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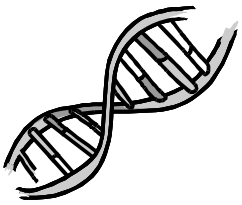
MutaREAL® DPD

real time PCR Kit



PCR test for analysis of DPD*2A (exon 14-skipping) polymorphism in the dihydropyrimidine-dehydrogenase gene (DPYD) involved in 5-Fluorouracil (5-FU) metabolism using *real time* capillary systems (e. g. LightCycler®, Roche).

REF KF291132 Σ 32

-20°C

CE For in vitro diagnostic use only

CONTENT

1. INTENDED USE

The **MutaREAL® DPD** Kit is a *real time* PCR test for the examination of *2A-mutation of the dihydropyrimidine-dehydrogenase gene (DPYD) from genomic DNA. The investigated variation is highly connected to a decrease in (or absence of) DPD enzyme activity, which in turn leads to an increased risk for severe side effects and toxicities during a 5-Fluorouracil (5-FU) containing chemotherapy.

2. INTRODUCTION

Next to age, gender and environmental influences such like diet and co medication, there are genetic factors that have a major impact on the catalytic activity of many enzymes. It is known that patients with decreased or totally absent function of the dihydropyrimidine dehydrogenase (DPD) enzyme, are at a higher risk to develop severe to lethal side effects upon chemotherapy with 5-Fluorouracil. Those are ranging between grades 3-4 according to the WHO. 5-FU is a cytostatic drug that is routinely used for the treatment of many solid tumours.
Analysis of the DPYD gene can help to reduce the risk of 5-FU related toxicities by anticipating the catalytic function of the DPD enzyme for each individual patient. With this assay patients bearing variants leading to a decreased enzyme activity can be identified prior to therapy initiation and can be treated accordingly with lower doses or alternative medications if applicable. Of all patients bearing one of the variants and treated with 5-FU, more than 40 % will experience severe side effects.
As many cases of 5-FU related side effects can be explained by mutations in the DPYD gene, genotyping of those variants can help to optimize and individualize chemotherapies to prevent undesired effects and lower the costs that emerge from prolonged hospitalization and the treatment of adverse reactions.

References:

1. Mol Cancer Ther. 2006; 5(11):2895-2904
2. Clin Cancer Res. 2004; 10(17):5880-5888
3. Clin Cancer Res. 2006; 12(13):3928-3934
4. Pharmacogenomics. 2001; J1:65-70
5. J Clin Oncol. 2008; 26(13):2130-2137

3. PRINCIPLE OF THE TEST

MutaREAL® DPD *real time* PCR Kit contains specific primers and additional material for the detection of DPD*2A (exon 14-skipping) polymorphism in the dihydropyrimidine-dehydrogenase gene (DPD) with the LightCycler® (Roche). The variable area of the DPD gene is amplified by PCR using LightCycler®-capillaries and **genomic DNA-template**.

The amplification product is subsequently analyzed by subjecting to melting curve including specifc hybridisation probes. This results in the final identification of genotype variants G/G, G/A or A/A.

The specific primers used in the kit flank the variable area of DPD gene and generate an **amplificate of 269 bp**.

Genotyping is performed by subsequent **melting curve analysis** of arised amplificates. This is due to the different melting points of the complexes formed by DNA template and "SNP-probes". The included "SNP-probe" is 100% homologous to the **exon-14-skipping wildtype (G/G)** variant. Therefore the hybridisation probe needs a higher temperature for complex-dissociation from G-allel than from the A-allel (containing a mismatch destabilizing the complex). Consequently, the melting curve for A-allel will be generated earlier (respectively at lower temperature). Samples with **heterozygous** genotype generate **both peaks** at different temperatures during the melting curve process.

4. KIT CONTENT

Each kit contains enough reagents to perform 24 tests. Each kit also contains a package insert.

Reference	**Type of Reagent**	**Volume** KF291132 (32 reactions)
Blue A1a	Enzyme Mix	10 µl
Blue A1b	Enzyme Buffer	65 µl
Yellow A2	Detection Mix	4 x 130 µl
Red A3	Positive Control	15 µl
Green A4	Negative Control	50 µl

5. REQUIRED MATERIALS

Provided:

- PCR reagents
- Package insert

Not provided:

- *real time* PCR capillary system (e. g. LightCycler® instrument, Roche)
- PCR reaction tubes (e. g. LightCycler® capillaries, Roche)
- Tabel centrifuge (e. g. LightCycler® capillary centrifuge, Roche)
- Cryo container for PCR reaction tubes (e. g. LightCycler® Cooling Block, Roche)
- Color Compensation Kit
- DNA extraktion kit for isolation of genomic DNA (ca. 10 ng/µl)
- Pipetts (0,5 – 200 µl)
- sterile filter Tipps for micro pipets
- sterile microtubes
- gloves (powder free)

6. STORAGE AND HANDLING

- All reagents (A1 to A4) should be **stored at <-20°C till immediate use** and then thawed **carefully** (at 8°C in refrigerator). Spin down kit components in their vials before long-term storage.
- **Avoid several freeze / thaw** cycles for the reagents A1, A2 and A3 (if necessary prepare suited aliquots and freeze again **immediately**).
- During preparation of PCR perform all working steps in a cryo-container (e.g. Light Cycler® Cooling block) or **cool all reagents** in suited manner.
- Primer-/ Probe-Mix (A2) should be **stored in the dark** (**light protection**).
- All reagents can be used until the expiration date (printed on the labels).

7. WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- This assay needs to be carried out by especially in molecular biology skilled personnel.
- Clinical samples should be regarded as potentially infectious materials.
- This assay needs to be run according to GLP (Good Laboratory Practice).
- Clinical samples should be regarded as potentially infectious materials.
- Mix all reagents carefully before use, but do not vortex.
- Do not use the kit after its expiration date.

8. TEST PERFORMANCE

Before start, **decontaminate** all working areas and used instruments. Thaw kit components **gently at 8°C** and handle detection mix (yellow, A2) in the dark. Prepare the necessary amount of LightCycler®-capillaries in a pre-cooled LightCycler®-Cooling Block and consider additional 2 capillaries for controls (red A3, green A4). Keep DNA samples ready and mix well before use.

Enzyme mix preparation (*ready to use*)

Centrifuge shortly both blue vials (A1a and A1b) to collect the solutions at the bottom of the vials. Transfer now the content of solution A1b (enzyme buffer) with sterile filter tip **quantitative** into vial A1a (enzyme) and mix well by pipetting (ca. **15x**, **do not vortex**!). *This ready to use enzyme mix is stable for about 3 month at -20°C; after freezing, this solution can be thawed twice at 8°C provided that it was not storedlonger than one hour (cooled) during the working steps.*

Master mix preparation

Following table shows the composition for **one reaction** (final volume: 20 µl). For analysis of several samples in parallel, a **master mix** should be prepared in a sterile vial **multiplying** each single volume by the number **N** of samples (incl. controls). *Additionally, 10% more volume should be calculated for reasons of inaccuracy*. The reagents should be pipetted in same order as indicated in the table:

Reagent	Volume	Master Mix Volume
Detection Mix (yellow, A2)	**16 µl**	**16 µl** x (N + 10%)
Enzyme Mix *ready to use* (blue, A1)	**2 µl**	**2 µl** x (N + 10%)

Mix prepared master mix well by gently pipetting (about **15 – 20 x**) and aliquot **18 µl** into each LightCycler®-capillary.

Samples

Add **2 µl** of each sample DNA in the corresponding LightCycler®-capillaries; use first two capillaries for the **both controls** (1. negative control, 2 µl and 2. positive control, 2 µl).

Close the filled LightCycler®-capillaries with their tips (if there is no capillary-tool from Roche use sterile tweezers to avoid contamination).

Transfer capillaries into the LightCycler® Carousel and keep position of capillaries (respectively samples).

Spin down samples in the LightCycler® Carousel-centrifuge (if a table centrifuge is used, insert the LightCycler®-Cooling Block with the capillaries inside and centrifuge at 3.000 rpm for 15 sec.).

Protocol

Insert the LightCycler®-Carousel with all sample-loaded capillaries into the LightCycler®-instrument. Activate following **PCR-protocol** and perform subsequently *real time* PCR:

Experimental Protocol

Program:	Denaturation			Type:	None	Cycles	1
Segment Number	Temperature Target (°C)	Hold Time	Stepe (C'/sec)	2° Target Temp (°C)	Stepsize (°C)	Step Delay (Cycles)	Acquisition Mode
1	**95**	**600**	**20**	0	0	0	**None**

Program:	Amplifikation			Type:	Quantification	Cycles	45
Segment Number	Temperature Target (°C)	Hold Time	Stepe (C'/sec)	2° Target Temp (°C)	Stepsize (°C)	Step Delay (Cycles)	Acquisition Mode
1	**95**	**10**	**20**	0	0	0	**None**
2	**58**	**10**	**20**	0	0	0	Single
3	**72**	**7**	**20**	0	0	0	**None**

Program:	Melting Curve			Type:	Melting Curves	Cycles	1
Segment Number	Temperature Target (°C)	Hold Time	Stepe (C'/sec)	2° Target Temp (°C)	Stepsize (°C)	Step Delay (Cycles)	Acquisition Mode
1	**95**	**20**	**20**	0	0	0	**None**
2	**40**	**20**	**20**	0	0	0	**None**
3	**80**	**0**	**0.2**	0	0	0	Continuous

Program:	Cooling			Type:	None	Cycles	1
Segment Number	Temperature Target (°C)	Hold Time	Stepe (C'/sec)	2° Target Temp (°C)	Stepsize (°C)	Step Delay (Cycles)	Acquisition Mode
1	**40**	**30**	**20**	0	0	0	**None**

9. ANALYSIS OF GENOTYPES AND INTERPRETATION OF RESULTS

Results of **melting curve** analysis for the G/A exon-14-skipping polymorphism are shown in **channel F2 at 640 nm** (choose **F2**/F1).

