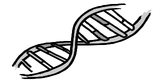
MutaGEL® OxStress II



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1. Intended Use — Code: KE09011

The kit "MutaGEL® Oxtress II" allows to detect the polymorphisms in the genes for the human protection enzymes against "oxidative stress": superoxide dismutase 2 (mangan-dependent), valin16alanin: T>C) and catalase (CAT promoter: C-262T). Starting material are human genomic DNA samples.

2. Introduction

Oxidative Stress is defined as pathological disequilibrium between the bodys molecules containing free electrons (e.g from food oxidation) 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 Superoxiddimutase 2 (**SOD2**) catalyses radicals of superoxide (from many different metabolic sources) to peroxide; the protein Catalase (**CAT** and also 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 reducing enzymes heritated variation. Primarily 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 relativ 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 proteins concentration in erythrocytes and blood: each T allele of a person increases the activity. This generates 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 and ND(P)H Oxidase (see PCR kit **Mutagel Oxstress I**).

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 a mix of restriction enzymes followed by a subsequent resolution of the produced DNA fragments by gelelectrophoresis. The resulting genotype of the sample can be recognised for both polymorphisms from the length of the resulting restriction fragments (RFLP method).

4. Material Supplied (24 determinations)

Component	Quantity	Colour	Description
PCR Mix (SOD2)	1 x 550 µl	(**green**)	PCR buffer, TAQ enzyme, dNTP`s, oligonucleotide primers specific for the region of the **eNOS** promotor which includes base -786
PCR Mix (Catalase)	1 x 550 µl	(**lilac**)	PCR buffer, TAQ enzyme, dNTP`s, oligonucleotide primers specific for the region of **NADOx**, which includes base 242
Positive control DNA	1 x 35 µl	(**red**)	buffered solution with DNA of T + C alleles of -786 eNOS as well as with DNA of C242T NADH Oxidase gene
Restriction enzyme 1 (SOD2)	1 x 20 µl	(**blue**)	restriction enzyme **eNOS** polymorphism
Restriction enzyme 2 (CAT)	1 x 10 µl	(**yellow**)	restriction enzyme **NADOx** polymorphism
Enzyme buffer 1	1 x 550 µl	(transparent)	buffer for restriction enzyme **eNOS** amplimer
Enzyme buffer 2	1 x 550 µl	(**brown**)	buffer for restriction enzyme **NADOx** amplimer

5. Materials Required but not Supplied

Reagents and Instruments:
- DNA extraction kit (e.g. BLOOD MINIPREP: KBR3005)
- Thermal cycler and 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 "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 sterile filter tips for pipetting and use special PCR pipettes for aerosol free pipetting.
- Routinely decontaminate your pipettes and the laboratory bench.
- Copyright: the intellectual property for this method owns to Dr.M.Eßrich, Denzlingen/Freiburg (Germany).

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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 / 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

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 "**Primers SOD2**" and **152 bp** length with "**Primers 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 present genotype consisting of protective (**pro**) respectively pathogen (**pat**) alleles:

SOD2: **C/C** = **199** bp **T/C** = **199** + **117** bp **T/ T** = **117** bp

Catalase: **T/T** = **152** bp **T/C** = **123** + **107** bp **C/C** = **107** bp

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 possesses for both loci the heterozygous genotype.

12. Restrictions

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