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MutaREAL® TPMT

Real-Time PCR Kit

Real-Time PCR kit for the analysis of allele variants *2, *3A, *3B und *3C of the TPMT gene based on the FRET technology on LightCycler 1.5 and 2.0

IVD

Only for in vitro diagnostics

CE

REF KF291332 Σ **32**

REF KF291396 Σ **96**

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Table of Contents

1 Intended Use

The TPMT Real-Time PCR Kit is a FRET-based test for the analysis of the alleles *2, *3A, *3B and *3C (corresponding to the point mutations G238C, G460A and A719G) of the TPMT gene.

2 Introduction

Thiopurine-based drugs, like the immunosuppressant Azathioprine and the anticancer agents Mercaptopurin and Thioguanin, are commonly used in the treatment of chronic inflammatory diseases. The enzyme thiopurine-methyltransferase (TPMT) is responsible for the methylation of thiopurine, a step that is necessary for the biotransformation of the latter.
TPMT mutations G238C, G460A and A719G (*2, *3A, *3B and *3C) are associated with a reduced TPMT enzyme activity. Reduced or no TPMT enzyme activity can lead to the accumulation of toxic degradation products after intake of thiopurine-containing drugs, which can cause severe adverse side effects, such as myelosupression, for example.

References:

- Skrzypczak-Zielinska et al., Molecular Diagnosis & Therapy, 2016, 20:493-9
- Yates et. al., Annals of Internal Medicine, 1997, 126(8):608-14
- Tamm et al., Clinical Pharmacology & Therapeutics, 22. October 2016, Accepted Article

3 Concept of the Assay

This sequence specific detection assay is based on fluorescence resonance energy transfer (FRET). The assay contains two specific primers flanking the target region as well as two hybridization probes. One of the hybridization probes binds to a specific target region known to carry the gene mutation of interest. The second hybridization probe binds to a sequence in close proximity to the first probe, not covering the mutation.
In order to achieve FRET, one of the hybridization probes is labeled with a donor-fluorophor, the other one is labeled with an acceptor-fluorophor. If both hybridization probes are in immediate proximity, the donor-fluorophor transfers energy to the acceptor fluorophor following excitation. Due to this energy transfer, the acceptor-fluorophor emits light of a longer wavelength. Since energy transfer can only occur if both hybridization probes are bound to the target sequence, the amount of hybridized probe pairs, and thereby the fluorescence signal proportionally increases to the amount of amplified PCR product. Following amplification, genotyping is then performed via melting curve analysis. For this purpose, the temperature is slowly increased after a short denaturation step and the dissociation behavior of the probe is monitored by constantly measuring the emitted fluorescence signal. If the targeted mutation is not present, the fluorescence signal decreases only at high temperatures since the perfectly matched hybridization probe dissociates late due to its high binding affinity to the target region. However, in the presence of the mutation, the fluorescence signal decreases earlier with increasing temperatures since the mismatched and therefore less stable probe dissociates earlier from the target region.

4 Kit Components

TPMT Real-Time PCR-Kit	Volume	
Reagent	**32-rxn**	**96-rxn**
Enzyme Mix **(blue)**	1300 µL	3 x 1300 µL
Detection Mix 1 **(yellow)** - *TPMT*2, G238C*	368 µL	3 x 368 µL
Detection Mix 2 **(brown)** - *TPMT*3B, G460A*	368 µL	3 x 368 µL
Detection Mix 3 **(purple)** - *TPMT*3C, A719G*	368 µL	3 x 368 µL
Positive Control 1 **(red)** - *TPMT*2 heterozygous*	25 µL	3 x 25 µL
Positive Control 2 **(orange)** - *TPMT*3B and *3C heterozygous*	50 µL	3 x 50 µL
Negative Control **(green)**	200 µL	200 µL

5 Required Material

Required Materials - not provided:

- Roche LightCycler® 1.5, 2.0 or 480 Real-Time PCR-System
  - The CE conformity is only given when one of the above mentioned components is used.
- LightCycler® capillaries, Roche or 96-well plate/stripes (white)
- LightCycler® cooling block, Roche or cryo container for PCR reaction tubes
- Pipettes (0,5 – 200 µL)
  - 0.5 - 10 µL
  - 10 - 200 µL
- 1.5 mL reaction tubes

6 Storage and Handling

- All reagents should be stored at -20 °C
- Avoid several freeze / thaw cycles of the reagents (if necessary prepare aliquots)
- The detection mixes have to be protected from exposure to light

7 Considerations and Precautions

The regulations and principles for working in a biomolecular laboratory have to be strictly followed.

- All steps have to be performed in an uninterrupted manner
- All PCR reagents have to be cooled while in use
- The DNA purity (A260/A280 ratio) should be between 1.8 and 2.0

8 Test Procedure

8.1 PCR Preparation

Gently thaw all components on ice. Mix them gently (do not vortex) and spin them down before use. Keep in mind to protect the detection mixes from exposure to light. During the PCR setup all reagents have to be kept cool.

For the amplification, three PCR reaction tubes (LightCycler® capillary) are needed (one for each mastermix) per sample, plus two additional tubes for each mastermix for the negative and positive controls. The following table shows the volumes of each reagent needed per mastermix. The mastermixes should be prepared for the number of samples (incl. negative and positive control) (N) plus 10 % to compensate for pipetting inaccuracies. The mastermixes should be prepared in the same order as indicated in the table.

Master Mix 1 - TPMT*2

Reagent	Volume per 25 µL Reaction	Master Mix Volume
Detection Mix 1 (yellow)	10.5 µL	10.5 µL * (N + 0.1)
Enzyme Mix (blue)	12.5 µL	12.5 µL * (N + 0.1)

- Thourougly mix the mastermix by pipetting up and down or inverting (do not vortex) and shortly spin down. Aliquot 23 µL into each PCR tube.
- For the negative control add 2 µL of the provided negative control (green).
- For the positive control add 2 µL of the provided positive control 1 (red).
- Add 2 µL of each sample DNA to the corresponding PCR tube.

Master Mix 2 - TPMT*3B

Reagent	Volume per 25 µL Reaction	Master Mix Volume
Detection Mix 2 (brown)	10.5 µL	10.5 µL * (N + 0.1)
Enzyme Mix (blue)	12.5 µL	12.5 µL * (N + 0.1)

- Thourougly mix the mastermix by pipetting up and down or inverting (do not vortex) and shortly spin down. Aliquot 23 µL into each PCR tube.
- For the negative control add 2 µL of the provided negative control (green).
- For the positive control add 2 µL of the provided positive control 2 (orange).
- Add 2 µL of each sample DNA to the corresponding PCR tube.

Master Mix 3 - TPMT*3C

Reagent	Volume per 25 µL Reaction	Master Mix Volume
Detection Mix 3 **(purple)**	10.5 µL	10.5 µL * (N + 0.1)
Enzyme Mix **(blue)**	12.5 µL	12.5 µL * (N + 0.1)

- Thourougly mix the mastermix by pipetting up and down or inverting (do not vortex) and shortly spin down. Aliquot 23 µL into each PCR tube.
- For the negative control add 2 µL of the provided negative control **(green)**.
- For the positive control add 2 µL of the provided positive control 2 **(orange)**.
- Add 2 µL of each sample DNA to the corresponding PCR tube.

Close the capillaries with the corresponding lids, transfer them into the LightCycler® carousel and spin them down in the LightCycler® centrifuge (if a table top centrifuge is used, spin the capillaries in the adapters of the cooling block at 3000 rpm for 15 s). Subsequently place the carousel in the LightCycler® and use the PCR protocol described in 8.2.

