MutaGEL® MTHFR

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1. Intended Use

Code: KT0001

The kit "MutaGEL® MTHFR" allows the detection of the most common C677T polymorphism of the human MTHFR gene encoding for the enzyme 5,10-methylene-tetrahydrofolatereductase. This mutation causes the substitution of alanine with valine within the enzyme (Ala 222 Val).

2. Introduction

Hyperhomocysteinemia is the result of a disturbed homocysteine metabolism often due to genetic defects. The increase of homocysteine plasma level is therefore a risk factor for cardio- or cerebrovascular complications as well as for venous thrombosis and mirgaine. Patients often carry the very common C677T mutation in the MTHFR gene coding for a thermolabile variant of the MTHFR enzyme with reduced activity. Also an A1298C mutation in the MTHFR gene increases the homocysteine plasma level additionally, but only in case of already present C677T heterozygotie. Nevertheless, it could be an option to analyse also this polymorphism in a separate PCR to determine complete genetic risk factors for hyperhomocysteinemia.

3. Test Principle

The kit **MutaGEL® MTHFR** contains a set of primers which amplify a specific sequence within the human MTHFR gene. The amplified product obtained from a wild type DNA will not be cut by the restriction enzyme included in this kit, whereas the fragment obtained from DNA carrying the very common C677T- mutation will be cut once. The generated amplification products and their cut-fragments are analyzed through gel electrophoresis.

4. Materials Supplied (24 determinations)

▪ Primer Mix (MTHFR)	1 x 100 µl (**green**)	solution of oligonucleotides specific for the MTHFR gene (including C677T).
▪ PCR Master Mix	1 x 250 µl (**violet**)	ready to use PCR reagents (hot start TAQ enzyme, $MgCl_2$, dNTP, buffer)
▪ MTHFR Positive Control	1 x 40 µl (**red**)	aqueous solution of human DNA pool with heterozygous genotype C677T.
▪ PCR water	1 x 400 µl (transparent)	(H_2O) deionized
▪ Restriction enzyme 677	1 x 25 µl (**blau**)	restriction endonuclease for the MTHFR C677T- mutation site.
▪ Restriction buffer (for C677T)	1 x 60 µl (white)	optimal restriction buffer for endonuclease 677 (for C677T digest).

5. Material required but not supplied

Reagents and Instruments:

- DNA extraction kit (e.g. with MutaCLEAN® DNA Blood, KG1033, Immundiagnostik)
- Reagents for gelelectrophoresis
- Thermal cycler (and optional mineral oil for thermal cyclers without heated lid)
- Pipettes (0,5 - 200 µl) and sterile pipette tips
- Sterile micro tubes suitable for the thermal cycler in use
- Instruments for gel electrophoresis

6. Storage and Stability

Storage at < -18°C. The reagents are stable in the unopened micro tubes until the expiration date indicated. In general, avoid several thawing/freezing cycles for the reagents in order to conserve their stability – if necessary, make suitable aliquots.

Before use: Spin tubes briefly before opening (contents may become dispersed during shipment).

Warnings and Precautions

For in vitro diagnostic use only.

- Specimens and controls should be handled as if potentially infectious.
- Don't use the kit after its expiration date.
- Set up (if possible) three separate working areas:
  1) DNA isolation
  2) Preparing amplification
  3) Detection of the amplification products
- Use different tips and wear separate coats and gloves in each area.
- Use sterile plugged tips for pipetting or use special PCR pipettes for aerosol free pipetting.
- Routinely decontaminate your pipettes and the laboratory benches and avoid aerosols.

Procedure

The complete procedure is divided in four steps:

1) Sample preparation/ DNA extraction.
2) Amplification with primers specific for the MTHFR gene.
3) Restriction enzyme digest for the identification of the C677T-polymorphism.
4) Analysis of genotype by gel-electrophoretical detection of the amplified/ restricted DNA.

