LightMix® in-vitro diagnostics kit

Multi-SNiP DPYD

Cat.-No.: 40-2002-64

Detection of DNA variations in DPYD gene:

1	DPYD*2A	*rs3918290*
2	DPYD*13	*rs55886062*
3	DPYD D949V	*rs67376798*
4	DPYD IVS10	*rs75017182*
5		
6		
7		
8		

Strip format

for use with the

Roche Diagnostics LightCycler® 480 Instrument family

Reagents for 64 reactions

Upon arrival:

Store Controls and Premixed PCR reagents (silver bag) refrigerate (2°C / 8°C) and protected from light (do not freeze)

Store FastStart DNA Master HybProbe reagents frozen (-20°C) (if included)

CE IVD

Manual Version V160121

Table of contents

1. Product Information

1.1 Contents:

Strips: Lyophilized premixed PCR reagents:

⚠ Store refrigerate (2°C / 8°C) and protected from light

	Description content	Total Reactions
8 x Strips each **two replicates of 4 SNP**	Each well contains premixed and lyophilized primers and probes for one reaction. <0,01pg unlabeled oligonucleotides (target specific primers); <0,01pg SimpleProbe 519 labeled (target specific probes) 4 independent targets for sample 2 samples for strip Strips are sealed in an Argon modified atmosphere.	64
8 x	Strip cover	

Standards (Control DNA):

⚠ Store refrigerate (2°C / 8°C) and protected from light

	Cap color	Label	Description content	Total Reactions
1 x	**Yellow**	**HT**	Positive Heterozygous Control <0,01pg plasmids target mix (synthetic) [about 10E4 genome equivalents]	40

Polymerase components:

⚠ Store at -20°C upon arrival

	Cap color	Label	Description content	Total Reactions
1 x	**Red**	**1a**	LightCycler® FastStart Enzyme	64
1 x	**White**	**1b**	LightCycler® FastStart Reaction Mix HybProbe	64
1 x	**Colorless**	**Water**	H_2O PCR grade	
1 x	**Blue**	**$MgCl_2$**	$MgCl_2$, 25 mM	

FastStart DNA Master HybProbe is included in kits supplied directly by TIB MOLBIOL for customers in Central Europe only (1).
The FastStart DNA Master HybProbe is not included in kits supplied through Roche Diagnostics or its local distributor.

(1) FastStart enzyme is shipped by TIB MOLBIOL at ambient temperature.

1.2 Intended Use

Starting with genomic human DNA from a nucleic acid extract obtained from peripheral blood, this kit allows the simultaneous detection of four single nucleotide polymorphisms (SNP) in the Dihydropyrimidine Dehydrogenase gene (DPYD) encoding the Dihydropyrimidine Dehydrogenase Protein (DPD).

SNP	allele	HGVS	Effect / protein change
rs3918290	**DPYD*2A**	c.1905+1G>A	IVS14 + 1G>A
rs55886062	**DPYD*13**	c.1679T>G	p.I560S
rs67376798		c.2846A>T	p.D949V
rs75017182		c.1129-5923C>G	IVS10 C>G

Lack, or reduced activity, of the DPD protein can cause profound toxicity in patients treated with 5-fluorouracil.

The patient´s mutations status should be considered to modulate 5-fluorouracil dosing; in some cases, a different drug must be selected.

5-fluorouracil treatment of patients with no mutations detected with this kit must be closely monitored because other mutations can also affect DPD activity.

Note: The performance of the assay can be guaranteed only when used with LightCycler® Instruments (see 1.3.2 for details).

1.3 Specifications

The "*LightMix® in-vitro diagnostic kit Multi-SNiP DPYD"* is an *in-vitro* diagnostic test and allows the parallel detection of four specified mutations as demonstrated with reference samples.
Other possible neighbored variants that may be detected, but not identified, by the kit are listed in section **'7.7 Melting peaks results for rare variants'**.

1.3.1 Clinical Samples

For each SNP detected by the kit the test requires 2 µl of purified genomic DNA in aqueous solution extracted from clinical specimen.

1.3.2 Detection range

2 µl of purified genomic DNA containing from 5 to 100 ng/µl of genomic DNA (10 ng – 200 ng total amount), as determined by UV spectrophotometry (1 OD = 50 µg DNA/ml).

1.3.3 Instruments, Software and Productivity

One kit contains reagents for 16 analyses of 4 SNPs, for a total of 64 reactions, each performed in a 10 µl volume.
Each biological sample is analyzed with SNP specific independent reactions.
The table below summarizes some features of the kit :

LightCycler® Instrument (1)	Software Version (or higher)	Maximum Productivity of the kit (2)	Max Samples per run (3)	Minimum Productivity of the kit (4)
LC480 (96 wells)	1.5	14+ 2 ctrl.	22 + 2 ctrl.	8
z 480 (open channel)	1.5	14+ 2 ctrl.	22+ 2 ctrl.	8
LC96	1.6	14+ 2 ctrl.	22+ 2 ctrl.	8

1 Each run is completed in about 100 minutes.
2 One standard and one negative control are required for each SNP detected by the kit.
3 It requires using more than one kit.
4 Calculated considering two clinical samples analyzed in each run.

1.4 Storage and Stability

Note the **different storage conditions** for reagents and polymerase mix!

