MutaGEL® ApoB100

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1. Intended Use

Code: KE09023

MutaGEL® ApoB100 (Codon 3500) is a RFLP-coupled PCR test kit for the inspection of codon 3500 of the human apolipoprotein-B gene, with the purpose to detect DNA base G (normal) or A (mutant), causative for tanslated arginine or glutamine at this position in the ApoB100 protein.

2. Introduction

Familial Hypercholesterolemia (FH) is a frequent cause of coronary artery disease in human beings. The normal removal of cholesterine from blood is interrupted, when DNA mutations of its liver receptor r-LDL make it defective or, as was found later, if the cholesterine carrier ApoB100 has gene mutations at or around codon 3500. Cd. 3500 Arg-Glu (G-A) is a mutation of Central European abundance and on average 1 of 250 persons is heterozygous for the dominant trait. The pathogenicity of the defective protein varies with further social and unknown genetic mutations: At age 50 20% of female mutation carriers have severe coronary artery disease and 40% of males. FH is easier screened for mutations of ApoB100 than for mutations of the LDL receptor gene.

3. Principle of the Test

With the 24-sample kit's PCR mix a piece of DNA sequence around codon 3500 can be amplified and lateron incubated at elevated temperature with a bacterial restriction enzyme, which can recognize and cut the amplimere, when an A-mutation is present and not in the case of a normal G. The effect of the restriction experiment can be controlled by comparing the length of uncut and cut fragments to DNA of known size with a fluorescently coloured electrophoresis gel and UV light. Copyright: the intellectual property for this method owns to Dr.M.Eßrich, Denzlingen/Freiburg (Germany).

4. Material Supplied (for 24 determinations)

▪ PCR Mix ApoB100 (Cd. 3500)	1 x 140 µl (**green**)	Ready-to-use PCR reagent (*hot start* Taq enzyme, $MgCl_2$, dNTP, buffer and nucleotides specific for the human Cd.3500-ApoB100 region.
▪ Positive control DNA "Cd.3500"	1 x 35 µl (**red**)	Buffered solution with amplified heterozygous DNA of the ApoB100 gene.
▪ Buffer for "Restriction Enzyme Cd. 3500"	1 x 110 µl (transparent)	Buffer for the Cd.3500 restriction enzyme digestions
▪ Restriction Enzyme Cd.3500	1 x 30 µl (**blue**)	Restriction enzyme, specific for mutation Cd.3500-A

5. Materials Required but not Supplied

Reagents and Instruments:

- DNA extraction kit (e. g. MutaCLEAN DNA Blood: KG1033)
- H_2O (deionized) for negative control
- Pipettes (0.5 - 200 µl) and sterile pipette tips
- Sterile micro tubes suitable for the thermal cycler in use
- Thermal cycler (and optional mineral oil for thermocylcer without heated lid) and instruments for gel electrophoresis

6. Storage and Stability

Store at < -18°C. The reagents are stable in the unopened micro tubes until the expiration date indicated (see print on the package).

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 only be performed only by skilled persons considering GLP (Good Laboratory Practice) guidelines.
- 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. 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.

Procedure

The complete procedure is divided in four steps:

1. Sample preparation.
2. Amplification with primers specific for the Cd.3500 region of the ApoB gene.
3. Digestion of the amplified product with a restriction enzyme.
4. Detection of the amplified and digested DNA by gel electrophoresis.

8. Sample Preparation

- Extract total genomic DNA (e.g. from 200µl EDTA-blood) using a commercial available DNA extraction kit according to the manufacturer's 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 control, and negative control the following Master-Mix (multiply the volumes necessary for each reaction with the number **N** of reactions and add 10% more volume).

PCR reagents	Reaction Volume: 10 µl	Volume Master Mix
PCR Mix "Cd.3500-ApoB100"	5 µl	5 µl x N + 10 %

- For each reaction aliquot 5**µl** of the PCR Mix in a sterile microtube suitable for the thermal cycler
- Samples: add **5 µl** of the **extracted DNA** to the PCR Mix in the tube
- Positive control: add **5µl** of the Cd.3500 **positive control DNA** to the PCR mix in the tube
- Negative control: add **5µl** of **H_2O** 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:	95°C for 5 min
35 cycles:	95°C for 30 sec / **58°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 (65°C)

Prepare a 65°C incubation facility. Prepare for each sample, and the positive control the following Digestion Mix (multiply the buffer volume necessary for each reaction with the number **N** of reactions, and add 10% more volume). The total volume is **10 µl**.

Reagents for DIGESTION	Reaction volume DIGESTION: 10 µl	Volume Digestion-Master Mix
Buffer for Cd.3500 restriction	**4 µl**	4 µl x N + 10%
Restriction Enzym Cd.3500	**1 µl**	1 µl x N

- Aliquot **5 µl** of the digestion mix into tubes suitable for the incubator (a thermal cycler may be used for the incubation too).
- Add **5 µl** of the amplification product to the digestion mix.
- Transfer the tubes to the incubator and incubate at **65°C** for **2-3** hours (or over night).

11. Detection of the Amplified and Digested DNA

- Carry out gel electrophoresis in **3,5%** agarose (or polyacrylamide 20%) for at least **150 Vh** (e. g. 90 min at 100Volt) in 1x TBE-buffer: mix **10 µl** of each digestion mix with **3 µl** loading buffer and load the gel. The length of the amplified DNA fragments can be determined with a suitable molecular weight standard (e. g. KBR311005). The separated DNA is coloured with ethidium bromide or SybrGreen (5µg/ ml) for **5min** and visualised under UV-light (312 nm).
- The PCR amplification produces for positive control and all samples a DNA fragment of **170 bp** (= amplificate before digestion).
- As a **control for the digestion efficiency** the amplificate is shortened by the restriction in each case (normal or mutated) by 30bp **to 140 bp**.
- The **mutated A-sequence** is restricted further by **30 bp to 110 bp,** the **normal G-sequence** is **not.**
- Such: The presence of the **protective gene variant (pro)** is identified by fragments of **140 bp**. The fragments of Cd.3500 mutations (**pat** = **pathogenic gene variant**) are identified by **110 bp** bands.
- In consequence, the following restriction enzyme patterns are obtained in relation to the genotype present:

GENOTYPE: CTR	fragment length (bp):
pro/pro	**140** bp
pro/ pat	**140** bp + **110** bp
pat/ pat	**110** bp

- The **Cd.3500-positive control DNA is heterozygous for the Cd. 3500-ApoB100 sequence**.
- In any case the negative controls must be negative for any amplification product of indicated length.

12. Restrictions

The PCR is resulting for all positive controls in DNA fragments of the indicated lengths and for samples at least in the amplification product of the 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 examined.

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