Sektion Experimentelle Anästhesiologie
Neubau Chirurgie NCKL C1.607
Schneider/Sander/Scheiber/Hein

Isolation of genomic DNA from whole blood using Maxwell 16®

DNA will be isolated from 300 µl of heparinized blood of the respective patient (pseudonymized by the KeyPat-Id number). The Maxwell 16 (*#AS1290,* Promega®) is an automated purification system, which isolates different nucleic acids from different samples. We will use the Maxwell 16 LEV Blood DNA Kit to isolate genomic DNA from blood samples. First, the blood is mixed for approximately five minutes. Then, 300 µl Lysis Buffer and 30 µl Proteinase K, which are supplied in the Kit, are added to 300 µl of the blood sample. The samples are mixed for about 10 seconds and incubated at 56 °C for 20 min. The high temperature and the detergents Guanidiniumthiocyanat (50-75 %) and Polyethylene glycol tert-octylphenyl ether (Triton-X, < 2 %), which are components of the lysis buffer, lead to lysis of cell membranes and nuclei of the cells. The Proteinase K catalyzes the denaturation of various proteins. Histones are also degraded, enabling the release of the genomic DNA. After the incubation time, the whole batch is pipetted into well one of the cartridge. The cartridges are placed into the cartridge holder with a plunger and an additional tube, containing 70 µl elution buffer. The cartridge holder is then placed inside the Maxwell 16® Instrument and the machine is started. The Maxwell 16 Instrument uses paramagnetic particles, the MagnaCel™ particle, which takes advantage of the cellulose binding capacity of nucleic acids. During the automated purification process, genomic DNA is bound to the paramagnetic particle, washed with ethanol and eventually released into the elution buffer.

DNA Quantification (Nanodrop®)

For quantification of the DNA, the photometric measurement at a wavelength of 260/280 nm is performed using a miniaturized spectrophotometer (Nanodrop® nd-1000, ThermoScientific). The purine and pyrimidine bases of nucleic acids absorb 260 nm light strongly. The specific extinction coefficient of a given DNA solution depends on various variables, like its sequence or the pH of the solution. In average the extinction coefficient of DNA is $\varepsilon = 0.02\ (\mu g/ml)^{-1} * cm^{-1}$. The concentration of a DNA solution can be calculated quite accurately with this value and the measured absorption value, using the following formula.

$$c\ (ng/\mu l) = A_{260\ nm} / (\varepsilon_{260} * d)$$

A = Absorption in AU at
e = extinction coefficient in $(\mu g/ml)^{-1} * cm^{-1}$ at 260 nm
b = path length in cm

The absorption maximum of aromatic side chains of amino acids is at 280 nm. In order to assess the purity of the nucleic acid solution, the ratio of absorption at 260 nm to 280 nm can be calculated. DNA is considered pure, if the ratio is approximately 1.8. In the presence of protein contamination, the ratio will strongly decrease. For further experiments, DNA is diluted with nuclease-free water to a concentration of 10 ng/ml.

Polymerase Chain Reaction (PCR)

The aim of the PCR is to amplify a desired sequence of the gDNA *in vitro*. Therefore, forward and reverse primers, flanking the target sequence are designed (Assay Design Software 1.0, Qiagen.com). They can initiate synthesis of DNA in the presence of a DNA polymerase and dNTPs from 5' to 3' direction. The premise for complementary binding of the primers to the bases of the DNA is the denaturation of the double-stranded DNA at 95 °C. The primers bind to the single-strand DNA at temperatures 2-5 °C below their melting temperature. During elongation, the Taq polymerase extends the primers from 5'→3' (**Fig 1**). The Taq polymerase is a heat-stable polymerase, hence its activity will only decrease marginally. The three steps of PCR are repeated 40 times, during these the targeted DNA sequence is increased exponentially (Wink M., 2004).