Reagents and Controls:
Store the lyophilized reagents (Strip and Standards) protected from light and refrigerate (2°C / 8°C).
Do not freeze these dried reagents. Expiration date is printed on the kit label.
Polymerase mix :
Store the LightCycler® FastStart DNA Master HybProbe at -15°C to -25°C.
See expiration date on the polymerase tube label.
Shipping:
Products are shipped at ambient temperature. Transport stability of reagents and enzyme components have been tested under shipping conditions.

2. Additional Devices and Reagents

2.1 Required

LightCycler® 480 Instruments	Roche Diagnostics
LightCycler® 480 Instrument (model I)	Discontinued
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
cobas z 480 Analyzer	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 8-Tube-Strip Adapter Plate	Cat.-No. 06 612 598 001
Or	
LightCycler® 96 Instrument	Roche Diagnostics
LightCycler® 96 Instrument	Cat.-No. 05 815 916 001
LightCycler® Software Version 1.0 or higher	Included with Instrument

2.2 Sample Preparation

Manual Sample Preparation:	Roche Diagnostics
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Nuclease-free PCR grade water	any supplier
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier

Automatic Sample Preparation:	Roche Diagnostics
MagNA Pure Instrument	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
Or	
MagNA Pure 2.0 Instrument	Cat.-No. 05 197 686 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
Or	
MagNA Pure Compact Instrument	Cat.-No. 03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001
Or	
MagNA Pure 96 Instrument	Cat.-No. 05 195 322 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001
Or	
MagNA Pure 96 IVD Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001

No Color Compensation is required for the use of this *LightMix® Kit Multi-SNiP*

3. Background Information

3.1 Methodology and Assay Principle

Using PCR methodology, a fragment of the genes of interest is amplified with specific primers. The fragment is detected with a mutation-specific detection probe internally labeled with SimpleProbe® 519 reagent.

SimpleProbe® probes are fluorescent only when bound to complementary DNA.

The probe binds to a part of the amplified fragment spanning the mutation site. During the melting curve analysis the temperature is slowly increased. The probe melts off at a specific melting temperature causing the fluorescence to decrease. Any mismatch covered by the probe destabilizes the hybrid producing a left-shift of the melting temperature.

In this product the probes that elicit the best discrimination possible have been selected. Whether the selected probes match the wild type or the mutant sequence is described elsewhere in the manual.

Reading of the genotype results is based on the melting temperatures compared to the supplied standards.

Tm calling results must be reviewed by eye for deviating curves and intermediate melting point temperatures. The genotype must be deducted from the melting temperatures following the criteria described in chapter 7.

The supplied control DNA standards allow a comparison with clinical samples.

3. 2 Performance Characteristics

Analytical Specificity
The specificity to the target gene and the suitability of the PCR amplification employed in the present test for the detection of the mutation site was demonstrated by direct sequencing of the amplicon.

Analytical Sensitivity
Detection of serial dilutions of several heterozygous human genomic DNAs has revealed that the limit of detection of the present kit is 250 copies (1.5 ng).
Perform analysis within detection range (see **1.3.2**)

Diagnostic Specificity and Sensitivity
More than 20 different genomic DNA samples from individuals of Caucasian origin were analyzed in parallel by sequencing and with the present kit.
The study compared results obtained with the kit with ABI 3730xl DNA sequencing data obtained by LGC Genomics GmbH, Berlin.

Study results:
Results for both analytical methods were in 100% concordance.
Specific results for each target gene are available upon request service@tib-molbiol.de.

Other variants:
Sometimes variants are present in the binding region of the probe. Synthetic targets, mimicking all the variants present in the GeneBank (Jan-2015), are used to demonstrate the ability of the assay to identify the correct genotype.
See section **'7.7 Melting peaks results for rare variants'**.

3.3 Medical Background

Dihydropyrimidine dehydrogenase (DPD), the enzyme encoded by the DPYD gene has a coding sequence of 4,399 nucleotides in 23 coding exons spanning 950 kb on chromosome 1p22.6 [(1)].

DPD is the rate-limiting enzyme for fluoropyrimidine catabolism and eliminates more than 80% of administered 5-fluorouracil [(2)].

In patients deficient in DPD, fluoropyrimidine drugs exhibit a profound toxicity with severe and sometimes fatal outcome with symptoms of myelosuppression, mucositis, neurotoxicity, hand–foot syndrome, and diarrhea.

The best known deficient variant IVS14+1G>A DPYD *2A causes the deletion of exon 14. Less frequent variants associated with 5-fluorouracil toxicity are c.2846A>T located in exon 22 and c.T1679G (DPYD*13) in exon 13.

In August 2013 the Clinical Pharmacogenetics Implementation Consortium has published guidelines to test patients for these three variants DPYD*2A, DPYD *13, and rs67376798 for fluoropyrimidine drug dosing [(3)]
http://www.pharmgkb.org/guideline/PA166109594
Sistonen in Clinica Chimica Acta 2012 identified rs75017182 a novel deep intronic polymorphism located in intron 10 that generates a splice site variant.
The inclusion of rs75017182 variant triples the proportion of risk variant carrier patients that can be identified.
Homozygous mutated patients (0.2% of the patients) and probably also any compound heterozygous patients exhibit a complete DPD deficiency and have to be treated with alternate drugs.
Heterozygous mutated individuals (up to 5% of all patients) may have a partial DPD deficiency and should be treated with a 50% reduced dose and monitored by a pharmacokinetic test.

