



<b>1. Intended Use</b>	<b>Code: KT0001</b>
<p>The kit "MutaGEL® MTHFR 2" allows the parallel detection of the both common point mutations in the position 677 and 1298 of the 5,10-methylenetetrahydrofolat-reduktase (MTHFR) gene. The substitution of cytosine against thymine and respectively adenine against cytosine causes a change in the amino acid sequence from alanine to valine (Ala222Val) and in case of A1298C from glutamine to alanine (Glu429Ala). For in vitro diagnostic use only.</p>	

<b>2. Introduction</b>
<p>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 migraine. Patients often carry the common C677T mutation in the MTHFR gene coding for a thermolabile variant of the MTHFR enzyme with reduced activity. The A1298C mutation in the MTHFR gene increases the homocysteine plasma level only in case of C677T heterozygotie. Due to this fact both mutations should be analysed in combination as genetically risk factors for hyperhomocysteinemia.</p>

<b>3. Principle of the Test</b>
<p>The kit "MutaGEL® MTHFR 2" contains different sets of primer for analysis of both mutations C677T and A1298C in the MTHFR gene. The primer sets are prepared in two separate mixes (for wildtype and mutation alleles) and are used for PCR directly with the extracted DNA. The resulting amplification products are subsequently identified with gelelectrophoretic methods. If there is no specific allele product detectable, the correct PCR procedure is proved by an internal control.</p>

<b>4. Material Supplied (for 24 determinations)</b>																		
<table border="0"> <tr> <td>▪ SSP-primer MTHFR (wt)</td> <td>24 x tubes</td> <td>aliquoted colored PCR tubes for the wildtype constellation C677T/ A1298 (blue).</td> </tr> <tr> <td>▪ SSP-primer MTHFR (mut)</td> <td>24 x tubes</td> <td>aliquoted colored PCR tubes for the mutation constellation 677T/ 1298C (orange).</td> </tr> <tr> <td>▪ PCR master mix</td> <td>1 x 350 µl</td> <td>ready to use PCR solution (inclusive buffer, dNTPs, cresol-red).</td> </tr> <tr> <td>▪ Positive control DNA</td> <td>1 x 30 µl</td> <td>aqueous solution of human gDNA of genotype wt/mut C677T/ A1298C.</td> </tr> <tr> <td>▪ PCR water</td> <td>1 x 750 µl</td> <td>deionized water</td> </tr> <tr> <td>▪ Taq polymerase (5 U/ml)</td> <td>1 x 8 µl</td> <td>PCR enzyme (<i>hot start</i>)</td> </tr> </table>	▪ SSP-primer MTHFR (wt)	24 x tubes	aliquoted colored PCR tubes for the wildtype constellation C677T/ A1298 (blue).	▪ SSP-primer MTHFR (mut)	24 x tubes	aliquoted colored PCR tubes for the mutation constellation 677T/ 1298C (orange).	▪ PCR master mix	1 x 350 µl	ready to use PCR solution (inclusive buffer, dNTPs, cresol-red).	▪ Positive control DNA	1 x 30 µl	aqueous solution of human gDNA of genotype wt/mut C677T/ A1298C.	▪ PCR water	1 x 750 µl	deionized water	▪ Taq polymerase (5 U/ml)	1 x 8 µl	PCR enzyme ( <i>hot start</i> )
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<b>5. Materials Required but not Supplied</b>
<p>Reagents:</p> <ul style="list-style-type: none"> <li>▪ DNA extraction kit (f.e. DNA Blood Miniprep, KBR3005)</li> <li>▪ reagents for gel electrophoresis</li> </ul> <p>Instruments:</p> <ul style="list-style-type: none"> <li>▪ thermal cycler</li> <li>▪ variable pipets (0.5 - 200 µl) and sterile pipette tips</li> <li>▪ sterile micro tube (for master mix preparation)</li> <li>▪ instruments for gel electrophoresis</li> </ul>

<b>6. Storage and Stability</b>
<p>The reagents are stable at 4°C for 30 d. For longer storage freeze at ≤ -18°C. The reagents are stable in the unopened micro tubes until the expiration date indicated (see print on the package). Do not thaw out the content of the "MTHFR 2 positive control DNA" for more than two times. If necessary, make suitable aliquots.</p> <p><i>Before use:</i> Spin tubes briefly before opening (contents may become dispersed during shipment).</p>

<b>7. Warning and Precautions</b>
<ul style="list-style-type: none"> <li>▪ For <i>in vitro</i> diagnostic use only.</li> <li>▪ Test should only be performed only by skilled persons considering GLP (Good Laboratory Practice) guidelines.</li> <li>▪ Don't use the kit after its expiration date.</li> <li>▪ After usage, dispose all reagents and test components included in the kit in conventional garbage.</li> <li>▪ PCR technology is extremely sensitive. The amplification of a single DNA molecule generates million identical copies. Therefore set up three separate working areas for a) sample preparation, b) PCR reagent preparation and c) DNA detection. For each working area a different set of pipettes should be reserved.</li> <li>▪ Wear separate coats and gloves in each working area.</li> <li>▪ Use sterile filter tips for pipetting and use special PCR pipettes for aerosol free pipetting.</li> <li>▪ Routinely decontaminate your pipettes and the laboratory benches.</li> <li>▪ Avoid aerosols.</li> </ul>



## Procedure

The complete procedure is divided in three steps:

1. Sample preparation (DNA extraction).
2. Amplification with primers specific for the MTHFR gene (as well as for the wild-type alleles as for the mutant alleles).
3. Analysis of genotype by gel electrophoretical resolution of amplified DNA-fragments.

