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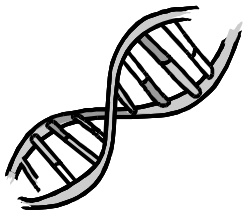
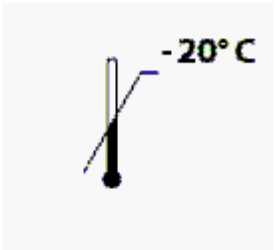


MutaPLATE® MTHFR (TM)

(TAQ-Man) real time PCR Kit

PCR test for analysis of the C677T Single Nucleotide Polymorphism (SNP) of the methylenetetrahydrofolatereductase (MTHFR) gene in open real time PCR systems (z. B. RotorGene, SmartCycler, Light Cycler, ABI, Amplifa, Stratagene) by Taq-Man technology.



IVD

Nur für in-vitro Diagnostik

CE

REF KF1901432 Σ **32**

REF KF1901496 Σ **96**

Table of content

1 Intended use

MutaPLATE ® MTHFR (TM) *real time* PCR kit is a molecular biological test for analysis of the C677T - Single Nucleotide Polymorphism (SNP) in the methylene-tetrahydrofolatereductase (MTHFR) - gene in open real time PCR systems (e. g. RotorGene, SmartCycler, Light Cycler, ABI, Stratagene, Amplifa). The C677T polymorphism leads to a change of alanin to valine in the flavin adenine dinucleotide (FAD) binding site. FAD is a cofactor for the MTHFR enzyme and this mutation prevents binding of this factor. In addition, it leads to a more thermolabile version of the enzyme. This in consequence reduces the enzyme activity, leading to increased homocystein levels inside the bloodstream.
The MTHFR C677T variant is associated with an increased risk for atherosclerosis and peripheral vascular disease. Also, a relation between the C677T genotype and low levels of the vitamins B6 and B12 as well as the occurrence of neural tube defects could be shown. Less clear are the associations of this polymorphism with Trisomy 21 or a general hypomethylation of DNA.

2 Introduction

Homocystein is produced in the body as by-product during the degradation of the amino acid methionine. Via several intermediates homocystein is formed, which is either converted back to methionine by remethylation or is further degraded to cystein. The clearance of the non-remethylated homocystein is performed by the kidneys. Homocystein is cytotoxic, for which reason the intracellular concentration has to be kept at a low level. Furthermore it forms aggregates with the low density lipoprotein (LDL), which leads to phagocytosis of these complexes and the release of reactive oxygen species that promote atherogenesis. High homocystein plasma levels cause vascular constriction, stimulate blood clotting and consequently increase the risk for thrombosis.
The enzyme methylenetetrahydrofolate reductase (MTHFR) plays a major role in the remethylation of homocystein to methionine and is thus directly linked to the plasma homocystein level. Other parameters influencing this level are vitamins B6 and B12. MTHFR reduces 5,10-methylentetrahydrofolate (5,10-MTHF) to 5-methylTHF, which is then available as methyl donor for the remeythlation of homocystein. Failures in this pathway, e.g. mutations in the MTHFR gene or vitamin B deficiency can lead to hypo methylation of the DNA, which in turn is a common feature of many cancer cells in solid tumors.
Genotyping the C677T polymorphism in the MTHFR gene can yield information about a possibly increased risk for thrombosis and atherosclerosis. Additionally, the risk of general DNA hypomethylation can be assessed which can be a clue for an existing or future cancer.

[1] van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van de Heuvel LP, Blom HJ. *A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects*? Am J Hum Genet. 1998 May; 62(5):1044-51.

[2] Khandanpour N, Willis G, Meyer FJ, Armon MP, Loke YK, Wright AJ, Finglas PM, Jennings BA. *Peripheral arterial disease and methylenetetrahydrofolate reductase (MTHFR) C677T mutations: A case-control study and meta-analysis*. J Vasc Surg. 2009 Mar; 49(3):711-8.

[3] Hertfelder HJ, Gnida C, Pötzsch B, Hanfland P. *MTHFR-Polymorphismus C677T: Sinn und Unsinn der Diagnostik*. Dtsch Arztebl 2004; 101(46): A-3101 / B-2625 / C-2501.

3 Principle of the test

MutaPLATE® MTHFR (TM) *real time* PCR Kit contains specific primers and additional material for the detection of the C677T polymorphism. The variable region of the flavin adenine dinucleotide (FAD) binding site in exon 4 of the methylenetetrahydrofolate reductase gene (MTHFR) is amplified by PCR using **genomic DNA template** as template. The specific primers used in the kit flank the variable area and generate an **amplificate of 180 bp** using Taq-Man technology.