Note: DPD deficiency can be caused by other mutations that are not tested by this device.

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.
Use the manual version which is delivered with the kit (see kit label).

Laboratory Procedures

All materials of human origin and related waste must be considered as potentially infectious. Thoroughly clean all work surfaces and treat them with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product employed (see chapter 2.3).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual.

Save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "PCR protocol" specific for your Instrument.

Do not touch the strip cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Programming

5.1 LightCycler® 480 Instruments

For details, see the LightCycler® Operator's Manual.

LightCycler® 8-Tube-Strip Adapter Plate can be extremely hot when removed from the instrument; use appropriate protection.

Detection Format: **SimpleProbe**
Reaction Volume: **10 µl**

Programming:
The protocol consists of four program steps (Tab.2):

1) **Denaturation** of sample and activation of the enzyme
2) **Cycling** PCR-amplification of the target DNA
3) **Melting** Identification of PCR amplified DNA sequence
4) **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	**1**	**45**			**1**			**1**
Target [°C]	**95**	**95**	**60**	**72**	**95**	**43**	**75**	**40**
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:30**	**00:02:00**	**00:00:00**	**00:00:30**
Ramp Rate [C°/ s] **96**	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.29**	**1.5**
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**
Sec Target [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Size [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**

Tab. 1: Programming of LightCycler® 480 Instruments and cobas z 480 Analyzer

Note: Store the program and the default values as 'RUN Template', which can be loaded to start every run.

Ensure to program 2 acquisitions per second instead of the default value 5; more acquisitions reduce the slope of the melting curve, increases experiment time and causes kit's malfunction.

5.2 LightCycler® 96 Instrument

For details, see the LightCycler® Operator's Manual.

Measurement:

Detection Format: **470/514 FAM**			General
Quant Factor	Melt Factor	Integration Time (S)	Volumes (µl)
10.00	1.20	Dynamic	**10**

Profile:
The protocol consists of four program steps (Tab.3):

1) **Preincubation** of sample and activation of the enzyme
2) **Step Amplification** PCR-amplification of the target DNA
3) **Melting** Identification of PCR amplified DNA sequence
4) **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Cycles	**1**	**45**			**1**			**1**
Ramp [°C/ s]	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.20**	**1.5**
Duration [s]	**600**	**5**	**10**	**15**	**30**	**120**	**1**	**30**
Target [°C]	**95**	**95**	**60**	**72**	**95**	**43**	**75**	**40**
Mode		Standard	Standard	Standard				
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Readings /°C							**5**	

Tab. 2: Programming of LightCycler® 96 Instrument
Note: Store the program and the default values as 'Experiment file', which can be loaded to start every run.

6. Experimental protocol

Program the Instrument before preparing the solutions (see 5. Programming and read the LightCycler® Operator's Manual for details).
The described performance of the assay can be guaranteed only when used with Roche Diagnostics LightCycler® systems.

6.1 Sample Preparation

For preparation of genomic DNA use human peripheral blood (EDTA, citrate). The use of heparin is strongly discouraged since this anticoagulant might interfere with the PCR.
Perform nucleic acid purification using the High Pure PCR Template Preparation Kit or the MagNA Pure LC Instruments using the extraction kit appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols.

6.2 Reagents Preparation

6.2.1 Preparation of the LightCycler® FastStart DNA Master

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30°- 35°C for 3 - 5 minutes.
3	Mix the solution carefully with a pipette.
4	Quickly spin tubes to collect drops.
5	The solution must be free of particles. Inspect carefully the side of the tube, if particles are present repeat from step 3. 
6	Add 60 µl of mix **1b** to the vial **1a**.
7 ⚠	Mix the solution carefully with a pipette. Do not vortex! Avoid the production of bubbles.
8	Spin the tubes to collect drops.
9	Use reagent to prepare the Reaction Mix (6.3).
10	Store left over reagent at 4°C.

6.2.2 Preparation of Positive Control

►	**HT Positive Control** tube is sufficient for 40 reactions.
1	Spin the tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 80 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **2 µl** of **Positive Control** for a 10 µl PCR reaction.
► **Positive Control** must be used in each run.
Please note: Opening the vials may cause contaminations of the workspace (aerosol).

6.3 Preparation of the Reaction Mix

Before starting, label the same number of vials for which you have samples, plus two more; one labeled **HT**, the second one **NTC**

6.3.1 Preparation of Reaction Mix for HT Positive Control

In the vial labeled **HT** mix:

Components	
H_2O, PCR-grade (colorless cap)	26.5 µl
Mg^{2+} solution 25 mM (blue cap)	3.5 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	4.0 µl
HT Positive Control	8.0 µl
Volume of reaction mix	42.0 µl

Tab. 3: Volumes of components for preparing positive control reaction mixture

6.3.2 Preparation of Reaction Mix for NTC Negative Control

In the vial labeled **NTC** mix:

Components	
H_2O, PCR-grade (colorless cap)	34.5 µl
Mg^{2+} solution 25 mM (blue cap)	3.5 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	4.0 µl
Volume of reaction mix	42.0 µl

Tab. 4: Volumes of components for preparing negative control reaction mixture

6.3.3 Preparation of Reaction Mix for biological sample

For each biological sample, in a clearly labeled vial, mix:

Components	
H_2O, PCR-grade (colorless cap)	26.5 µl
Mg^{2+} solution 25 mM (blue cap)	3.5 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	4.0 µl
Biological Sample	8.0 µl
Volume of reaction mix	42.0 µl

Tab. 5: Volumes of components for preparing a single reaction mixture

Gently pipette up and down each reaction mix
A high percentage of experimental failure is due to a non homogeneous reaction mix!