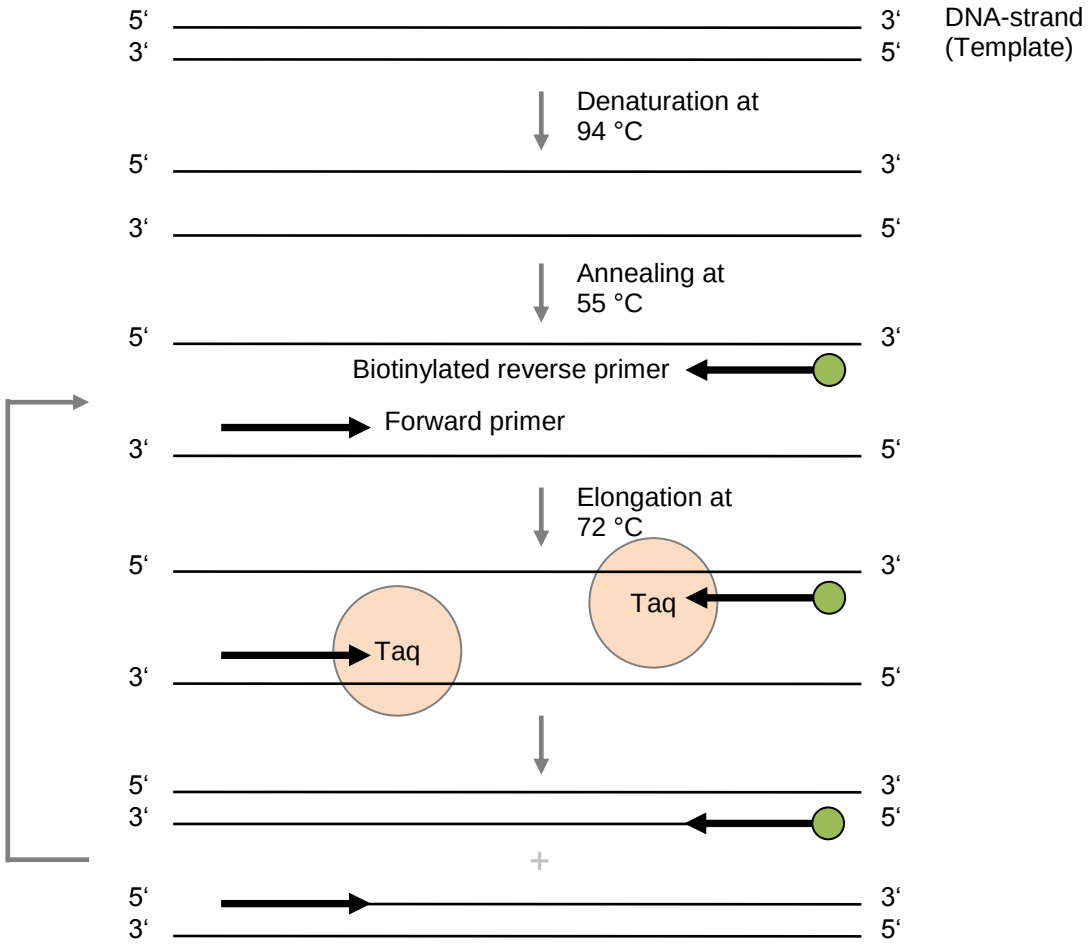

Fig 1: *Schematic reaction process of a PCR. Initial denaturation, followed by annealing of primer at a DNA single-strand. Elongation by Taq-polymerase and incorporation of dNTPs.*

The PCR will be performed for the amplification of the desired region in the TNF-a promotor and the IL-6 promotor, respectively.

TNFα 308G>A primers

Primer Set 1			**Score: 75** **Quality: Medium**		
Primer	Id	Sequence	Bp	Tm, °C	%GC
PCR ↘	F1	CGCAGGGACCCAAACACA	18	73.2	61.1
PCR ↖	R1	TTCTGGGCCACTGACTGATTT	21	70.9	47.6
Sequencing ➡	S1	GTTTTGAGGGGCATG	15	52.9	53.3
Target Polymorphisms	Position 1				
Sequence to Analyze	**G/A**GGACGGG GTTCAGCCTC CAGGGTCC				

IL-6 -174 C>G primers

Primer Set 1			**Score: 93** **Quality: High**		
Primer	Id	Sequence	Bp	Tm, °C	%GC
PCR ↘	F1	TAAGCTGCACTTTTCCCCCTAGTT	24	72.2	45.8
PCR ↖	R1	ATTGTGCAATGTGACGTCCTTTAG	24	71.2	41.7
Sequencing →	S1	GTGACGTCCTTTAGCAT	17	50.7	47.1
Target Polymorphisms	Position 1				
Sequence to Analyze	**C/G**GCAAGAC ACAACTAGGG GGAAAAGT				

