



<b>1. Intended Use</b>	<b>Code: KT0002</b>
The kit "MutaGEL® HFE" allows the parallel detection of the three common point mutations C282Y, H63D and S65C in the haemochromatosis gene HFE. For in vitro diagnostic use only.	

<b>2. Introduction</b>
Haemochromatosis is a genetic disease associated with progressive iron overload in different organs. The disease is common among populations of northern European origin (prevalence about 0.3 – 0.5 %). HFE is a recently reported candidate gene for this condition. Three mutations have been identified in the HFE gene: a substitution of cysteine for tyrosine at amino acid 282 (C282Y, nucleotide G845A), a substitution of histidine for aspartate at amino acid 63 (H63D, nucleotide C187G) and a substitution of serine for cysteine at amino acid 65 (S65C, nucleotide A193T). Over 90% of haemochromatosis patients are homozygous for the C282Y mutation. The H63D as well as the S65C mutation increase the risk of haemochromatosis for C282Y heterozygotes (= compound heterozygote).

<b>3. Principle of the Test</b>
The kit „MutaGEL® HFE“ contains different sets of lyophilised primer for analysis of the three mutations in the HFE gene using the Amplification Refractory Mutation System (ARMS). The primer sets are used in separate ARMS-mixes for each mutation (in case of mutation C282Y two ready to use tubes are provided). The mixes contain both allele-specific primer pairs for the wildtype and the mutation allele, as well as two consensus primer (latter ones generate the internal control fragment). All resulting amplification products are subsequently identified by gelelectrophoretic methods. Besides the internal control at least one (in case of heterozygote both) allele-specific DNA-fragment is visible. The present genotype is analysed by interpretation of the specific DNA-fragment pattern in the gel.

<b>4. Material Supplied (for 24 determinations)</b>
<ul style="list-style-type: none"> <li>▪ SSP-Primer HFE (282 wt)            24 x tubes            aliquoted colored PCR tubes for wildtype-constellation of C282Y (green).</li> <li>▪ SSP-Primer HFE (282 mut)        24 x tubes            aliquoted colored PCR tubes for mutation-constellation of C282Y (blue)</li> <li>▪ SSP-Primer HFE (63)                24 x tubes            aliquoted colored PCR tubes for mutation H63D (yellow).</li> <li>▪ SSP-Primer HFE (65)                24 x tubes            aliquoted colored PCR tubes for mutation S65C (orange).</li> <li>▪ PCR master mix                        1 x 600 µl            ready to use PCR solution (inclusive buffer, dNTPs, cresolred).</li> <li>▪ positive control DNA 1,                1 x 40 µl            aqueous solution of cloned human DNA (genotype C282Y)</li> <li>▪ positive control DNA 2, (3)        2 x 20 µl            aqueous solution of cloned human DNA (genotype H63D respectively S65C)</li> <li>▪ Taq polymerase (5U/µl)                1 x 13 µl            PCR enzyme (hot start Taq-polymerase)</li> <li>▪ Water (aqua dest.)                    1 x 1 ml              PCR water</li> </ul>

<b>5. Materials Required but not Supplied</b>
<p>Reagents and Instruments:</p> <ul style="list-style-type: none"> <li>▪ DNA extraction kit (f. e. "BLOOD MINIPREP" kit; Code: KBR3005)</li> <li>▪ reagents for gel electrophoresis</li> <li>▪ thermal cycler</li> <li>▪ pipettes (0.5 - 1000 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>
The reagents are stable at 4°C for 30 d. For longer storage freeze at ≤ -20°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 "HFE positive control DNA" for more than two times. If necessary, make suitable aliquots. <i>Before use:</i> Spin tubes briefly before opening (contents may become dispersed during shipment).

<b>7. Warning and Precautions</b>
<ul style="list-style-type: none"> <li>▪ For in vitro 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.
2. Amplification with different primer sets specific for the HFE gene (wild type- and mutation-allels for all three point mutations).
3. analysis of genotype by gelelectrophoretic separation of the amplified DNA-products.

## 8. Sample Preparation

- Extract total genomic DNA f.e. from 200 µl using a commercial available DNA extraction kit according to the manufacturers manual.
- Start immediately with the amplification procedure or store the extracted DNA at  $\leq -20^{\circ}\text{C}$ .

## 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	reaction volume: 15 µl	master mix-volume
PCR master mix	4.5 µl	4.5 µl x (N + 0.5)
aqua (dest)	7.5 µl	7.5 µl x (N + 0.5)
Taq polymerase (5 U/µl)	0.12 µl	0.12 µl x (N + 0.5)

- add 12 µl of the prepared master mix to each PCR-tube (with lyophilysed SSP-HFE) use for each sample in parallel the four PCR-tubes for all three mutations (green/blue, yellow and orange)!
- samples: add 3 µl of the **extracted gDNA** (about 20 ng/µl) to each PCR tube (**green, blue, yellow, orange**).
- positive controls: add 3 µl of **HFE positive control DNA** (1 or 2 or 3: each to the corresponding PCR tube) for positive references.
- negative control: add 3 µl of aqua (dest.) to the PCR tubes for neagative reference.
- 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 (2% agarose or 10% polyacrylamid) with about 8 µl of each amplified material (products from **green/blue, yellow** and **orange** tubes side by side = four lanes for one DNA-sample). Due to the included loading buffer the PCR-solution can be added directly to the gel without any further preparation. Length of detected DNA fragments can be equalized with a suitable molecular weight standard. The separated DNA is coloured by ethidium bromide (5 µg/ml) for 5 min and visualised under UV-light (312 nm).
- In dependence of present genotype the samples show a defined pattern of allel-specific DNA fragments as well as an internal control fragment (*Please consider: if the mutation allels are present the internal control could be weaker or even lack completely !*). See following table for interpretation of obtainedresults:

allel	bp	C282	282Y	bp	H63D			bp	S65C		
control fragment	400	+	+	460	+	+	+	460	+	+	+
mutation	260		+	300		+	+	300		+	+
wild type	200	+		200	+	+		200	+	+	
genotype		C282	282Y		H63	H63/63D	63D		S65	S65/65C	65C

- Each separate **HFE positive control DNA** has the genotype **wt/mut**.
- In any case the negative controls must be negative for any amplification product.

## 11. Restrictions

The allel-specific PCR results for the positive control in DNA fragments of indicated length and for samples at least in the internal control fragment of 400 (respectively 460) 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.

