



1. Intended Use

Code: KE09012

The **MutaGEL[®] Collagen** test kit allows the detection of the regulatory variant (G→T mutation, **Sp1**-polymorphism) in intron 1 of the type1 alpha1 collagen gene (COL1A1) encoding for the alpha 1 (I) protein chain of type I collagen, the major protein of the bone.

2. Introduction

Type 1 α 1- Collagen is the major protein of bone and its structure is essential for the development of osteoporosis. In several clinical studies an influence of the naturally variation of the DNA sequence from collagenase protein was shown. The pathogen variant results in lower mineral density of bone - especially for femoral neck and lumbar spine. Therefore the osteoporotic fracture risk increases with age in patients carrying this gene variant. The investigated COL1A1- polymorphism is biallelic and changes a regulatory binding site (for the transcription factor Sp1). The more rare allele (pathogen) transfers the disease factor independent and also additional to the variability of other genetic factors involved in alteration of whole osteoporotic risk (f. e. mutations in receptors for calcitonin, vitamin D3 or estrogen). The more frequent allele has protective properties.

3. Principle of the Test

The kit **MutaGEL[®] Collagen (AS)** is an amplification refractory mutation system (ARMS) containing two sets of primers for both allele-specific sequences within the human collagen gene COL1A1. The allele specific primers can be used directly for PCR with extracted DNA and generate amplicates only in case of presence of one from both sequence possibilities (normal or pathogen). The resulting amplification products are subsequently identified with gelelectrophoretic methods. If there is no specific allele-product detectable, the correct PCR procedure is proved by an internal control. The present genotype of unknown samples is interpreted by detection of corresponding DNA-amplicates for normal- and pathogen-constellation in two separate lanes of the gel (method by Dr. Essrich, Biologisches Labor, Denzlingen).

4. Material Supplied (for 24 Determinations)

▪ PCR Mix 1 (normal)	1 x 550 μ l (green)	ready to use PCR reagent (<i>hot start</i> Taq enzyme, MgCl ₂ , dNTP, buffer) with oligonucleotides specific for the normal human collagen type 1 alpha 1 gene.
▪ PCR Mix 2 (pathogen)	1 x 550 μ l (violett)	ready to use PCR reagent (<i>hot start</i> Taq enzyme, MgCl ₂ , dNTP, buffer) with oligonucleotides specific for the pathogen human collagen type 1 alpha 1 gene.
▪ positive control DNA	1 x 30 μ l (red)	buffered solution with (amplified) DNA of the heterozygous COL1A1 gene.

5. Materials Required but not Supplied

Reagents:

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

Instruments:

- thermal cycler
- pipettes (0.5 - 200 μ l) 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 $\leq -18^{\circ}\text{C}$. 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 "COL1A1 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 three steps:

1. Sample preparation.
2. Amplification with two sets of primers allele-specific for the COL1A1 gene.
3. Digestion of the amplified product and subsequent analysis of genotype.



8. Sample Preparation

- Extract total genomic DNA (e. g. 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).
- For each sample are prepared **two parallel** reactions in **separate** PCR tubes.

PCR reagents	Reaction Volume: 25 µl	Master Mix Volume
PCR Mix 1 respectively PCR Mix 2	(2 x) 20 µl	(2x) 20 µl x N + 10 %

- aliquot **20 µl** from each PCR Mix 1 and Mix 2 in separate sterile micro tube suitable for the thermal cycler.
- Samples: add **5 µl** of the **extracted DNA** to each PCR Mix in the tubes.
- Positive control: add **5 µl** of the **COL1A1 positive control DNA** to each PCR Mix in the tubes.
- Negative control: add **5 µl** of **H₂O** to each PCR Mix in the tubes.
- Transfer the micro tubes 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 90 sec
Final Hold:	74°C for 5 min, 4°C follow up

11. Analysis of Genotype and Interpretation of Results

- Carry out a gel electrophoresis in **2,5 %** agarose (or polyacrylamide 20 %) for about **130 Vh** (f. e. 90 min at 90 volts): mix about **15 µl** of each digestion mix with **4 µl** loading buffer (e.g. KAN01070) and load the gel. The length of the amplified DNA fragments can be equalized with a suitable molecular weight standard (e.g. 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 both alleles in positive control (not negative control) and all samples to clinical relevant DNA-fragments of **270 bp**.
- Additionally an **internal control** fragment for PCR performance is detectable at **140 bp**. (*Please consider: if the diagnostic relevant fragment of 270 bp is present, the internal control can be weaker or even absent*).
- The presence of protective gene variant (**pro: S**) is indicated by detection of the DNA-fragment (270 bp) in **Mix 1**, whereas the presence of **pathogene gene variant (pat: s)** is indicated by detection of the DNA-fragment (270 bp) in **Mix 2**. Therefore the following pattern are possible:

GENOTYP: COL1A1 (Intron 1)	fragment length (bp):		
pro: S / pro: S	(internal control 140 bp +)	270 bp (Mix 1-lane)	+ no DNA band (Mix 2-lane)
pro: S / pat: s	(internal control 140 bp +)	270 bp (Mix 1-lane)	+ 270 bp (Mix 2-lane)
pat: s / pat: s	(internal control 140 bp +)	no DNA band (Mix 1-lane)	+ 270 bp (Mix 2-lane)

- The **positive control DNA** possesses for the analysed gene the heterozygous genotype.
- The **internal amplification control** appears at **400 bp**.
- In any case the negative controls must be negative for any amplification product of indicated lengths.

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.