Table 2: ***Overview on mastermix preparation for PCR reaction (PyroMark, Qiagen).***

PCR Mastermix	**1x [µl]**	**7x [µl]**	**8x [µl]**
PyroMark Master Mix (2x)	12,5	87,5	100
CoralLoad Concentrate (10x)	2,5	17,5	20
Q-Solution (5x)	5	35	40
10pMol forward primer	0,5	3,5	4
10pMol reverse primer	0,5	3,5	4
Water	2	14	16
MasterMix-Volume	**23**	**161**	**184**

The mastermix is calculated for the number of samples, one negative control where DNA is replaced by RNase-free water and one back up (Table 2). 23 µl of mastermix are pipetted into a 96-well plate, then 2 µl of DNA (10 ng) are added. For the PCR we use the T Professional Thermocycler (AnalytikJena, Biometra) (Table 3).

*Table 3****: time- and temperature-conditions for PCR***

	temperature	***time***	***Number of cycles***
1. Denaturation	95 °	15 min	1
Denaturation	94 °	30 s	45
Annealing	57 °	30 s	45
Extension	72 °	30 s	45
Final Extension	72 °	10 min	1
Cooling	4°C	90 min	1

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Agarose gel electrophoresis

Agarose gel electrophoresis can be used to separate DNA fragments, based on their size and conformation. The underling mechanism is the migration of DNA in an electric field. The phosphate backbone of nucleic acids leads to a negative charge. When exposed to an electric field, DNA will therefore migrate to the anode. Since DNA has an identical mass/charge ratio, the distance traveled of the molecules is inversely proportional to their size (linear DNA). (Lee P.Y., 2012). Agarose is a linear, uncharged polysaccharide, which interacts with nucleic acids and proteins only marginally. After gelation, agarose polymerizes through non-covalent binding and a network of double helices is formed. Pores with the size of 100 - 300 nm are formed, where the size is determined by the amount of agarose used (Brown T.A., 1998). In our experiment, a band of around 300 bp should be detected and therefore a 2.2 % agarose gel is used (Lonza).

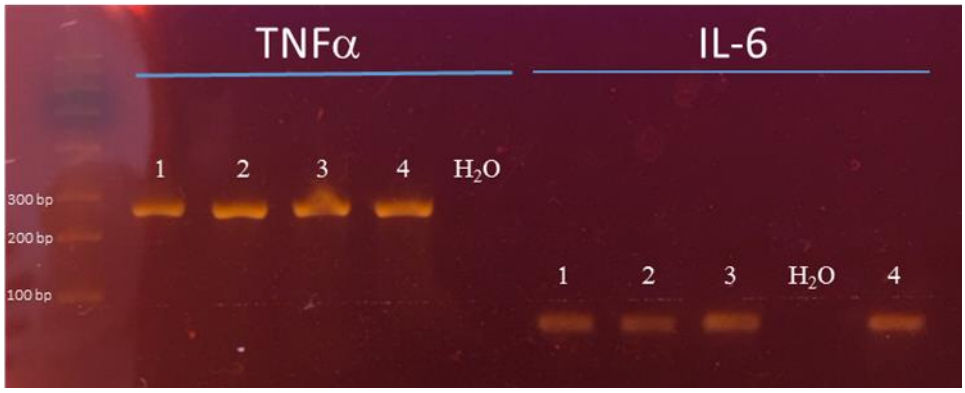

Fig. 2. *Typical Flash-Gel of cDNA generated by TNFα sequence / IL-6 sequence analysis to detect the SNP rs1800629 (-308G/A) (TNFα gene) and* rs1800795 (-174C/G) (*IL-6 gene)*, respectively.

Pyrosequencing

In order to analyze a specific sequence of bases on a desired segment of DNA or of a gene, pyrosequencing can be applied. Pyrosequencing is a sequencing method based on the detection of pyrophosphate, which is formed during DNA-synthesis. Therefore, single nucleotides are added to a single-strand DNA matrix, consecutively. A DNA-Polymerase elongates the sequencing strand with the added dNTP. This incorporation event of the specific dNTP leads to the equimolar release of inorganic pyrophosphate (PPi) (**Fig 3**).

