MutaGEL® OxStress II

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1. Intended Use

Code: KE09011

The PCR kit MutaGEL Oxstress II has been developed for the diagnosis of the variability in the human enzyme genes of superoxide dismutase 2 (alleles valine16alanine) and catalase (promotor alleles C-262T) responsible for protectivity against metabolic "oxidative stress" tested on isolated DNA.

2. Introduction

Oxidative stress is defined as a pathological disequilibrium between the body's molecules (e.g from food oxidation), which have free electrons and the reducing agents who normally react with them. The anions of superoxide and peroxide are mainly concerned and their surplus acts as aging cause and may especially generate diabetes or cancer.

The protein superoxide dimutase 2 (manganese-dependent **SOD2**) catalyses radicals of superoxide (from many different metabolic sources) to peroxide; the protein catalase (**CAT** together with glutathion S-transferase) transforms peroxide to water and oxygen. The higher or lower serum concentration of reactive molecules - NO, ox-LDL, H2O2, and many others - as result of ecological agents (e.g. poor or well living conditions) is therefore "framed" by the heritated variation of their reducing enzymes. Firstly our test diagnoses the polymorphism Val16Ala of SOD2, which changes the enzymes recognition by mitochondria, in whom it acts on ROS (reactive oxygen species): diseased people with certain cancers have more often valine alleles of SOD2 than healthy people (the relative abundance of Val/Val homozygotes in lung cancer patients is 1,7x the number in healthy persons; in bladder cancer the deviation is still higher). Because valine alleles are 1/3 less active than alanine alleles, its reduced decomposition of oxidative stress molecules can generate cancer. The results are complicated by the fact, that the alanine allele of SOD2 may also act as pathogenic factor in breast cancer (in unfavourable circumstances like with women with high body mass index and with many menstruation years), and this may point on a neoplastic potential of ROS metabolites produced by the more active enzyme.

Secondly we diagnose the promotor polymorphism C-262T of human catalase - the enzyme acts downstream of SOD2 (s.a.) -, which changes the protein's concentration in erythrocytes and blood: each T allele of a person increases the activity. This is generating a protective effect of its counterpart with a C against breast or pancreas cancer and here also a mirror effect exists with a pathogenic effect for CC homozygotes in the pathogenesis of diabethic neuropathy.Thus the enzymes allelic variants in oxidative stress generate pathogenic or protective effects depending on the examined disease. Further influence on the reduction of oxidative stress comes from the null mutants of glutathion S-transferase M1 and T1 (see PCR kit **Mutagel GST M1/T1**) and sequence changes in the genes for endothelial NO synthase + NAD(P)H oxidase (see PCR kit **Mutagel Oxstress I**), (M.E.).

3. Principle of the Test

With “MutaGEL® Oxtress II” two DNA regions specific for the critical gene parts of superoxide dismutase 2 (SOD2) and catalase (CAT) are amplified in parallel reactions. The amplification products are then treated with their respective restriction enzymes followed by a subsequent resolution of the produced DNA fragments by gel electrophoresis. The resulting genotype of the sample can be recognised from the length of the resulting restriction fragments for both polymorphisms (RFLP).

4. Material Supplied (24 determinations)

▪ PCR Mix SOD2	1 x	550 µl (**green**)	PCR buffer, hotstart Taq polymerase, dNTP`s, oligonucleotide primers specific for the region of the SOD mutation in the codon of amino acid 16.
▪ PCR Mix Catalase	1 x	550 µl (**lilac**)	PCR buffer, hotstart Taq polymerase, dNTP`s, oligonucleotide primers specific for the promotor region of the catalase gene, which includes base 262
▪ Positive control DNA	1 x	35 µl (**red**)	buffered solution with DNA of T and C alleles of SOD2 as well as with DNA of Cand T alleles of catalase.
▪ Restriction enzyme 1 (SOD2)	1 x	20 µl (**blue**)	restriction enzyme **SOD2 mutation**
▪ Restriction enzyme 2 (CAT)	1 x	10 µl (**yellow**)	restriction enzyme **CAT** mutation
▪ Restriction enzyme buffer 1	1 x	550 µl (transparent)	buffer for restriction enzyme SOD2 amplimer
▪ Restricition enzyme buffer 2	1 x	550 µl (**brown**)	buffer for restriction enzyme CAT amplimer
▪ Negative control	1 x	50 µl (white)	PCR water (deionized)

5. Materials Required but not Supplied

Reagents and Instruments:

- DNA extraction kit (e.g. BLOOD MINIPREP: KBR3005)
- Thermal cycler and Pipettes (0.5 - 200 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 “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 (e.g. 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 special PCR pipettes + sterile filter tips for aerosol free pipetting).
- Routinely decontaminate your pipettes and the laboratory bench.
- Method and scientific text: Dr.Michael Eßrich, Denzlingen/ Freiburg (Germany).