The standard PCR contains additionally **two sequence specific oligonucleotides** marked with fluorescence dye (TaqMan probes). Both probes bind at the amplificated target-DNA which includes the single nucleotide polymorphism (SNP). Due to this, a fluorescence signal is generated and detected by the **optical unit** of the used *real time* PCR instrument. The TaqMan probe for the C-allele (wildtype) is marked with **FAM (510 - 530 nm, green)** and the TaqMan probe for the T-allele (mutation) is marked with **YAK (555 - 560 nm, yellow)**.

The following three discriminations are possible:

1. Homozygous **C/C**:
   Increase of the fluorescent signal from the **FAM** labeled TaqMan probe, no increase of the fluorescent signal from the **YAK** labeled TaqMan probe.
2. Heterozygous **C/T**:
   Increase of the fluorescent signal from the **FAM** labeled TaqMan probe and increase of the fluorescent signal from the **YAK** labeled TaqMan probe.
3. Homozygous **T/T**:
   No increase of the fluorescent signal from the **FAM** labeled TaqMan probe, increase of the fluorescent signal from the **YAK** labeled TaqMan probe.

4 Kit content

Each kit contains enough reagents to perform **32** respectively **96** tests. Each kit also contains a package insert.

Reference	Type of reagent	Volume (32x)	Volume (96x)
Blue	Enzyme Mix	435 µl	3 x 435 µl
Yellow	Detection Mix **T - Allele**	175 µl	3 x 175 µl
White	Detection Mix **C - Allele**	175 µl	3 x 175 µl
Red	Positive Control	15 µl	3 x 15 µl
Green	Negative Control (Water)	50 µl	3 x 50 µl

5 Required materials

Provided:

- Reagents for real-time PCR
- Package insert

Not provided:

- *real time* PCR capillary system (e. g. RotorGene)
- PCR reaction tubes
- Cryo container for PCR reaction tubes
- DNA extraktion kit for isolation of genomic DNA (ca. 10 ng/µl), **e.g. MutaCLEAN Universal RNA/DNA, KG1037**
- Pipetts (0,5 – 200 µl) with sterile filter Tipps for micro pipets
- sterile microtubes
- gloves (powder free)

6 Storage and handling

- All reagents should be **stored at <-20°C till immediate use**. Spin down kit components in their vials before long-term storage.
- **Avoid several freeze / thaw** cycles for the reagents (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 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: 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 procedure

Before start, **decontaminate** all working areas and used instruments. Thaw kit components **gently at 5-8°C** and handle detection mixes in the dark. Prepare the necessary amount of PCR reaction tubes in a pre-cooled cooling block and consider additional 2 tubes for controls. Keep DNA samples ready and mix well before use.

Enzyme mix (*ready to use*)

This ready to use enzyme mix is stable for about 3 month at -20°C; after freezing, this solution can be thawed twice at 5-8°C provided that it was not stored longer than one hour (cooled) during the working steps.

Master mix preparation

Following table shows the composition for **one reaction** (final volume: 25 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)	**5 µl**	**5 µl** x (N + 10%)
Detection Mix (**white**)	**5 µl**	**5 µl** x (N + 10%)
Water (**green**)	**0.5 µl**	**0.5 µl** x (N + 10%)
Enzyme Mix *ready to use* (**blue**)	**12.5 µl**	**12.5 µl** x (N + 10%)

Mix prepared master mix well by gently pipetting (about **15 – 20 x, do not vortex**) and aliquot **23 µl** into each PCR reaction tube.

Samples

Add **2 µl** of each sample DNA in the corresponding PCR reaction tube; use first **both controls** (1. negative control, 2 µl and 2. positive control, 2 µl). Close the tubes and transfer them into the real time PCR instrument (keep position of samples).

Protocol

Activate following **PCR-protocol** and perform subsequently the *real time* PCR:

Experimental Protocol

Program:	**Denaturation**		1
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	94	120	None

