



## 1. Intended Use

Code: KE09005

The MutaGEL<sup>®</sup> r-Estrogen test kit allows the detection of the codon changing DNA variability ("PX"-haplotype) in intron 1 of the human estrogen receptor gene (ESR1) which encodes for the corresponding protein involved in the steroid receptor signal cascade.

## 2. Introduction

Estrogen receptor is a molecule for the functional transmission of activity generated from the hormonal ligand estrogen. It's thereby involved in reproduction metabolism as well as in growth processes. Furthermore, several clinical studies showed significant associations between variabilities of DNA sequence of estrogen receptor and the onset of osteoporosis. Especially the risk for fractures of the vertebral bones was altered in dependence of present polymorphisms (also in different populations). The investigated haplotype is defined by two point mutations causative protective or respectively pathogenic properties. The major alleles "PX" (in big letters) encode for the **protective** genotype "PX/PX", whereas the more rare alleles "px" (in small letters) define the **pathogenic** genotype "px/px".

## 3. Principle of the Test

The kit MutaGEL<sup>®</sup> r-Estrogen contains a set of primer for amplification of the specific sequence within the human estrogen receptor gene ESR1. Amplificates of varying "PX"-haplotypes are characterized by subsequent specific restriction enzyme digestion. The **rare (pathogen)** DNA-variant possesses restriction sites for specific endonucleases, whereas the **major (protective)** DNA-variant does not. The amplified products obtained from the pathogen DNA-variant will therefore be cut into smaller fragments, whereas the major protective DNA-variant will not be cut. The identification of the present genotype is done through subsequent analysis of present DNA-fragments by gel electrophoretic methods (Dr. Essrich, Biologisches Labor, Denzlingen).

## 4. Material Supplied (for 24 Determinations)

▪ PCR Master Mix	1 x 480 µl (yellow)	- ready to use PCR reagent (buffer, <i>hot start</i> Taq enzyme, Mg <sup>2+</sup> , dNTP)
▪ primer ESR	1 x 80 µl (green)	- solution with oligonucleotides specific for the human estrogen receptor gene.
▪ positive control DNA	1 x 50 µl (red)	- buffered solution with (amplified) DNA of the estrogen receptor gene
▪ enzyme ESR	1 x 20 µl (blue)	- restriction enzyme mix
▪ buffer for enzyme ESR	1 x 350 µl (white)	- buffer for restriction enzyme mix

## 5. Materials Required but not Supplied

Reagents and Instruments:

- DNA extraction kit (f.e. BLOOD MINIPREP: KBR3005)
- H<sub>2</sub>O (deionized)
- Mineral oil (optional, for thermocycler without heated lid)
- thermal cycler
- pipettes (0.5 - 200 µl) and sterile pipette tips
- sterile micro tubes 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 micro tubes until the expiration date indicated (see print on the package). Do not thaw out the content of the "ESR positive control DNA" for more than two 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.
- 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.
- Use sterile filter tips for pipetting and use special PCR pipettes for aerosol free pipetting.
- Routinely decontaminate your pipettes and the laboratory benches.
- Avoid aerosols.

## Procedure

The complete procedure is divided in four steps:

1. Sample preparation.
2. Amplification with primers specific for ESR gene.
3. Digestion of the amplified product with the restriction enzyme mix.
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 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: 25 µl	Master Mix Volume
PCR Mix (ESR)	20 µl	20 µl x N + 10 %

- For each reaction aliquot **17 µl** of the PCR Mix in a sterile microtube suitable for the thermal cyclor
- Primer: add **3 µl** of primer solution to each PCR microtube and mix by pipetting up an down several times
- Samples: add **5 µl** of the **extracted DNA** to the Master-Mix
- Positive control: add **5 µl** of the **ER positive control DNA** to the master mix
- Negative control: add **5 µl** of **H<sub>2</sub>O** to the master mix
- Transfer the micro tubes into the thermal cyclor (if necessary overlay the Mix with 60 µl of mineral oil)
- Perform the following amplification protocol:

<b>Initial Hold:</b>	95°C for 5 min
<b>37 cycles:</b>	95°C for 30 sec / 58°C for 30 sec / 72°C for 60 sec
<b>Final Hold:</b>	72°C for 5 min, 4°C follow up

## 10. Digestion of the Amplified DNA

Prepare for each sample, and the positive control 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 ESR	0,7 µl	0,7 µl x N + 10 %
buffer for enzyme ESR	11,8 µl	11,8 µl x N + 10 %

- aliquot **12,5 µl** of the Digestion Mix into tubes suitable for the incubator (a thermal cyclor may be used for the incubation too).
- add **12,5 µl** of the amplification product to the Digestion Mix.
- transfer the tubes to the incubator.
- incubate at **37°C for minimal 3 hours** (optional over night).

## 11. Detection of the Amplified and Digested DNA

- Carry out gel electrophoresis in **2,5%** agarose (or polyacrylamide 20%) for at least **110 Vh** (f.e. 70 min at 90 volt) in 1xTBE-buffer: mix about **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 PCR amplification leads for positive control and all samples to a DNA-fragment of **213 bp** (= amplicate before digestion).
- Two variable loci **N1** and **N2** (closly together) of the estrogen receptor gene are analysed. The presence of the major **protective gene variants** (**pro** = "**P**" resp. "**X**" – big letters) is identified by lack of corresponding restriction sites in the gene for estrogen receptor. Due to this, the amplicates generated from these alleles are **not cut** by the restriction enzymes. In contrast, the amplicates generated from the rare **pathogen gene variants** (**pat** = "**p**" resp. "**x**" – small letters) are **cut**, resulting in smaller DNA-fragments .
- In consequence, the following restriction enzyme patterns are obtained in relation to the present genotype:

GENOTYPE: ESR	Fragment Length (bp):
N1 pro / N2 pro = "PX"	213
N1 pro / N2 pat = "Px"	178 / 31
N1 pat / N2 pat = "px"	131 / 48 / 35
(N1 pat / N2 pro = "pX" <b>not found</b> )	131 / 82

- INTERPRETATION**  
The result (individual genotype) is composed by **both** alleles). The relative risk of the patient increases rises with increasing quantity (1-6) of pathogen variants in the patient fragment combination (PFC). The relative risk is indicated far as known.
- PFC: 213/ 213 = Genotyp: PX / PX (relative risk: **1,0**)
  - PFC: 213/ 178 = Genotyp: PX / Px
  - PFC: 213/ 131 = Genotyp: PX / px (relative risk: **1,9**)
  - PFC: 178/ 178 = Genotyp: Px / Px
  - PFC: 178/ 131 = Genotyp: Px / px
  - PFC: 131/ 131 = Genotyp: px / px (relative risk: **3,9**)
- The **ESR-positive control DNA** is an artificial mixture leading (after restriction digestion) to all diagnostic relevant DNA fragments (213, 178 and 131 bp) for both polymorphisms in estrogen receptor gene (**P + X + p + x**). It is a non natural existing but a mixture of genotypes".
  - In any case the negative controls must be negative for any amplification product of indicated length.

## 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.