Procedure

The complete procedure is divided into four steps:

1. Sample preparation.
2. Amplification with primers appropriate for the SOD2 and CAT genes (in two tubes parallel).
3. Digestion of the amplification products of the two amplifications with a restriction enzyme preparation (in two tubes parallel).
4. Size resolution and detection of the amplified and digested DNA by gel electrophoresis.

8. Sample Preparation

- Extract total genomic DNA (for example from 200 µl of whole blood) using a commercially available DNA isolation kit.
- 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 about 10% more volume).
- The total PCR reaction volume (inclusive sample DNA) is 25 µl.
- Two amplifications in parallel are performed for SOD2 resp. catalase for each probe in separate tubes.

PCR Reagents	Reaction Volume: 25 µl	Master Mix Volume
PCR Mix (SOD2) **resp.** (CAT)	(2x) 20 µl	(2x) 20µl x N + 10 %

- Aliquot 20 µl of the respective PCR Mix into a sterile micro vessel suitable for the thermal cycler
- For samples: add 5 µl of the **extracted DNA** to the PCR Mix
- For positive controls: add 5 µl of the **SOD2 resp. CAT positive control DNA** to its respective PCR Mix
- For negative control: add 5 µl of **H_2O** to the respective Master Mix
- 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 5 min
37 cycles:	**94°C** for 30 sec / **60°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 amplified sample from SOD2 **resp.** CAT and the positive controls the following Digestion Mix (multiply the volumes necessary for each reaction with the number **N** of reactions, and add 10% more volume) **in parallel** for both amplificates **in separate** tubes:

Reagents for DIGESTION	Total volume for each DIGESTION: 25 µl	Volume in the Digestion-Mix
Restriction enzymes for SOD2 **resp**. CAT	0.6 µl respect. 0.3 µl	0.6 µl respect. 0.3 µl x N + 10 %
Buffer for restriction enzymes	19.4 µl respect. 19.7 µl	19.4 µl respect. 19.7 µl x N + 10 %

- For each sample aliquot **20 µl** of the Digestion Mix into tubes suitable for the incubator (a thermal cycler may be used for the incubation too).
- Add **10 µl** of SOD2 **and in parallel** CAT amplification product to the respective Digestion Mix.
- Transfer the tubes to the thermoblock and incubate **SOD2-** reactions at **60°C** for **3 hours** (or optional over night) and **CAT**-reactions at **37°C** for **3 hours** (or optional over night).

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

- Carry out a gel electrophoresis in **2,5 %** agarose (or polyacrylamide 20 %) for about **130 Vh** (e.g. 80 min at 100 volts) in 1x TBE-buffer: add about **4 µl** loading buffer (e.g. KAN01070) to each Digestion Mix (for each sample load Digestion Mix from SOD2 and CAT in separate lanes) and load about **15 µl** of each the gel: The length of the amplified/ restricted DNA fragments can be equalized with a suitable molecular weight standard (e.g. KBR311005). The separated DNA is coloured by ethidium bromide (5 µg/ml) or SybrGreen for 5 min and visualised under UV-light (312 nm).
- The PCR amplifications produce a fragment of **199 bp** length with PCR primers for SOD2 and **152 bp** length with PCR primers for CAT. In any case the negative controls must be negative for each amplification product.
- Treatment with the restriction enzymes generate the following fragments allowing interpretation of a genotype of a probe consisting of protective and/or pathogenic alleles:

GENOTYPE: SOD2	corresponding fragment length (bp)	GENOTYPE: CAT	corresponding fragment length (bp)
C / C	199	**T / T**	152
C / T	199 / 117 (+ 82)	**T / C**	152 / 107 (+ 45)
T / T	117 (+ 82)	**C / T**	107 (+ 45)

The positive control contains the heterozygous genotype for both loci.

12. Restrictions

This PCR method results for positive controls and for samples in DNA fragments of the indicated length of the amplified or restricted products. 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 PCR conditions chosen have to be inspected.

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