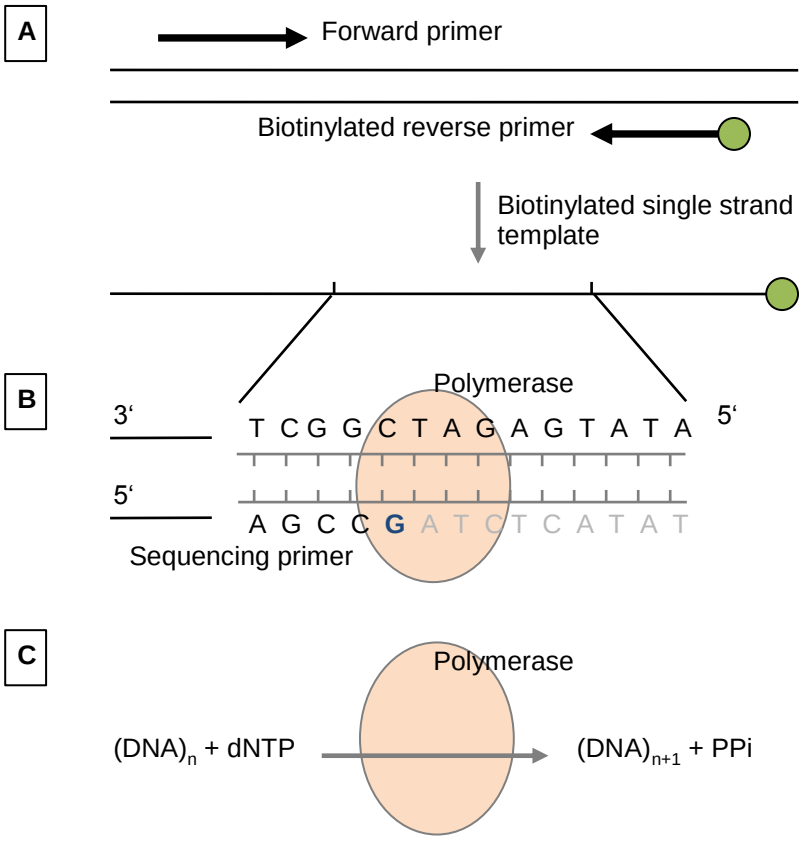

Fig 3: *After amplification of a specific DNA sequence with forward and a biotinylated reverse primer [A], the biotinylated strand can be isolated. This isolated single strand is subjected to the pyrosequencing reaction, where the sequencing primer anneals to the strand and single nucleotides are added. DNA polymerase catalyzes incorporation of dNTP if it is complementary to the base in the template strand [B]. Upon incorporation of a dNTP, PPi is released, in a quantity equimolar to the amount of incorporated nucleotides.*

The presence of an ATP sulfurylase and its substrate adenosine 5' phosphosulfate (APS) leads to the transformation of the released PPi to ATP (**Fig 4**).

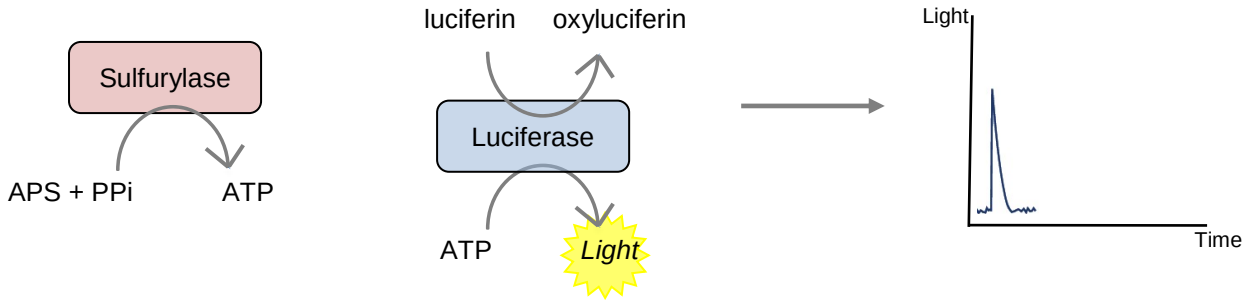

Fig 4: *Sulfurylase converts PPi and APS to ATP, which drives luciferase driven conversion of luciferin to oxyluciferin. With this, visible light is generated which is proportional to the amount of ATP. CCD sensors detect the light which is produced and generate a pyrogram.*