6.4 Handling of the strips' bag

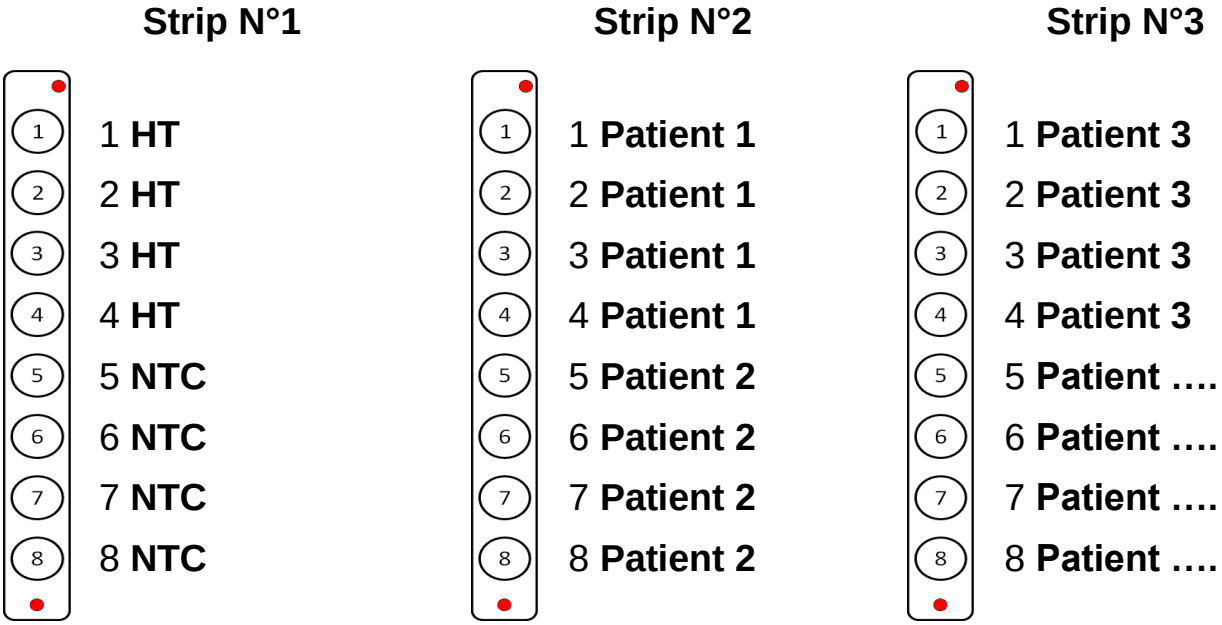
To allow the Argon to reach the reagents, the strips inside the bag are open and loose. Cut a side of the aluminum bag; using gloves extract the strips from the bag and immediately seal with the included strip covers; strips are stable for 30 days when stored refrigerated (4°C - 8°C) protected from light.

6.5 Well Loading Procedure

At least two strips must be used for each run.

Two small holes indicate the strip orientation; the asymmetrical hole indicate position one; mark strips before filling.

Dispense **10 µl** per well of each reaction mixes prepared in **6.3** as described in the figure below:



►	Use gloves; do not touch the optical part of the strip cover.
1	Close the strip.
2	Spin the strip to eliminate bubbles.
3	Load strip into adapter and place in the instrument.
4	Start Run

6.6 Storage and Stability of Diluted Components

LightCycler® **FastStart DNA Master** HybProbe:
The combined FastStart DNA Master HybProbe master mix (1a+1b) can be stored refrigerated (4°C - 8°C) for 30 days.

Positive Control:
The dissolved Positive Control is stable for 30 days when stored refrigerated (4°C - 8°C).

Reaction Mix / prefilled strip:
Strip can be filled up to 6 hours in advance before amplification, maintain sealed strips stored refrigerated (4°C - 8°C) protected from light.

Strips:
Once removed from the aluminum bag, strips closed with the cover strip are stable for 30 days when stored refrigerated (4°C - 8°C) protected from light.

7. Data Analysis and Interpretation

7.1 Limits and Interferences

The present assay is specific for the intended targets.
No interferences for these assays are known.

7.2 Calibration

Calibration has to be performed following the procedure described in 6.3.1, 6.3.2, 6.5, 7.3.1, 7.3.2 and 7.6.

7.3 Quality Control – Acceptance Criteria

In order to perform a reliable genotyping analysis, it is essential that Positive Control **HT** and Negative Control **NTC** are included in each run.

NOTE: The test is performed at an annealing temperature of 60ºC at which the probes will not bind well, yielding low or even no signals in 'quantification'.
For this reason, **the acceptance criteria are based only on the definition of the melting-curve patterns** as described below.

7.3.1 HT Positive Control DNA

HT is mimicking a clinical sample that is heterozygous in all SNPs under analysis. (See **7.6**).
Tm-Calling analysis must always show two melting peaks for SNP.
In table 7 (chapter **7.6**) the expected melting temperatures are described.
Melting temperature can vary of ±2.5°C between runs;
The peaks difference can vary of ±1.5°C between runs.
If **HT** does not show two melting peaks for each SNP, the run is invalid and the procedure has to be repeated.

