



1. Intended Use

Code: KE09003

The **MutaGEL[®] r-Calcitonin** test kit allows the detection of the codon changing DNA variability (T→C mutation) in the gene of calcitonin receptor (CTR) which encodes for the corresponding protein involved in bone metabolism.

2. Introduction

Calcitonin receptor is a membrane protein produced by osteoclasts and it functions as transmitter of the peptide hormon calcitonin leading to decreased bone resorption processes. Sequence analysis detected an frequently amino acid changing variability (Leu → Pro) in the receptor gene. This polymorphism alters the bone density and fracture frequency if healthy persons are compared with osteoporotic patients or even between populations with different skeletal measurement. It could be shown that the polymorphism changes a phosphorylation region of the 4th intracellular domain. By this, the in Caucasian population more frequent allele "T" (Leucin) protects from the described pathogen effect (due to the in Caucasian population more rare allele "C", Prolin). The "T" allele can be detected by specific restriction digest due to creation of a new restriction site in the gene.

3. Principle of the Test

The kit **MutaGEL[®] r-Calcitonin** contains a set of primer for amplification of the specific sequence within the human calcitonin receptor gene **CTR**. Amplificates of varying genotypes (T → C polymorphism) are characterized by subsequent specific restriction enzyme digestion. The variant (T, protective) possesses a restriction site for the endonuclease whereas the other variant (C, pathogen) does not. The amplified products obtained from the protective DNA-variant will therefore be cut into two fragments, whereas the pathogen DNA-variant will not be cut. The identification of the present genotype is done by analysis of present DNA-fragments by gel electrophoretic methods (Dr. Essrich, Biologisches Labor, Denzlingen).

4. Material Supplied (for 24 Determinations)

▪ PCR Mix (CTR)	1 x 550 µl (green)	ready to use PCR reagent (<i>hot start</i> Taq enzyme, MgCl ₂ , dNTP, buffer) with oligo-nucleotides specific for the human calcitonin receptor gene.
▪ positive control DNA	1 x 30 µl (red)	buffered solution with (amplified) DNA of the calcitonin receptor gene CTR.
▪ enzyme CTR	1 x 22 µl (blue)	restriction enzyme.
▪ buffer for enzyme CTR	1 x 320 µl (white)	buffer for restriction enzyme mix.

5. Materials Required but not Supplied

Reagents:

- DNA extraction kit (f.e. BLOOD MINIPREP: KBR3005)
- H₂O (deionized)
- Mineral oil (optional, for thermocycler without heated lid)

Instruments:

- thermal cycler
- pipettes (0.5 - 1000 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 "CTR 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 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.
- 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 CTR gene.
3. Digestion of the amplified product with a restriction enzyme.
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 (CTR)	20 µl	20 µl x N + 10 %

- For each reaction aliquot **20 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 **5 µl** of the **CTR positive control DNA** to the PCR mix in the tube
- Negative control: add **5 µl** of **H₂O** 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:	94°C for 15 min
35 cycles:	94°C for 30 sec / 58°C for 30 sec / 72°C for 90 sec
Final Hold:	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 CTR	0.8 µl	0.8 µl x N + 10 %
buffer for enzyme CTR	11.7 µl	11.7 µl x N + 10 %

- aliquot **12,5 µl** of the Digestion Mix into tubes suitable for the incubator (a thermal cycler may be used for the incubation too).
- add **12,5 µl** of the amplification product to the Digestion Mix.
- transfer the tubes to the thermoblock.
- incubate at **37°C for 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 **228 bp** (= amplicate before digestion).
- The presence of the **protective gene variant (pro = T)** is identified by presence of a restriction site in the gene for calcitonin receptor. Due to this, the amplicate generated from this allele is **cut once** by the restriction enzyme resulting in two smaller DNA-fragments. In contrast, the amplicate generated from the **pathogen gene variant (pat = C)** is **not cut**.
- In consequence, the following restriction enzyme patterns are obtained in relation to the present genotype:

GENOTYPE: CTR	fragment length (bp):
pat: C / pat: C	228
pat: C / pro: T	228 / 120 / 108
pro: T / pro: T	120 / 108

- The **CTR positive control DNA** possesses for the polymorphism in calcitonin receptor gene the genotype **pat/ pro (C/ T = heterozygous)**.
- 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 be proven/ corrected.

