



1. Intended Use **Code: KE09007**

The kit **MutaGEL® ApoE** allows to detect the famous Apo E genotypes **e2**, **e3** and **e4**. Each genotype is characterised by its DNA sequence in amino acid **codons 112 and 158**, which leads to a different behaviour in incubation with a specific restriction enzyme. This method is called RFLP (restriction fragment length polymorphism) and has been used widely for detection of gene variations. The diagnostic ApoE-RFLP method was optimized under practical aspects for this kit to obtain a very reduced number of fragments, which should allow an easier interpretation of the results.

2. Introduction

Morbus Alzheimer is one of the most common causes of dementia (leading to loss of orientation and judgment capacity). The apolipoprotein E (ApoE) is involved in the development of this disease. The enzyme exists in three different isoforms and functions as regulator in lipid metabolism. Due to its role in lipid transport of injured neurones, ApoE causes plaques in the brain of Alzheimer patients. Only carriage of the phenotypes of ApoE4 (heterozygously and homozygously) is associated with the development of these plaques and a genetical analysis of the ApoE-genotype present can be used as prediction factor for individual Morbus Alzheimer risk. Additionally the isoforms functions in plasma are differentially associated to Type III hyperlipoproteinemia (e2) and LDL cholesterol (e4).

3. Principle of the Test

The kit **MutaGEL® ApoE** amplifies in 2 separate reactions two different regions of the ApoE gene (one with "**Primers ApoE 112**" includes codon 112, and the other with "**Primers ApoE 158**" includes codon 158). Digestion with restriction enzyme of both amplicons **unified in one tube** with the specific "**Enzyme ApoE**" leads to genotype-specific DNA fragments. Analysis of the restriction fragments obtained allows the correct identification of the sample's genotype. The amplification Master Mix contains chemical additives for a productive amplification in this DNA region with its 73% GC content.

4. Material Supplied (24 determinations)

▪ PCR Mix (ApoE 112)	1 x 550 µl (green)	ready to use PCR reagent (<i>hot start</i> Taq enzyme, MgCl ₂ , dNTP, buffer) with oligonucleotides specific for the human ApoE gene (codon 112).
▪ PCR Mix (ApoE158)	1 x 550 µl (lilac)	ready to use PCR reagent (<i>hot start</i> Taq enzyme, MgCl ₂ , dNTP, buffer) with oligonucleotides specific for the human ApoE gene (codon 158).
▪ Positive control DNA	1 x 30 µl (red)	buffered solution with DNA of the ApoE alleles e2 + e3 + e4.
▪ Restriction enzyme ApoE	1 x 50 µl (blue)	restriction enzyme mix.
▪ Buffer for restriction enzyme ApoE	1 x 260 µl (white)	buffer for restriction enzyme mix + BSA (10 x concentrated).

5. Materials Required but not Supplied

Reagents and Instruments:

- DNA extraction kit (f.e. BLOOD MINIPREP: KBR3005)
- H₂O (deionized)
- Thermal cycler and mineral oil (optional, for thermocycler without heated lid)
- Pipettes (0.5 - 1000 µl) and sterile pipette tips
- Sterile microtubes suitable for the thermal cycler in use
- Thermoblock and instruments for gel electrophoresis

6. Storage and Stability

Store at < -18°C. The reagents are stable in the unopened microtubes until the expiration date indicated (see print on the package). Do not thaw out the content of the "ApoE positive control DNA" for more than five times. If necessary, make suitable aliquots.
Before use: Spin tubes briefly before opening (contents may become dispersed during shipment).

7. Warning and Precautions

- For in vitro diagnostic use only.
- Test should be performed only by skilled persons considering GLP (Good Laboratory Practice) guidelines.
- It's recommended to store enzyme mixes on ice during pipetting – especially if room temperature is more than 25°C (f.e. during summer time).
- Don't use the kit after its expiration date.
- After usage, dispose all reagents and test components included in the kit in conventional garbage.
- 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.
- Wear separate coats and gloves in each working area and avoid aerosols.
- Use sterile filter tips for pipetting and use special PCR pipettes for aerosol free pipetting.
- Routinely decontaminate your pipettes and the laboratory benches.
- Copyright: the intellectual property for this method owns to Dr.M.Eßrich, Denzlingen/Freiburg (Germany). The primer used in this MutaGEL® ApoE testkit are covered by patent N° 198 55 469.9-44 owned by Immundiagnostik AG (Bensheim, Germany).

Procedure

The complete procedure is divided into four steps:

1. Sample preparation.
2. Amplification with primers appropriate for the ApoE gene codons 112 and 158 in two tubes parallelly.
3. Digestion of the unified products of the two amplifications with a restriction enzyme preparation.
4. Detection of the amplified and digested DNA by gel electrophoresis (size resolution).