7.3.2 Negative Control

NTC Negative Control.
Tm-Calling analysis of the Negative Control must provide a negative result: no assay-specific melting peaks (see **7.6**) must be detected.
In case that the **NTC** should report one or more specific peaks (compare signal with **HT** results to avoid that the software enlarges background noise to window size suggesting the presence of phantom melting peaks), a contamination or a pipetting error has occurred; the session is not valid and the procedure has to be repeated. If the problem sustains, change water and/or reagents and repeat. Feel free to ask for assistance at service@tib-molbiol.de.

7.3.3 Samples

Tm-Calling analysis must always show one or two melting peaks.

No more than two peaks per sample are expected

The melting peak profiles must be conformable to the acceptance criteria described in the present chapter and in chapter **7.6 Interpretation of results**.
Otherwise, the result is not valid and the whole procedure has to be repeated (sample preparation, amplification and detection).
See also **7.7 Melting Peak Results for Rare Variants.**
If one of the polymorphism in the panel do not generate any melting curve, the sample must be considered not valid and the whole procedure has to be repeated (sample preparation, amplification and detection).

Before repeating a run consider common errors; check in particular the amplification profile, correct master-mix and $MgCl_2$ concentration used, and keep in mind that also inadequate storage of reagents may cause a failure of the device.

7.3.4 Abnormal Melting Curves

Rare variants generating unexpected melting curves, are described in chapter **7.7 Melting Peak Results for Rare Variants**
If an abnormal melting curve persists, it can be due to a defect in the product or can be caused by other variations (mutations) in the probe-binding region. In the latter case, another method must be used for comparison / verification of the sequence.
Submit the PCR fragment for DNA sequencing to confirm the sequence or identify any unknown mutations. Report deviations to service@tib-molbiol.de
Feel free to send deviant melting samples to TIB MOLBIOL Berlin laboratories to confirm the obtained results and/or identify other mutations by DNA sequencing.

7.5 Reading the Results

Perform data analysis as described in the LightCycler® Operator's Manuals.

7.5.1 Detection channels

Instrument	Select channel
LightCycler® 480	483-533
LightCycler® 480 II	465-510
cobas z 480	465-510
LightCycler® 96	470/514 FAM

Tab. 6: Detection channels

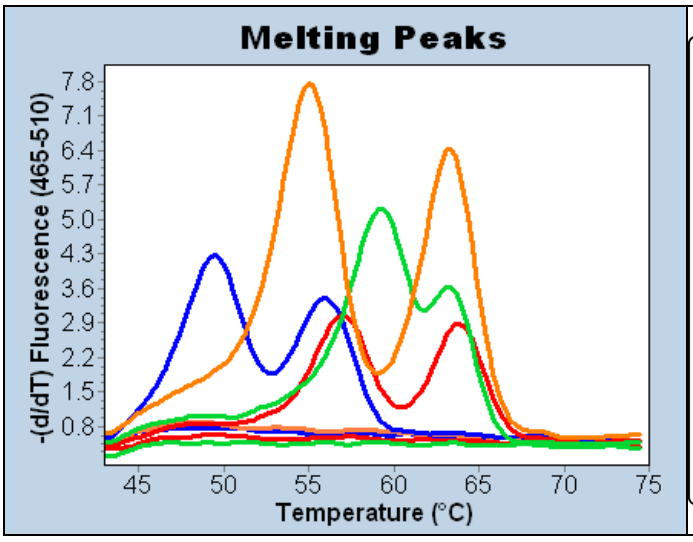
7.5.2 Typical Data for Amplification

The amplification curves do not contain any analytical information.
Depending on the sample's specific genotype and the structure of the probe the amplification might not be visible (see 7.3).

7.5.3 Melting Analysis: LightCycler® 480 Instruments

The melting-curve peaks (Fig. 1) discriminate between wild type, mutant and heterozygous genotypes.

View data for Melting with **"Tm Calling"** analysis mode:



	Color	Gene	
1	Blue	rs3918290	DPYD*2A
2	Red	rs55886062	DPYD*13
3	Green	rs67376798	
4	Orange	rs75017182	IVS10
5	Blue	rs3918290	DPYD*2A
6	Red	rs55886062	DPYD*13
7	Green	rs67376798	
8	Orange	rs75017182	IVS10

Fig 1: Typical analysis results of **HT** Positive Control (position 1-4) and **NTC** Negative Control (position 5-8).