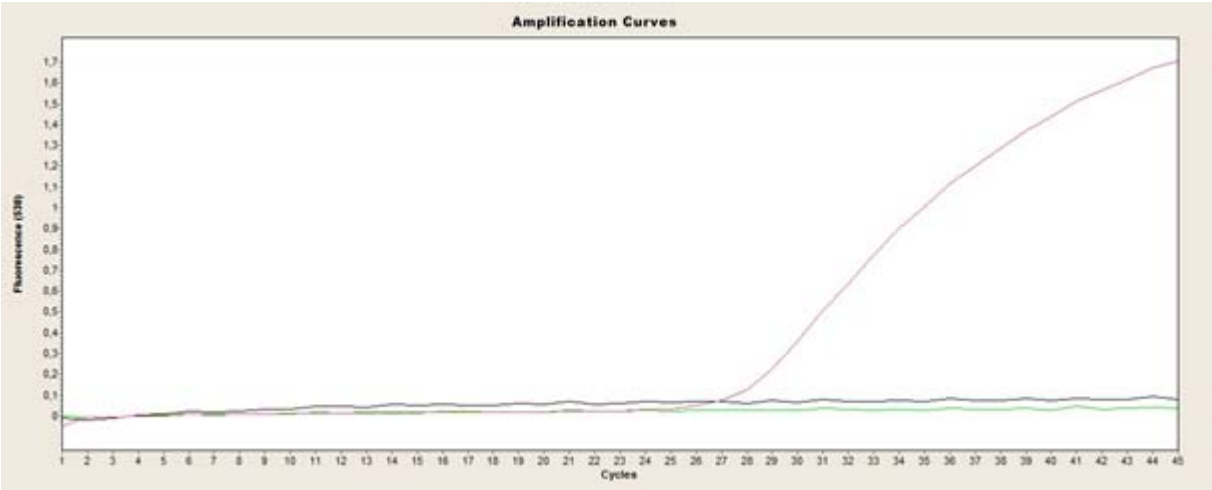
Program:	**Amplifikation**		45
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	94	30	None
2	58	60	**Single**
Program:	**Cooling**		1
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	40	30	None

9 Analysis of genotype and interpretation of results

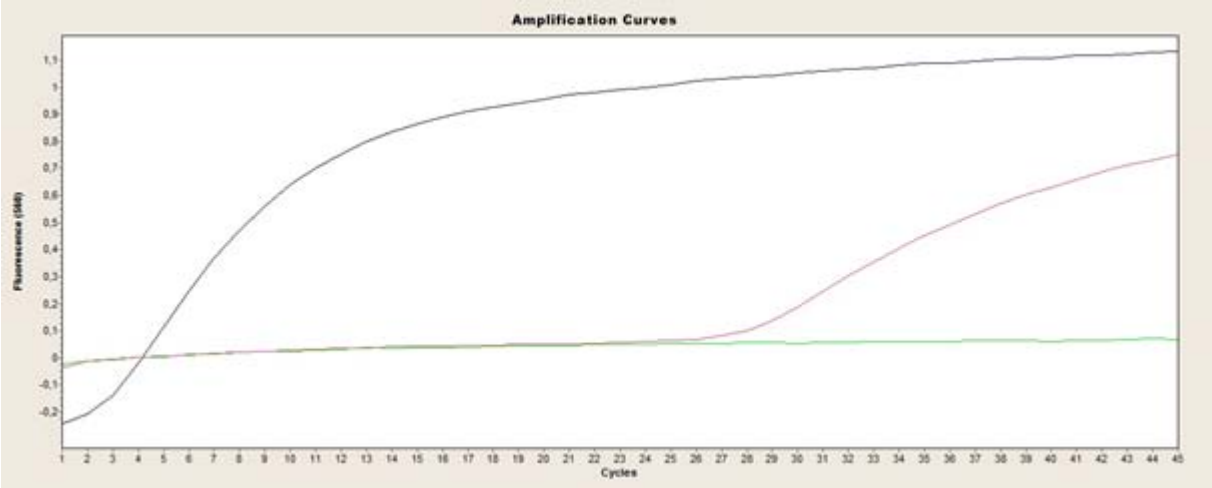
Results of the analysis for the C677T polymorphism are shown for at **510 - 530 nm / green** and **550 - 560 nm / yellow** (choose corresponding channel of your real time PCR instrument). The **positive control** contains template **heterozygous** for C677T polymorphism (one allel carries the **T**, another allel carries the **C**).

Following **figures** shows typical **examples** for **homozygous** as well as **heterozygous** samples on the LightCycler 2.0. A **color compansation file** might be required on some *real time* PCR devices, e.g. LightCycler 2.0.

C-Allele at 530 nm



T-Allele at 560 nm



10 Troubleshooting

No fluorescence peak with positive control or samples at about **510-530 nm or 550-560 nm:**

- Proof PCR-program of the real time PCR instrument in use:

⇒ repeat analysis with corrected protocol.

- MutaPLATE® MTHFR (TM) kit was thawed/ frozen more than twice or stored longer than four days at 2-8 °C:

⇒ consider storage recommendations. Repeat analysis with new MutaPLATE® MTHFR (TM) reagents.

- low quality of DNA -template:

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

Low fluorescence peak at about **510 - 530 nm or 550 - 560 nm**:

- 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) Block is recommended.

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