	None
C3	Water	FAM	NTC	3006.119	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:



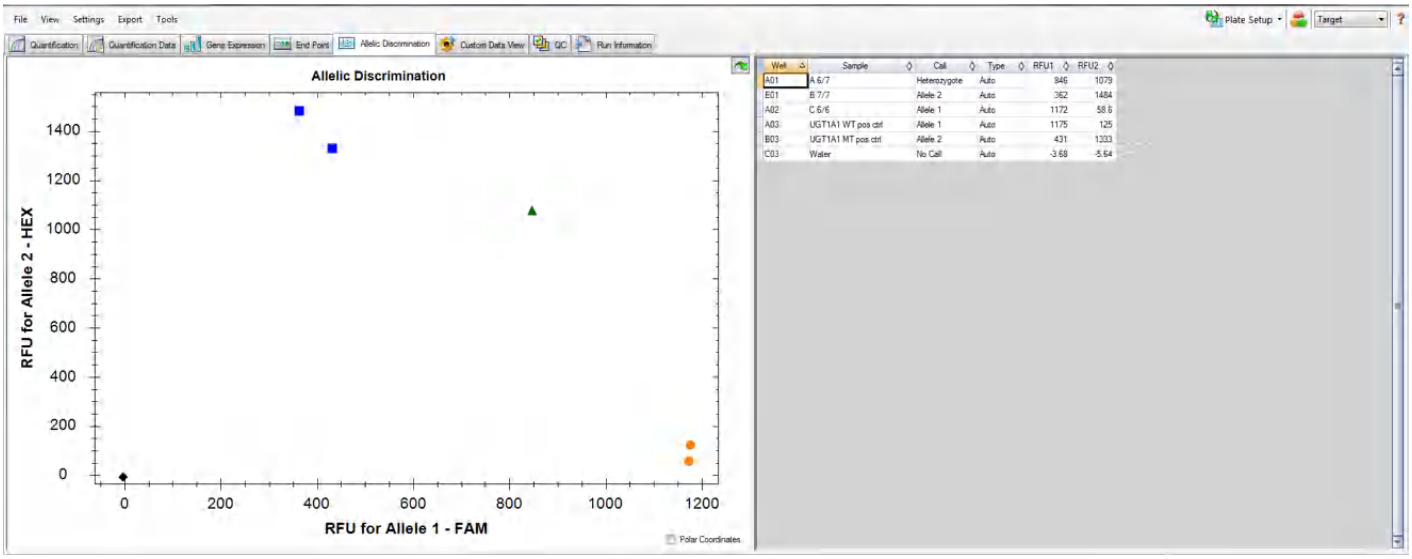
2. False heterozygous:



ⓘ 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.

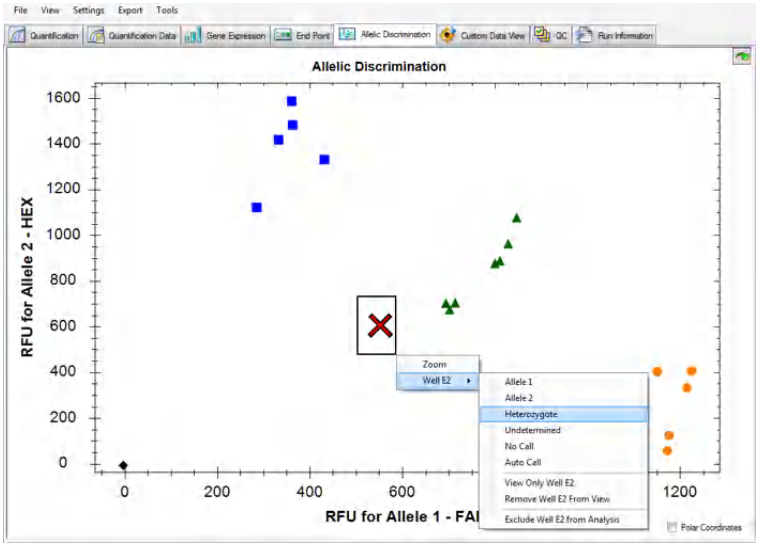
CFX96

- 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 **UGT1A1 WT pos ctrl** and **UGT1A1 MT pos ctrl**.
- Select "NTC" for **WATER**.
- Select the empty wells and click on Exclude wells in analysis.
- 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: FAM and Y: HEX, 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 **UGT1A1 WT pos ctrl** and **UGT1A1 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":