Note: The values of the melting temperatures (TM) may vary ±2.5°C between different runs. The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions:

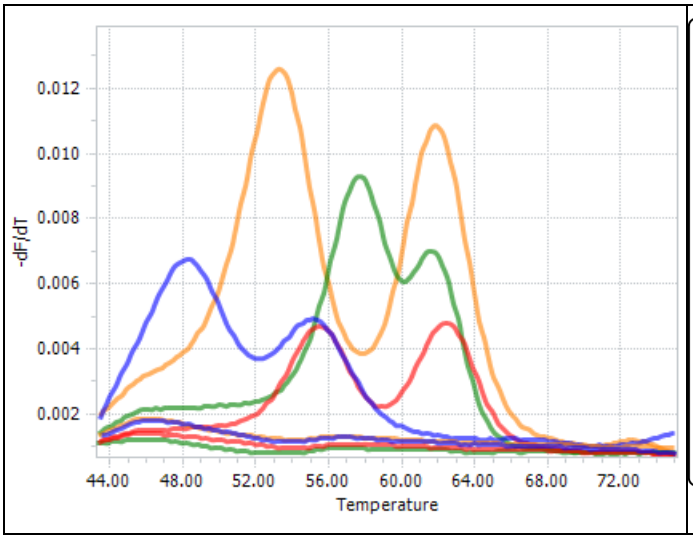
- **7.7. Melting Peak Results for Rare Variants**
- **7.3.5 Abnormal Melting Curves**

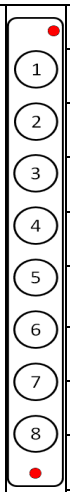
7.5. 4 Melting Analysis: LightCycler® 96 Instrument

The melting-curve peaks (Fig. 2) discriminate between wild type, mutant and heterozygous genotypes.

Add Analysis: **Tm Calling**
View data in: **Melting peak**
Select peaks by using the: **Area marker tool**

Note: The LightCycler® 96 Instrument needs a manual input to mark the region that will be used for determining the melting peak; if the peak is not contained in the box (marker tool) the software will not report a Tm value.
Visually analyze the melting curve of each patient in comparison with the melting curve of **Positive Control**.
Use also table 7 in chapter **7.6. Interpretation of the Results** for comparison.





	Color	Gene	
1	Blue	rs3918290	DPYD*2A
2	Red	rs55886062	DPYD*13
3	Green	rs67376798	
4	Orange	rs75017182	IVS10
5	Blue	rs3918290	DPYD*2A
6	Red	rs55886062	DPYD*13
7	Green	rs67376798	
8	Orange	rs75017182	IVS10

Fig 2: Typical analysis results of **HT** Positive Control (position 1-4) and **NTC** Negative Control (position 5-8)

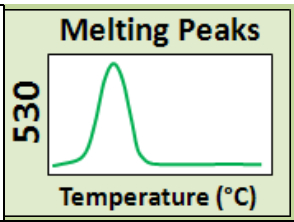
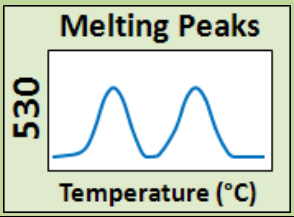
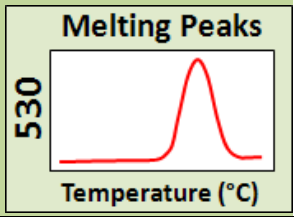
Note: The values of the melting temperatures (TM) may vary ±2.5°C between different runs. The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions:

- **7.7. Melting Peak Results for Rare Variants**
- **7.3.5 Abnormal Melting Curves**

7.6. Interpretation of the Results

After analysis validation (see **7.3.1**, **7.3.2**) select one strip at a time and compare the obtained temperatures with the expected values described below.
One strip contains 2 replicates of the 4 tests, starting from the asymmetrical hole.

For simplicity WildType Heterozygous Mutant are color coded		Melting Peaks  	Melting Peaks  	Melting Peaks 
		Left Peak	**Two peaks**	**Right Peak**
1	**rs3918290 DPYD*2A**	GG **50°C** WildType	Δ 7°C Heterozygous	AA **57°C** Mutant
2	**rs55886062 DPYD*13**	CC **58°C** Mutant	Δ 7°C Heterozygous	AA **65°C** WildType
3	**rs67376798**	TT **60°C** WildType	Δ 4°C Heterozygous	AA **64°C** Mutant
4	**rs75017182 IVS10**	CC **55°C** WildType	Δ 8°C Heterozygous	GG **64°C** Mutant
5	**rs3918290 DPYD*2A**	GG **50°C** WildType	Δ 7°C Heterozygous	AA **57°C** Mutant
6	**rs55886062 DPYD*13**	CC **58°C** Mutant	Δ 7°C Heterozygous	AA **65°C** WildType
7	**rs67376798**	TT **60°C** WildType	Δ 4°C Heterozygous	AA **64°C** Mutant
8	**rs75017182 IVS10**	CC **55°C** WildType	Δ 8°C Heterozygous	GG **64°C** Mutant

Tab. 7 Typical analysis results

Note: The values of the melting temperatures (TM) may vary ±2.5°C between different runs.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.
The right melting peak derives from the perfect match between probe and amplicon; the probes are selected to maximize the distance of the two peaks (heterozygous) and not according to the genotyping status.
In case of variations see instructions:

- **7.7. Melting Peak Results for Rare Variants**
- **7.3.5 Abnormal Melting Curves**