8.2 PCR Protocol

Step	Temperature [°C]	Time [s]	Ramp rate [°C/s]	Cycles	Acquisition
Initial Denaturation	94	120	20	1 x	none
Denaturation	94	10	20	45 x	none
Primer Annealing	55	25	20		**single**
Elongation	72	25	20		none
Melting Curve	95	20	20	1 x	none
	47	20	20	1 x	none
	75	0	0,2	1 x	**constant**
Cooling	40	30	20	1 x	---

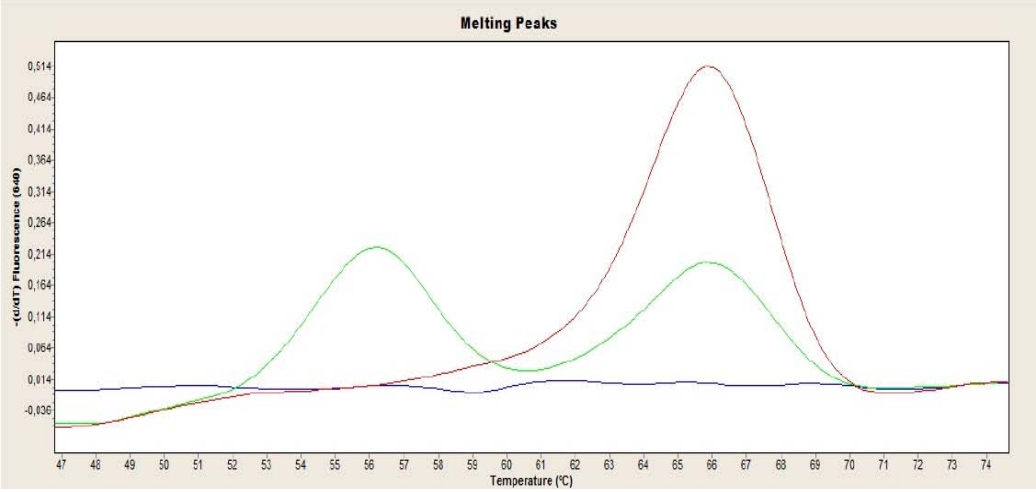
9 Evaluation

For the evaluation of the melting curves add an analysis of the type "genotyping". Thereby the derivation of the fluorescence curve is generated. The detection wavelength is 640 nm.

TPMT G238C (*2)

Temperature mutated allele: **56,0 °C** (+/-2 °C)
Temperature wildtype allele: **66,0 °C** (+/-2 °C)

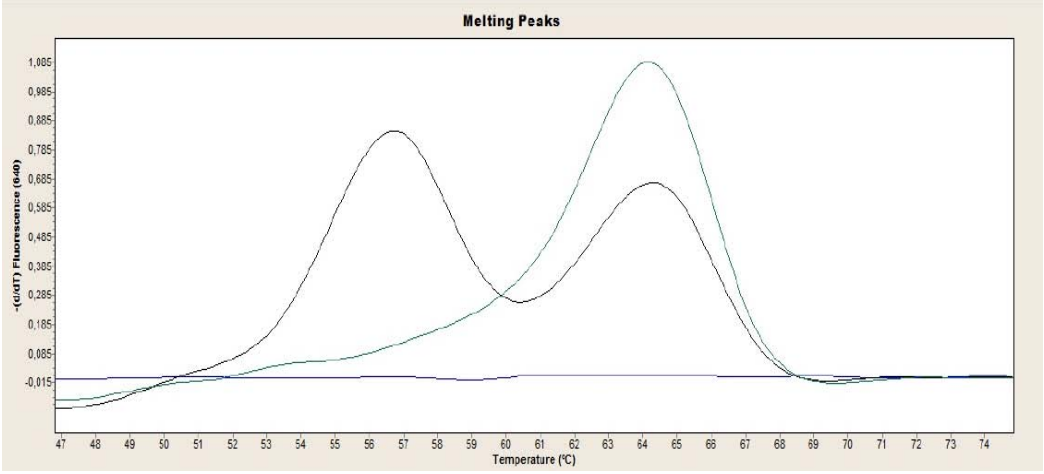
The following graphic shows the typical results for TPMT*2: **blue curve** - negative control, **red curve** - homozygous wildtype, **green curve** - heterozygous mutation



