MutaGEL® OxStress I

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1. Intended Use

Code: KE09008

The kit "MutaGEL® Oxtress I" allows to detect the genotypes T-786C (promotor region) of the endothelial NOSynthase gene (NOS3) and C242T (His72Tyr) of the NAD(P)H Oxidase subunit CYBA in human genomic DNA probes.

2. Introduction

Within the metabolic status named "oxidative stress" the production of molecules with free reactive electrones from e.g. glycolysis is not equalled by its normal potent lipidic or enzymatic electron acceptors. This causes diabetes, cancer and aging.

The levels of several indicative molecules vary under the influence of some common DNA polymorphisms of enzymes, which metabolize the agonists of the radicalic or nonradicalic oxidants - mainly superoxide and hydroxide anion - such as T-786C of the NOSynthase promotor, C242T (His72Tyr) in a subunit of the NAD(P)H oxidase macromolecule. Additionally, the common complete deletion of glutathion-S-transferase genes M1 and T1 (see "MutaGEL® GST-M1/T1") as well as sequence variation of superoxide-dismutase SOD2 and catalase (see "MutaGEL® Oxstress II") influence the radicalic situation in the body.

3. Principle of the Test

With "MutaGEL® Oxtress I" two DNA regions specific for the critical gene parts of eNOS and NAD(P)H Oxidase are amplified in parallel reactions. The amplification products are then treated with a mix of restriction enzymes, which are sensitive for the "negative" sequence variant of each gene - in parallel as well - such that in the following gel the genotype of the probe can be recognised for both polymorphisms from the length of the resulting restriction fragments (RFLP method).

4. Material Supplied (24 determinations)

▪	PCR Mix eNOS -786	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 NAD(P)H Oxidase 242	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 (eNOS)	1 x 10 µl (**blue**)	restriction enzyme **eNOS** polymorphism
▪	Restriction enzyme 2 (NADOx)	1 x 30 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
- 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 benche.
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Procedure

The complete procedure is divided into four steps:

1. Sample preparation.
2. Amplification with primers appropriate for the eNOS and NADOx genes (in two tubes parallely).
3. Digestion of the amplification products of the two amplifications with a restriction enzyme preparation (in two tubes parallely).
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 eNOS -786 resp. NADOx 242 for each probe in separate tubes.

PCR Reagents	Reaction Volume: 25 µl	Master Mix Volume
PCR Mix (-786 eNOS **resp.** 242 nadOx	(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 **eNOS resp. NADOx 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
35 cycles:	94°C for 30 sec / 55°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 eNOS **resp.** NADOx 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 **eNOS resp. NADOx**	**0.3 µl** respect. **1 µl**	**0.3 µl** respect. **1µl** x N + 10 %
Buffer for restriction enzymes	**19.7 µl** respect. **19 µl**	**19.7 µl** respect. **19 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 eNOS- **and in parallel** NADOX- amplification product to the respective Digestion Mix.
- Transfer the tubes to the thermoblock and incubate 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 **100 Vh** (e.g. 70 min at 90 volts) in 1x TBE-buffer: add about **4 µl** loading buffer (f.e. KAN01070) to each Digestion Mix (for each sample load Digestion Mix from eNOS -786 and NADOx 242 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 (f.e. 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 **151 bp** length with "**Primers -786eNOS**" and **123 bp** length with "**Primers NADOx**". 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**) allels:

eNOS -786: **T/T** = **151** bp **C/T** = **151** + 116 bp **C/C** = 116 bp

NADOx 242: **C/C** = **123** bp **C/T** = **123** + 95 bp **T/T** = 95 bp

GENOTYPE: eNOS -786	corresponding fragment length (bp)	GENOTYPE: NADOx 242	corresponding fragment length (bp)
pro: **T** / pro: **T**	151	pro: **C** / pro: **C**	123
pro: **T** / pat: **C**	151 / 116	pro: **T** / pat: **C**	123 / 95
pat: **C** / pat: **C**	116	pat: **T** / pat: **C**	95

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.

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