The ATP supplies the energy for the oxidation of luciferin to oxyluciferin, which is catalysed by luciferase. With this reaction, visible light is generated that is proportional to the amount of ATP and therefore also proportional to the amount of incorporated dNTPs (**Fig 4**). Since desoxyadenosine triphosphate (dATP) could serve as a substrate for the luciferase, which could possibly lead to false-positive results, deoxyadenosine alpha-thio triphosphate (dATPαS) is used as a substitute for dATP. The light produced is detected by a charge coupled device camera (CCD camera). In order to ensure that only one nucleotide at a time is present, an apyrase is continuously degrading non-incorporated nucleotides and ATP. Therefore, the recorded light signal can definitively be assigned to a specific dNTP and eventually to the actual DNA sequence. The light is visualized as a peak in a Pyrogram and analyzed by the PSQ HS 96A 1.2 software (Pyrosequencing, Qiagen.com).

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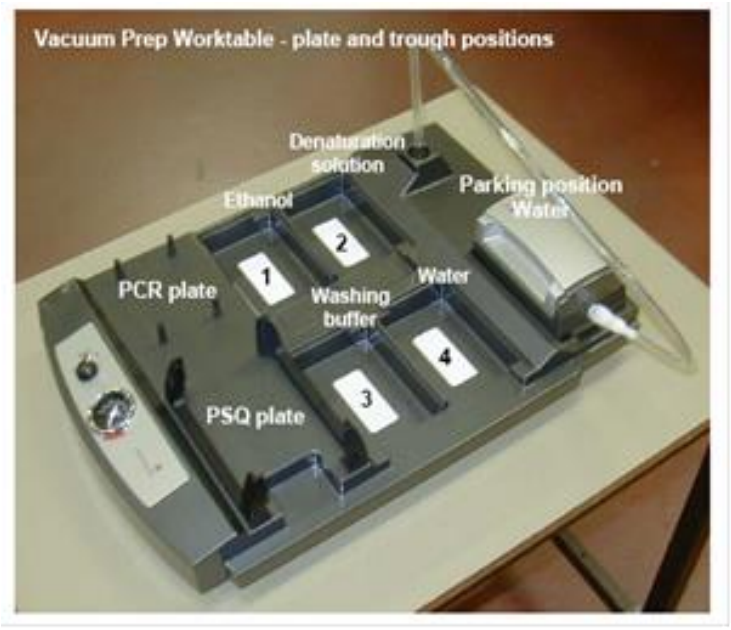
Separation and Purification of the amplicon

The separation of the double-strand DNA and its purification is performed with the PSQ™ 96 sample preparation kit according to the manufacturers' standard protocol. 20 µl of the PCR product are pipetted with 17 µl of the Binding Buffer (Biotage, Qiagen.com) and 3 µl Streptavidin-Sepharose Beads in a 96 well plate. The 96 well plate is incubated in a shaker for 10 minutes at 1400 rpm. The double-strand PCR amplificates, which are bound to the Streptavidin-Sepharose Beads, are soaked to the glassfibre grid of the vacuum prep tool (Pyrosequencing, Qiagen.com), denatured in 0.2 M Sodium Hydroxide Solution and washed in washing buffer (Biotage, Qiagen.com).



Sequencing reaction

Eventually, the single-strand PCR amplicon is released into a PSQ HS 96 well plate, which contains 38 µl of an Annealing Buffer per well (Biotage, Qiagen.com) and 2 µl of the sequencing primer. For annealing of the sequencing primer to the DNA template, the PSQ HS 96 well plate is incubated at 80°C for 2 minutes on a hot metal plate. Before insertion into the PSQ 96 MA machine, the plate is cooled to room temperature for 5 minutes (Pyrosequencing Technology and Platform Overview, Qiagen.com).