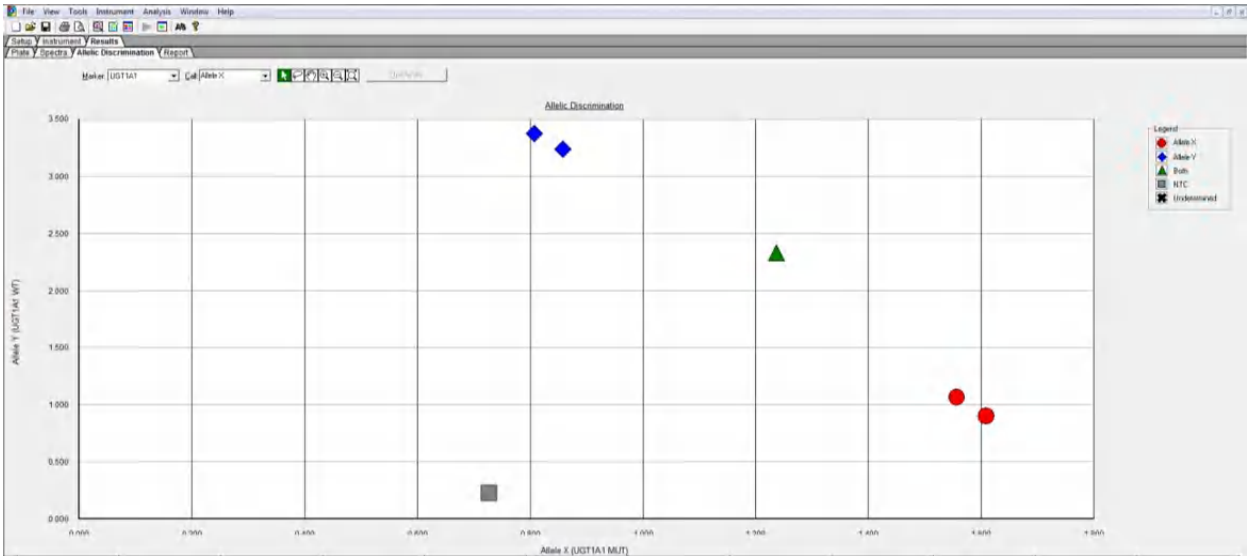


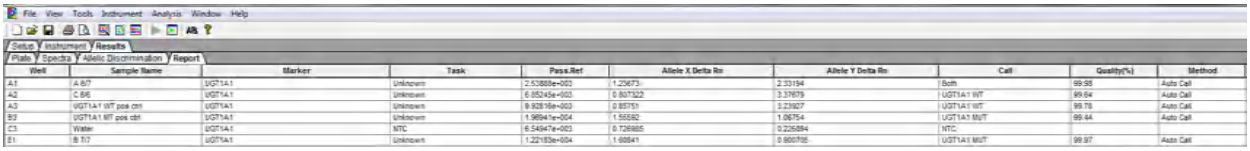
ABI 7300

- At the end of the Post-Read run, click Setup – Plate, select the wells to be analyzed and load samples names.
- Select “Unknown” for samples, **UGT1A1 WT pos ctrl** and **UGT1A1 MT pos ctrl** and “NTC” for **WATER**.
- Select the empty wells, click Analysis and Omit wells.
- 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: “UGT1A1”, Automatic Allele Calling and set Quality Value: 90%.
- Click Apply, Ok & Reanalyze then Yes.
- In Results – Report, in the column “Call” check that:

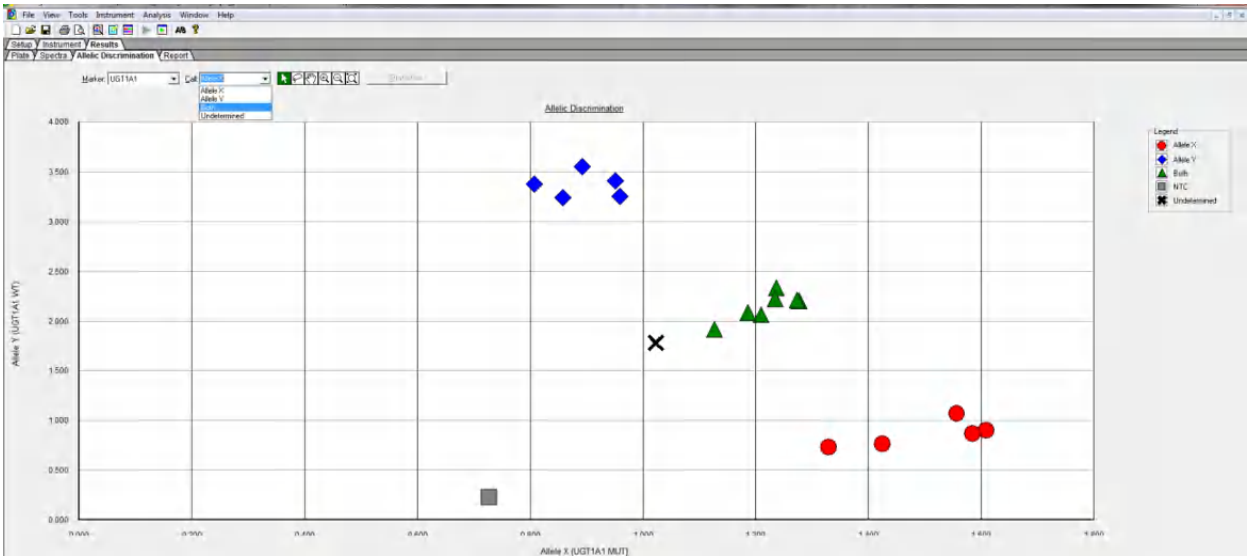
1) negative control **WATER** is identified as “NTC”
2) positive controls **UGT1A1 WT pos ctrl** and **UGT1A1 MT pos ctrl** are identified respectively as “UGT1A1 WT” and “UGT1A1 MT”.

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



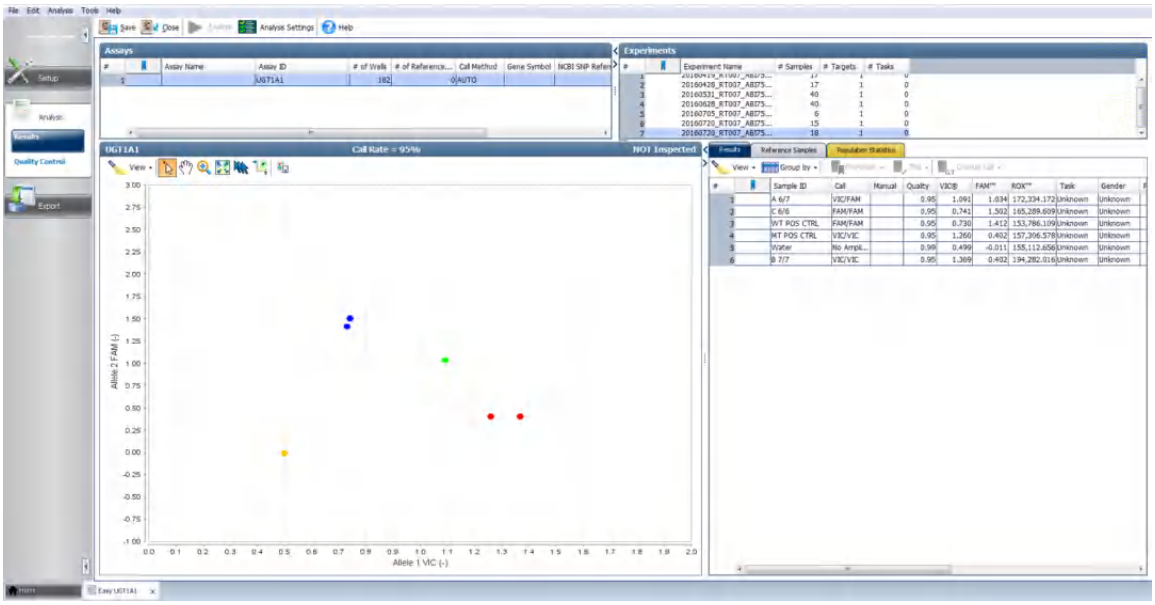


ⓘ If an “Undetermined” sample shows a graphic pattern similar to a wild-type sample (Y axis), mutant sample (X axis) or heterozygous sample (diagonal axis), assign the correct genotype in the “Call” drop-down menu. The graph below shows an heterozygous sample identified as “Undetermined”:

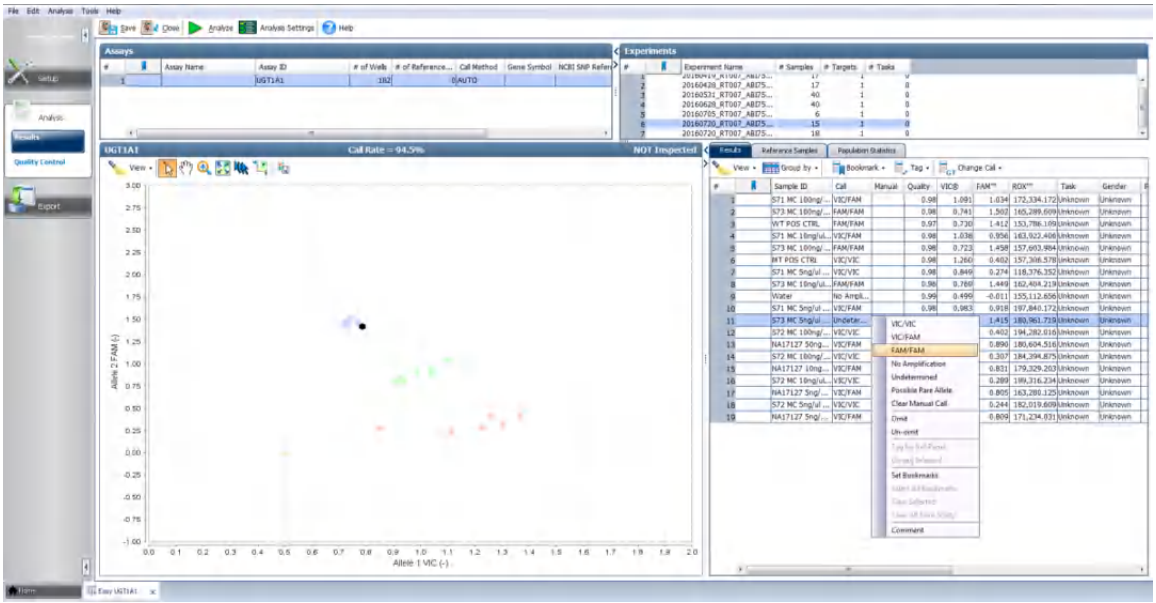


ABI 7500, ABI 7500 Fast

- 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 **UGT1A1 WT pos ctrl**, “Positive Control Allele 2/Allele 2” for **UGT1A1 MT pos ctrl** and “Negative Control” for **WATER**.
- Select empty wells right-click and then Clear.
- 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 UGT1A1, 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, click Analysis settings and in QC settings set Genotype quality low: 0.9. Click Apply and OK.
- In Analysis, Results, in “Call” column check that:
  1) negative control **WATER** is identified as “No Amplification”;
  2) positive controls **UGT1A1 WT pos ctrl** and **UGT1A1 MT pos ctrl** are identified respectively as “FAM/FAM” and “VIC/VIC”.
- Samples genotype is reported in the “Call” column.



ⓘ If an “Undetermined” sample shows a graphic pattern similar to a wild-type sample (Y axis – Allele 2), mutant sample (X axis – Allele 1) 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 in the channel "VIC/Yellow/HEX" for both **UGT1A1 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 UGT1A1 mix into Amp-Mix.	▪ Samples show no signal also for the FAM/Green channel. ▪ Repeat amplification.
Low or absent amplification signal in the channel "FAM/Green" for both **UGT1A1 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 UGT1A1 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 input 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 input 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 5ng/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 10-20FI for "Green" and between 30-40FI for "Yellow".
Low or absent amplification signal.	Degradation of the fluorophores due to improper storage of the UGT1A1 mix.	▪ Store UGT1A1 mix protected from light. ▪ Store UGT1A1 mix at -35/-20°C and avoid thawing and re-freezing the reagents more than twice. ▪ Do not store UGT1A1 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 **UGT1A1 WT pos ctrl** and/or **UGT1A1 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 the **Amp-Mix** in a dedicated area. Carefully decontaminate benches, pipettes and instruments. ▪ Close the cap of reaction tube after the addition of each reagent.
	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, 7500 Fast** - 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 ("**IRINOTECAN response®**", cod. UP021 Diatech Pharmacogenetics), Mass Spectrometry using MassArray® platform ("**Myriapod® ADMET**" cod. SQ040 Diatech Pharmacogenetics) or direct sequencing. Also samples with known genotype from Coriell Institute have been analyzed.
Results are reported in the following table:

	Rotor-Gene		Stratagene Mx3000P, Mx30005P		CFX96		ABI 7300		ABI 7500/ABI 7500 Fast	
Results	**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**
UGT1A1 wild-type(1)	10	10/10	8	8/8	9	9/9	9	9/9	9	9/9
UGT1A1 heterozygous(2)	15	15/15	13	13/13	12	12/12	12	12/12	12	12/12
UGT1A1 mutant(3)	4	4/4	4	4/4	4	4/4	4	4/4	4	4/4
Total	29	29/29	25	25/25	25/25	25/25	25/25	25/25	25/25	25/25

(1) UGT1A1 wild-type: UGT1A1*1/*1, UGT1A1*36/*36 or UGT1A1*1/*36 samples.
(2) UGT1A1 heterozygous: UGT1A1*1/*28, UGT1A1*1/*37, UGT1A1*36/*28 or UGT1A1*36/*37 samples.
(3) UGT1A1 mutant: UGT1A1*28/*28, UGT1A1*37/*37 or UGT1A1*28/*37 samples.

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 serial dilutions of quantified DNA samples with known genotype as reported in the following table:

Sample	Genotype	Tested concentration (ng/µl)
S73	UGT1A1*1/*1	100 - 10 - 5
S71	UGT1A1*1/*28	100 - 10 - 5
S72	UGT1A1*28/*28	100 - 10 - 5
NA17039	UGT1A1*1/*36	50 - 10 - 5
NA17130	UGT1A1*1/*37	50 - 10 - 5
NA17120	UGT1A1*36/*28	50 - 10 - 5
NA17118	UGT1A1*28/*37	50 - 10 - 5
NA17127	UGT1A1*36/*37	50 - 10 - 5

Wild-type and mutant samples have been tested in duplicates at the highest concentration (100 or 50 ng/µl), while heterozygous samples have been tested in duplicates at lowest concentration (5 ng/µl).
Three independent sessions have been performed on each instrument (Rotor-Gene, Mx3000P/Mx3005P, ABI 7300, ABI 7500/7500 Fast and CFX96).
Given the above criteria, the sensitivity limit was 5 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 three independent sessions. Results are reproducible and repeatable in terms of correct genotypization.

Results are reported in the following table:

Sample	Genotype	N° replicates	N° sessions	Results
S73	UGT1A1*1/*1	4	3	12/12 wild-type
S71	UGT1A1*1/*28	4	3	12/12 heterozygous
S72	UGT1A1*28/*28	4	3	12/12 mutant
NA17039	UGT1A1*1/*36	4	3	12/12 wild-type
NA17130	UGT1A1*1/*37	4	3	12/12 heterozygous
NA17120	UGT1A1*36/*28	4	3	12/12 heterozygous
NA17118	UGT1A1*28/*37	4	3	12/12 mutant
NA17127	UGT1A1*36/*37	4	3	12/12 heterozygous

Robustness

Lot to lot consistency

Different batches of **Taq PreMix 310** have been tested with the same DNA samples. Results from the different lots are comparable.

Different batches of primers and probes have been tested with the same DNA samples. Results from the different lots are comparable.

APPENDIX A – RT007 96 well plate sample grid A – Easy® UGT1A1

DATE		RUN NAME	

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

UGT1A1 mix

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

diatech pharmacogenetics

APPENDIX B - RT007 Rotor-Gene sample grid B – Easy® UGT1A1

DATE		RUN NAME	

	UGT1A1 mix									
WATER	1 ●	9 ●	17 ●	25 ●	33 ●	41 ●	49 ●	57 ●	65 ●
WT POS CTRL	2 ●	10 ●	18 ●	26 ●	34 ●	42 ●	50 ●	58 ●	66 ●
MT POS CTRL	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 ●

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

OPERATOR NOTES

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