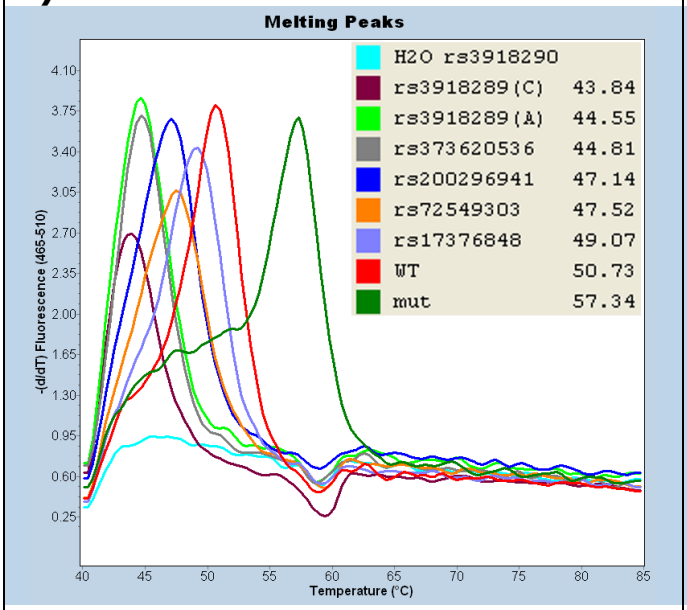
Melting Peak Temperatures °C observed														
50	**50/57**	**50**	**50**	**50**	**50/57**	**50/57**	**50/57**	**50**	**50**	**50**	**57**	any	any	any
65	**65**	**58/65**	**65**	**65**	**58/65**	**65**	**65**	**58/65**	**58/65**	**65**	any	**58**	any	any
60	**60**	**60**	**60/64**	**60**	**60**	**60/64**	**60**	**60/64**	**60**	**60/64**	any	any	**64**	any
55	**55**	**55**	**55**	**55/64**	**55**	**55**	**55/64**	**55**	**55/64**	**55/64**	any	any	any	**64**
WT	**One site Heterozygous : Reduce Dose**				**Compound Heterozygous : Use alternate drug**						**Homozygous mutated : Use alternate drug**			

Tab. 8 Possible Melting Peak Temperature combinations

7.7. Melting Peak Results for Rare Variants

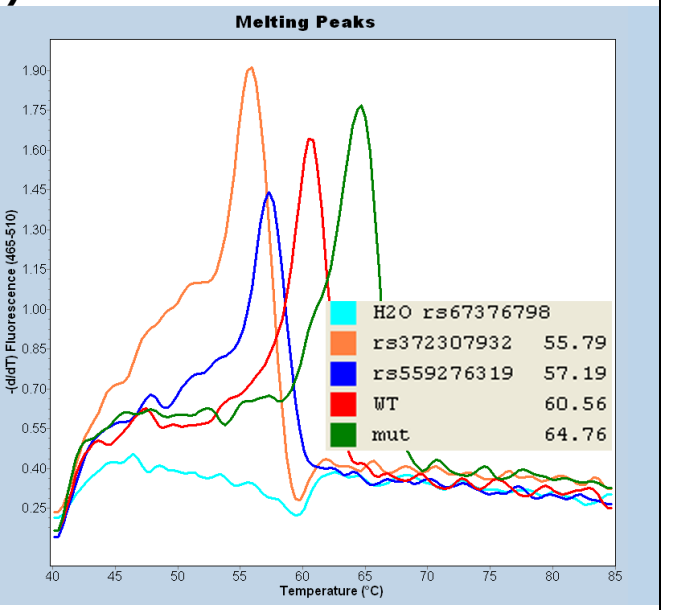
Using synthetic targets the melting temperature of all SNPs positioned under the probes (revision NCBI 01/01/2015) are analyzed.

1) rs3918290 DPYD*2A



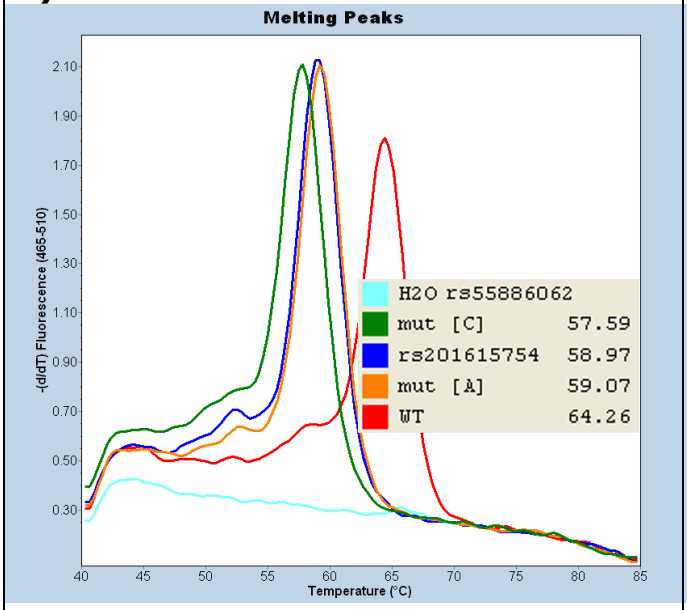
rs3918289 (C) MAF: NA
rs3918289 (A) MAF: C=0.0002/1
rs373620536 MAF: NA
rs200296941 MAF: C=0.0002/1
rs72549303 MAF: NA
rs17376848 MAF: G=0.0521/261
rs3918290 MAF: T=0.0030/15