## 8. Sample Preparation

- For template use total genomic DNA which can be extracted (f. e. from about 200 µl whole blood) using commercial available DNA extraction kits according to the manufacturers instruction.
- Start immediately with the amplification procedure or store the extracted DNA at ≤ -20°C.

## 9. Amplification

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

PCR reagents	reaktion volume: 12 µl	master mix volume
PCR master mix	6 µl	6 µl x (N+ 0.5)
aqua (dest.)	10 µl	10 µl x (N + 0.5)
Taq polymerase (5 U/µl)	0.12 µl	0.12 µl x (N+ 0.5)

- add **16 µl** of the prepared master mix (**red**) to each PCR-tube (with lyophilysed SSP-MTHFR) use for each sample in parallel a wildtype-PCR-tube (**blue**) and a mutation-PCR-tube (**orange**)!
- samples: add **4 µl** of the **extracted gDNA** (50 ng/µl) to the wildtype-tube (**blue**) and to the mutation-tube (**orange**).
- positive control: add **4 µl** of the **MTHFR positive control DNA** to the wildtype-tube (**blue**) and to the mutation-tube (**orange**).
- negative control: add **4 µl** of aqua (dest.) to the Master-Mix
- transfer the micro tubes into the thermal cycler
- perform the following amplification protocol (about 1 h 20 min):

<b>Initial hold:</b>	95°C for 7 min
<b>10 cycles:</b>	96°C for 15 sec / 65°C for 1 min
<b>20 cycles:</b>	96°C for 10 sec / 61°C for 50 sec / 72°C for 30 sec
<b>Final hold:</b>	10°C

## 10. Detection of the amplified DNA and Interpretation of the Results

- Carry out gel electrophoresis in **2 %** agarose (or 10 % polyacrylamid) for about **75 Vh** (f.e. 50 min at 90 volts) in 1x TBE-buffer: load after amplification about **15 µl** of each amplified material (products from **blue** and **orange** tubes side by side = two lanes for one DNA-sample) to the gel (due to the included loading buffer the PCR-solution can be added directly to the gel without any further preparation). The length of detected DNA fragments can be equalized with a suitable molecular weight standard (f.e. KBR311005). The separated DNA is colored by ethidium bromide or SybrGreen (5 µg/ml) for 5 min and visualised under UV-light (312 nm). The detected DNA pattern should be documented in suited way (f.e. digital photo).
- In dependence of the present genotype the samples show a defined pattern of **300 bp**- or respectively **230 bp**- amplifcats as well as a internal control fragment of **1100 bp**. (*Please consider: if the wildtype and mutation alleles are present, the internal control is expressed much weaker or could also lack completely !*).
- wildtype alleles are determined by the SSP-Primer MTHFR (**wt = blue** tubes) and the mutation alleles by the SSP-Primer MTHFR (**mut = orange** tubes): The fragment of **300 bp** length corresponds to analysed cDNA position **677**; the fragment of **230 bp** length corresponds to the analysed cDNA position **1298**.
- The **MTHFR 2 positive control DNA** has (for both point mutations) the genotype **wt/mut**.
- In any case the negative controls must be negative for any amplification product.
- The determination of the genotype present in the analysed sample is done according to the table for interpretation (showing the expectable DNA pattern for both MTHFR mutations C677T and A1298C).

## 11. Restrictions

The allelspecific PCR results for the positive control in DNA fragments of indicated length and for samples at least in the internal control fragment (1100 bp). 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 be proven/ corrected.



**Table for interpretation of MTHFR-677/1298- genotype**

MTHFR-SSP	1(wildtype)	2 (mutation)	genotype	allele frequency Caucasian [%] <sup>(*)</sup>
SSP-Mix	# 300 bp C677 * 230 bp A1298	# 300 bp 677T * 230 bp 1298C		
677	#		CC677	13,2
1298	*		AA1298	
677	#		CC677	30,7
1298	*	*	AC1298	
677	#		CC677	9,6
1298		*	CC1298	
677	#	#	CT677	19,3
1298	*		AA1298	
677	#	#	CT677	14,9
1298	*	*	AC1298	
677	#	#	CT677	>0,1
1298		*	CC1298	
677		#	TT677	10,5
1298	*		AA1298	
677		#	TT677	>5
1298	*	*	AC1298	
677		#	TT677	>0,1
1298		*	CC1298	

(\*) **Literatur:** Skibola CF et al., 1999, PNAS, Vol. 96 (no. 22): 12810-815.