8. Sample Preparation

- Extract total genomic DNA f.e. from 200 µl whole blood using a commercial available DNA extraction kit according to the manufacturers' manual.
- 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, positive controls and negative control the following Master Mix (multiply volumes necessary for each reaction with number N of reactions and add 10% more volume). Two amplifications in parallel are performed for **ApoE 112** respectively **158** for each sample.

PCR reagents	Reaction Volume: 25 µl	Master Mix Volume
PCR Mix (ApoE 112 resp. ApoE 158)	20 µl	20 µl x N + 10 %
<ul style="list-style-type: none"> For each reaction aliquot 20 µl of the PCR Mix into a sterile microtube suitable for the thermal cycler Samples: add 5 µl of the extracted DNA to the PCR Mix in the tube Positive controls: add 5 µl of the ApoE positive control DNA to the PCR Mix in the tube Negative control: add 5 µl of H₂O to the PCR Mix in the tube Transfer the microtubes into the thermal cycler (if necessary overlay the mix with 60 µl of mineral oil) Perform the following amplification protocol: 		
Initial Hold:	94°C for 15 min	
35 cycles:	94°C for 30 sec / 66°C for 30 sec / 72°C for 60 sec	
Final Hold:	72°C for 5 min, 4°C follow up	

10. Digestion of the Amplified DNA

Prepare for each sample, and the both positive controls the following Digestion Mix (multiply the volumes necessary for each reaction with the number N of reactions, and add 10% more volume). The total volume for each Digestion Mix is 25 µl.

Reagents for DIGESTION	Volume DIGESTION each reaction: 12,5 µl	Volume DIGESTION Master Mix
enzyme ApoE	1.8 µl	1.8 µl x N + 10 %
buffer for enzyme ApoE	9.2 µl	9.2 µl x N + 10 %
<ul style="list-style-type: none"> For each sample aliquot 11 µl of the Digestion Mix into tubes suitable for the incubator (a thermal cycler may be used for the incubation too). add 2 x 7 µl (this combines 10 µl of each "112" and "158" in the same mix) of the amplification products to the Digestion Mix. transfer the tubes to the thermoblock and incubate at 37°C for 3 hours (optional over night). 		

11. Detection of the Amplified/ Digested DNA and Interpretation of the Results

- Carry out gel electrophoresis in **2,5%** agarose (or polyacrylamide 20%) for at least **130 Vh** (f.e. 90 min at 90 volt) in 1x TBE-buffer: mix up to **15 µl** of each digestion mix with **4 µl** loading buffer (f.e. KAN01070) and load the gel. The length of the amplified 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 separated DNA is coloured by ethidium bromide (5 µg/ml) or SybrGreen for about 5 min and visualised under UV-light (312 nm).
- The PCR amplifications leads to a fragment of **191 bp** length with "primers ApoE 112" and **118 bp** length with "Primers ApoE 158". In any case the negative controls must be negative for any amplification product.
- Sequencing of the alleles has shown the following polymorphism for ApoE codons 112 and 158:

	codon 112	enzyme ApoE	codon 158	enzyme ApoE
ApoE2:	TGC – cysteine	cut (142+49 bp)	TGC – cysteine	no cut (118 bp)
ApoE3:	TGC – cysteine	cut (142+49 bp)	CGC – arginine	cut (93+25 bp)
ApoE4:	CGC – arginine	no cut (191 bp)	CGC – arginine	cut (93+25 bp)

6 combinations exist:

ApoE2/ApoE2	ApoE2/ApoE3	ApoE2/ApoE4	ApoE3/ApoE3	ApoE3/ApoE4	ApoE4/ApoE4
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These combinations lead to the following RFLP patterns (amplicon 112 treated with enzyme ApoE / amplicon 158 treated with enzyme ApoE), diagnostically conclusive fragments are underlined:

ApoE2/ApoE2	ApoE2/ApoE3	ApoE2/ApoE4	ApoE3/ApoE3	ApoE3/ApoE4	ApoE4/ApoE4
<u>142+49</u> / <u>118</u>	<u>142+49</u> / <u>118+93+25</u>	<u>191+142+49</u> / <u>118+93+25</u>	<u>142+49</u> / <u>93+25</u>	<u>191+142+49</u> / <u>93+25</u>	<u>191</u> / <u>93+25</u>

- Primers have been selected to additionally enable the detection of the rare genotype **apoE Leiden** (Arg112/ duplication 121-127/ Arg158).
- The presence of this allele leads to a 212 bp amplicon with "Primers ApoE 112":

Leiden/Leiden	Leiden/ApoE2	Leiden/ApoE3	Leiden/ApoE4
<u>212</u> / <u>93+25</u>	<u>212+142+49</u> / <u>118+93+25</u>	<u>212+142+49</u> / <u>93+25</u>	<u>212+191</u> / <u>93+25</u>

The positive control is an artificial mixture which contains all three alleles e2, e3 and e4.

12. Restrictions

The PCR results for all positive controls in DNA fragments of indicated length and for samples at least in the amplification product of indicated length. 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.