3) rs67376798



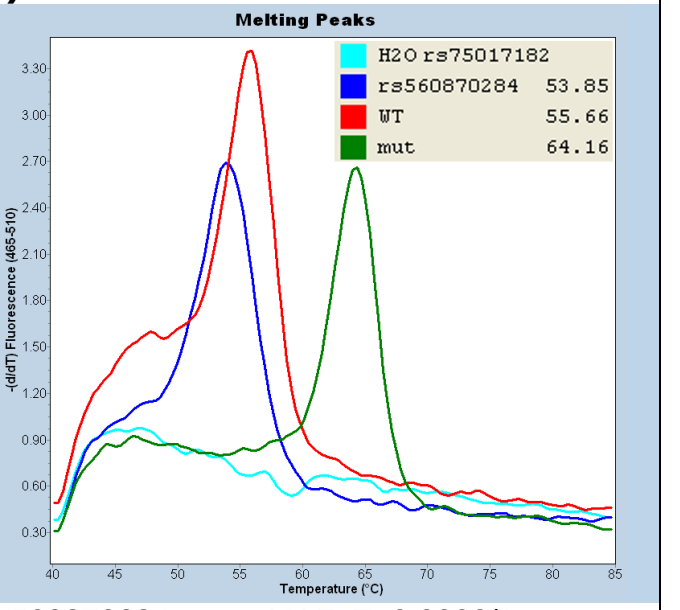
rs372307932 MAF: NA
rs559276319 MAF: G=0.0002/1
rs67376798 MAF: A=0.0022/11

2) rs55886062 DPYD*13



rs55886062 [C] MAF: C=0.0002/1
rs201615754 MAF: NA
rs55886062 [A] MAF: NA

4) rs75017182 IVS10



rs560870284 MAF: T=0.0002/1
rs75017182 MAF: C=0.0096/48

MAF: Minor Allele Frequency. NA= Not Available

This device is intended to identify only the SNP described in chapter 1.2. Expert users, alerted by the deviances of the melting curves hereby depicted, must confirm the results by alternative methods.

8. Troubleshooting

Event	Possible Reason	Solution
All negative PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Inconsistent baseline among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Strip badly sealed	Ensure that strip is properly sealed
Baseline "Saw teeth like"	Bubble in the well	Centrifuge plate before run
No signal in HT Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect $MgCl_2$ concentration	Repeat assay
	Positive Control degradation	Use a new aliquot.
Positive signal in NTC Negative Control	Error while setting the instrument	Check the position settings of the Negative Control
	Dispensing error	Comply with the work sheet when dispensing Positive Controls, Negative Controls and samples
		Always change tips among samples
		Avoid spilling the contents of the sample test tube
	Contamination of PCR-grade water.	Use a new aliquot
	Contamination of $MgCl_2$	Use a new aliquot
	Contamination of the enzyme	Use a new aliquot
	Contamination of the extraction /preparation area	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
		Add LightCycler® Uracil-DNA Glycosylase (Cat.-03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
Melting curve outside the expected temperature range	With peaks TM concordant with Positive Control: Incorrect reagent concentration	Manually assign results accordingly to Positive Control
	With peaks TM discordant with Positive Control: Possible extraction inhibitor	Repeat assay diluting the DNA 1:3
	With peaks TM discordant with Positive Control: Possible different mutation	Repeat assay by sequencing and report unexpected variant to service@tib-molbiol.de

9. References

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Characterization of the human dihydropyrimidine dehydrogenase gene.
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PharmGKB summary: fluoropyrimidine pathways
Pharmacogenet Genomics 2011 April ; 21(4): 237–242.

3) KE Caudle, CF Thorn, TE Klein, JJ Swen, HL McLeod, RB Diasio, and M Schwab
Clinical Pharmacogenetics Implementation Consortium Guidelines for Dihydropyrimidine Dehydrogenase Genotype and Fluoropyrimidine Dosing
Clin Pharmacol Ther. 2013 Dec;94(6):640-5
http://www.pharmgkb.org/guideline/PA166109594

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A new DPYD genotyping assay for improving the safety of 5-fluorouracil therapy
Clinica Chimica Acta 414 (2012) 109–111

5) Steven M. Offer, Adam M. Lee, Lori K. Mattison, Croix Fossum, Natalie J. Wegner, and Robert B. Diasio
A DPYD variant (Y186C) in individuals of African ancestry associated with reduced DPD enzyme activity
Clin Pharmacol Ther. 2013 July ; 94(1)

Classification / Reference

N°	Gene Name	COSMIC	AA Mutation	CDS Mutation	EDMA	EAN
1	DPYD		non (Intron)	c.1905+1G>A	16 01 01 90 00	
2	DPYD		p.I560S	c.1679T>G	16 01 01 90 00	
3	DPYD		p.D949V	c.2846A>T	16 01 01 90 00	
4	DPYD		non (Intron)	c.1129-5923C>G	16 01 04 90 00	
5						
6						
7						
8						

CPV 33694000-1

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When the present kit is distributed through Roche Diagnostics or their local distributors, FastStart® DNA Master HybProbe is supplied as a separate product:

Roche Diagnostics Cat.-No. 03 003 248 001 kit for 96 reactions
Roche Diagnostics Cat.-No. 12 239 272 001 kit for 480 reactions

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Product is not hazardous, not toxic, and not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotides primers and probes.

Version History

Notes in red mark events that require to change laboratory procedures
Notes in blue mark improvements and changes in composition

Version	Event	Date
V140309	Release version	07-03-2014
V150220	Substituted rs115232898 with rs75017182 Clarification on the components stability	12-02-2015
V160121	Corrected genotype of rs75017182 WT in Tab. 7 and temperature in Tab. 8	21-01-2016

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