



1. Intended Use	Code: KE09004
The MutaGEL[®] r-Vitamin D3 test kit allows the detection of the codon changing DNA variability (start codon polymorphism) in the gene of vitamin D3 receptor (VD3R) which encodes for the corresponding protein involved in calcium metabolism.	

2. Introduction
Vitamin D3 (cholecalciferol) receptor is a protein involved in the regulation of blood calcium levels and also in bone turnover processes. Sequence analysis detected an frequently amino acid changing variability in the vitamin D3 receptor gene (due to a second ATG-triplett 3 codons before). This start codon polymorphism alters vertebral bone fracture frequency of elderly persons, also in different populations.

3. Principle of the Test
The kit MutaGEL[®] r-vitamin D3 contains a set of primer for amplification of the specific DNA sequence within the human vitamin D3 receptor gene VD3R. Amplificates of varying genotypes (start codon polymorphism) are characterized by subsequent specific restriction enzyme digestion. The rare variant (f, pathogen) possesses a restriction site for the specific endonuclease, whereas the major variant (F, protective) does not. The amplified products obtained from the pathogen DNA-variant will therefore be cut into two fragments, whereas the protective DNA-variant will not be cut. The identification of 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)
<ul style="list-style-type: none"> ▪ PCR Mix (VD3R) 1 x 550 µl (green) ready to use PCR reagent (<i>hot start</i> Taq enzyme, MgCl₂, dNTP, buffer) with oligo-nucleotides specific for the human VD3-Receptor gene. ▪ positive control DNA 1 x 30 µl (red) buffered solution with (amplified) DNA of the VD3R gene. ▪ enzyme VD3R 1 x 35 µl (blue) restriction enzyme mix. ▪ buffer for enzyme VD3R 1 x 320 µl (transparent) buffer for restriction enzyme mix.

5. Materials Required but not Supplied
Reagents and Instruments:
<ul style="list-style-type: none"> ▪ DNA extraction kit (f.e. BLOOD MINIPREP: KBR3005) ▪ H₂O (deionized) ▪ thermal cycler and Mineral oil (optional, for thermocycler without heated lid) ▪ 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 "VD3R positive control DNA" for more than two times. If necessary, make suitable aliquots. <i>Before use:</i> Spin tubes briefly before opening (contents may become dispersed during shipment).

7. Warning and Precautions
<ul style="list-style-type: none"> ▪ 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:
<ol style="list-style-type: none"> 1. Sample preparation. 2. Amplification with primers specific for VD3R gene. 3. Digestion of the amplified product with a restriction enzyme. 4. Detection of the amplified and digested DNA by gel electrophoresis.



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	Volume Master Mix
PCR Mix (VD3R)	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 60 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: 25 µl	Volume DIGESTION Master Mix
enzyme VD3R	1.2 µl	1.2 µl x N + 10 %
buffer for enzyme VD3R	11.3 µl	11.3 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 incubator and 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 1x TBE-buffer: mix up to **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 **150 bp** (= amplicate before digestion).
- The presence of the **protective gene variant (pro = F)** 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 = f)** is **not cut**.
- In consequence, the following restriction enzyme patterns are obtained in relation to the present genotype:

GENOTYPE: VD3R	fragment length (bp):
pro: F / pro: F	150
pro: F / pat: f	150 / 110 / 40
pat: f / pat: f	110 / 40

- The **VD3R-positive control DNA** possesses for the polymorphism in vitamin D3 receptor gene the genotype **pro/pat (Ff = 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.