TPMT G460A (*3B)

Temperature mutated allele: **56,5 °C** (+/-2 °C)
Temperature wildtype allele: **64,0 °C** (+/-2 °C)

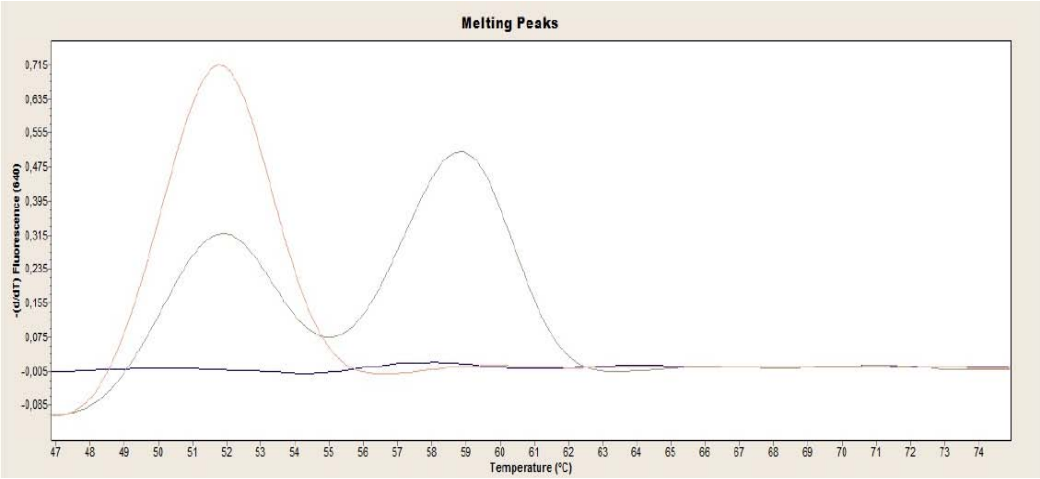
The following graphic shows the typical results for TPMT*3B: **blue curve** - negative control, **green curve** - homozygous wildtype, **black curve** - heterozygous mutation



TPMT A719G (*3C)

Temperature wildtype allele: **52,0 °C** (+/-2 °C)
Temperature mutated allele: **59,0 °C** (+/-2 °C)

The following graphic shows the typical results for TPMT*3C: **black curve** - negative control, **beige curve** - homozygous wildtype, **grey curve** - heterozygous mutation



Special cases - TPMT G460A and A719G
If both mutations are present on one allele, the allele is called TPMT*3A. This results in the following execptions for the evaluation of the analysis:

TPMT*3B und TPMT*3C heterozygous: The genotype is either TPMT*3B/*3C or TPMT*1/*3A (if both mutations are present in the same allele). This method cannot distinguish between these two possible genotypes.

TPMT*3B heterozygous and TPMT*3C homozygous mutation: The genotype is TPMT*3A/*3C.

TPMT*3B homozygous mutation and TPMT*3C heterozygous: The genotype is TPMT*3A/*3B.

TPMT*3B and TPMT*3C homozygous mutation: The genotype is TPMT*3A/*3A.

The provided positive control 1 contains a template heterozygous for the point mutation G238C. The positive control 2 contains a template heterozygous for G460A and A719G.

10 Troubleshooting

Problem	Solution
No or low fluorescence peak with positive control or samples	Check PCR-program of the real time PCR instrument in use and repeat with corrected protocol
	PCR reagents were thawn / frozen more than twice or stored longer than four days at 2-8 °C. Repeat analysis with a fresh aliquot or new PCR reagents
	Quality of DNA template is not sufficient. Use freshly extracted DNA and measure the concentration/purity before use.
	The detection mixes were not protected from light. Repeat analysis with a fresh aliquot or new PCR reagents.

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