8. Sample preparation

- Extract total genomic DNA (e.g. from 200 µl whole blood) using a commercial extraction kit after the manufacturer´s instruction.
- Start immediately with the amplification procedure or store the extracted DNA at < -18°C.

9. Amplification

- Every set of amplifications should include a positive and a negative control.
- Prepare for each sample and positive as well as negative control the following Master-Mix (multiply the volumes necessary for each reaction with the number **N** of reactions, and add one volume).

Reagents for PCR	Total volume for each reaction: 25 µl	Volume in the Master-Mix
Primer MTHFR	4 µl	4 µl x (N+1)
PCR Master Mix	10 µl	10 µl x (N+1)
PCR water	6 µl	6 µl x (N+1)

- aliquot **20 µl** of the master mix in sterile micro tubes suitable for the thermal cycler.
- **Samples**: add **5 µl** of the extracted DNA to the master mix (about 10 - 20 ng/ µl).
- **Positive control**: add **5 µl** of the "MTHFR positive control DNA" to the master mix.
- **Negative control**: add **5 µl** of PCR water to the master mix.
- Transfer the micro tubes into the thermal cycler (if necessary overlay the Mix with 60 µl of mineral oil).
- Perform the following cycles:

Initial Hold:	95°C for 10 minutes
40 cycles:	**95°C** for 45 seconds / **61°C** for 50 seconds / **72°C** for 45 seconds
Final Hold:	72°C for 10 minutes, 20°C follow up

10. Digestion of the amplified DNA

Prepare for each sample as well as for the controls (positive and negative) the following Digestion-Mix (multiply the volumes necessary for each reaction with the number **N** of reactions, and add about 10% surplus).

Reagents for DIGESTION	Total volume for each DIGESTION: 20 µl	Volume in the Digestion-Mix
restriction enzyme 667	1 µl	1 µl x (N+1)
Buffer for restriction digest C677T	2 µl	2 µl x (N+1)

- Aliquot **3 µl** of the Digestion-Mix into tubes suitable for the incubator (a thermal cycler may be used for the incubation too).
- Add **15 µl** of the amplification product from the PCR to the Digestion-Mix.
- Transfer the tubes to the thermoblock-incubator.
- Incubate at **37°C** for at least **3 hours** (or over-night).

11. Detection of the amplified/ restricted DNA

- Carry out gel electrophoresis in at least **2.5 %** (optimal **3 – 3.5 %**) agarose (or polyacrylamide 20%) for about **120 Vh** (e. g. 80 min at 90 volt) in 1x TBE-buffer: mix **18 µl** of each digestion mix with about **4 µl** loading buffer and load the gel. The length of the amplified DNA fragments can be equalized with a suitable molecular weight standard – or alternative: use the undigested amplification product for each patient in a corresponding second lane as direct reference. Please consider that a high gel concentration is indicated due to the small fragment size differences!
- The separated DNA is coloured by ethidiumbromide or SybrGreen (5 µg/ ml) for 5 min and visualised under UV-light (312 nm).
- The amplification produces following amplification products with indicated fragment-lengths corresponding to the present genotype:

GENOTYPE	Length of the amplified DNA (in base pairs)
wt / wt	**198** bp
wt / mut	**198** **175** (+ 23) bp
mut / mut	**175** (+ 23) bp

- The **MTHFR positive control DNA** has the **genotype wt / mut** and shows therefore all diagnostic relevant DNA bands (please consider that the small 23 bp fragment is too short to be detected in the agarose gel).
- In any case the negative controls must be negative for any amplification product.

12. Restrictions

The PCR results for positive control in DNA fragments of indicated length and for the samples at least one of both amplification products must be detectable. If this is not the case, the sample must be tested a second time or the complete analysis must be repeated with freshly isolated DNA. If there are no positive control DNA fragments present, the amplification was incorrect and the chosen PCR conditions have to been proven/ corrected.

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