Loading a **Color Compensation File** is not necessary!

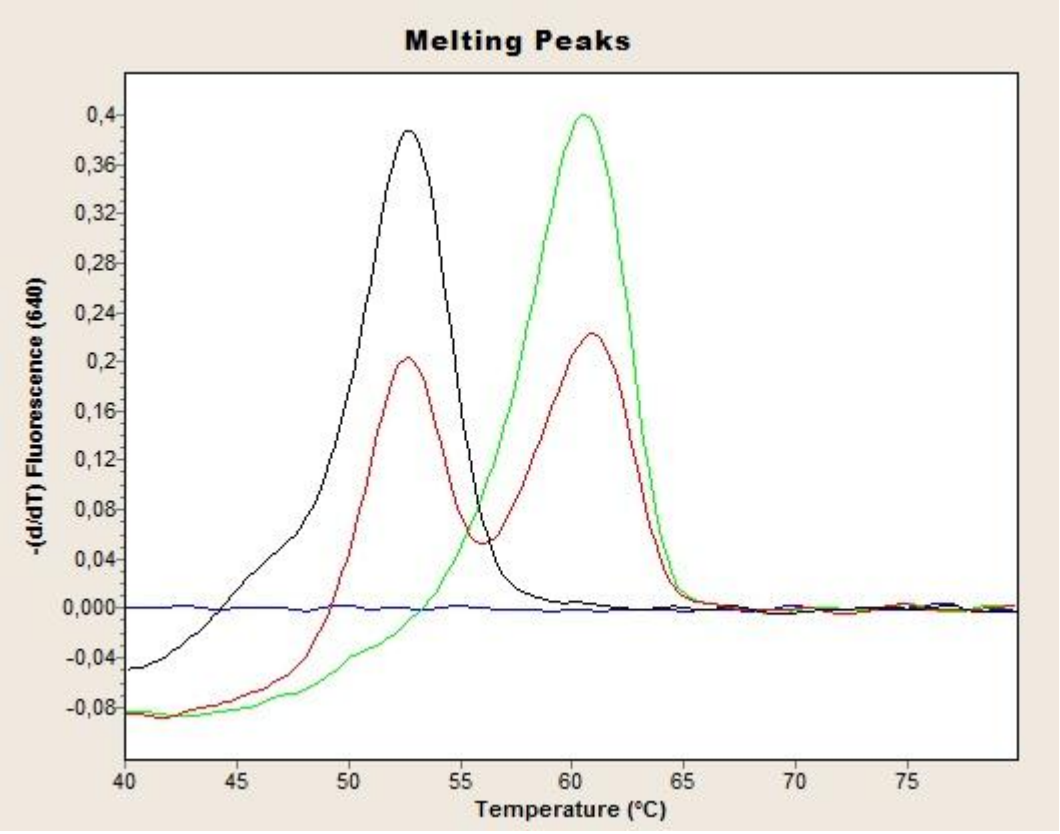
The melting curve analysis should be performed with following settings:

- Calculation mode: polynominal
- Digital filter: enabled
- Degrees to average: 9

The **positive control (A3)** contains template **heterozygous** for G/A exon-14-skipping polymorphism (one allel carries G-allel, the another allel carries the A-allel).

Following **figures** shows typical **examples** for **homozygous** as well as **heterozygous** samples - indicated temperatures should be found again within +/- 1°C:

Figure: G/A exon-14-skipping polymorphism



temperature **Mutation (A-allel)**: 52.5 °C
temperature **Wildtype (G-allel)**: 60.5 °C

10. TROUBLESHOOTING

No fluorescence peak with positive control or samples at 640 nm (F2):

- Proof PCR-program of the LightCycler®:

⇒ repeat analysis with corrected protocol.

- MutaREAL® DPD kit was thawed/ frozen more than twice or stored longer than four days at 2-8 °C:

⇒ consider storage recommendations. Repeat analysis with new MutaREAL® DPD reagents (LightCycler® PCR Kit).

- low quality of DNA -template:

⇒ exactly follow the manufactorer`s manual for DNA extraction.

Low fluorescence peak at 640 nm (F2):

- mix single components carfully before use (only by pipetting several times - do not vortex!).
- cool all stock solutions during the working steps in suited manner and protect the detection mix from light.
- Working on ice or with cooled (4°C) LightCycler®-Cooling Block is recommended.

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