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Dispensation order

The sequencing primer binds one base upstream of the SNP *rs1800629, -308 (G/A)*. The PSQ HS 96A 1.2 software automatically generated the dispensation order of the dNTPs. Here, the dispensation order was CGAGTCTCT, with “C” serving as negative control, because the sequence to analyze was: **G/A**GGACGGGGTTC. The amount of dNTPs, which needed to be supplied, was calculated with the following formula:

dNTP [µl] = 50 µl + (0,22 µl * number of dispensations of dNTP * # of wells)

To calculate the amount of substrate and enzyme (both Qiagen), the following formula is used:

Substrate/Enzyme [µl] = 50 µl + (5,5 µl * # of wells)

The amount of dNTPs and substrate/enzyme are calculated for each run.

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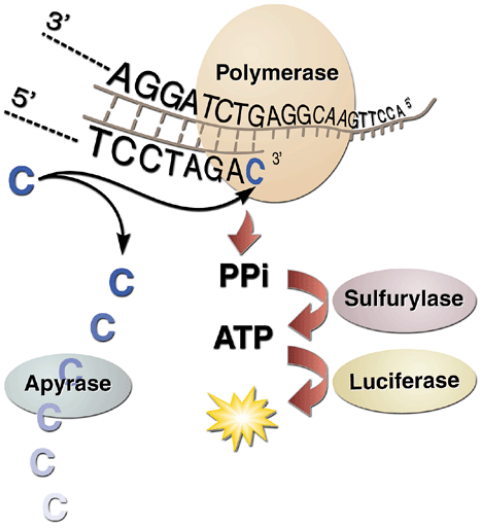
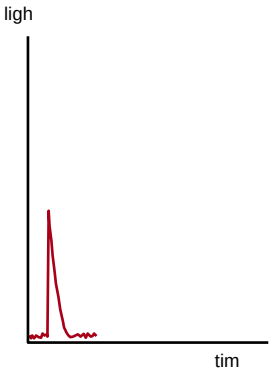
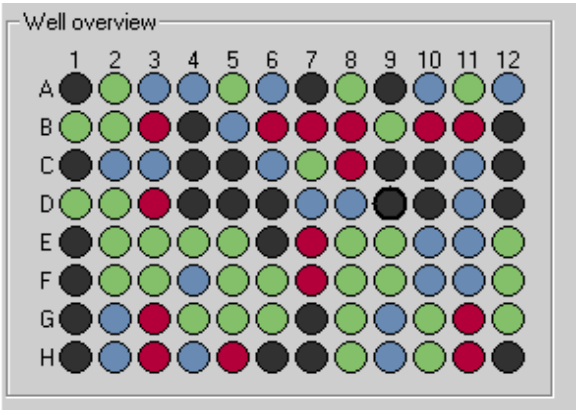
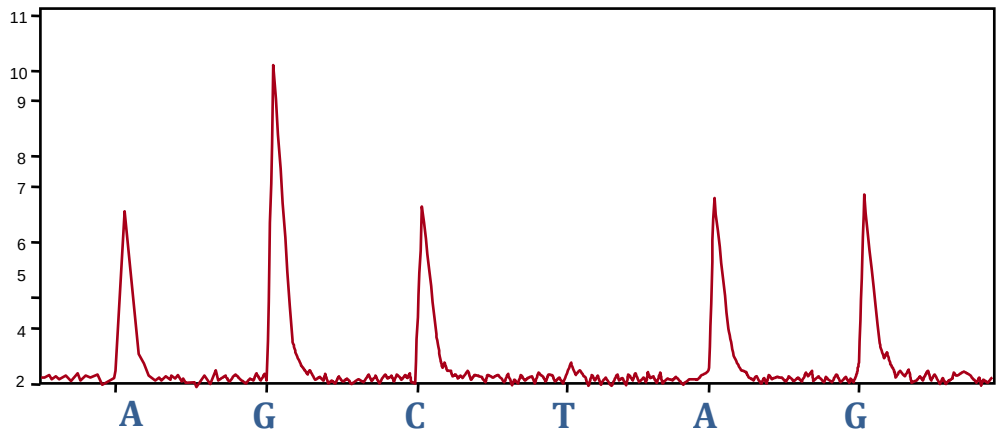






Raw data of a pyrosequencing assay



The sequence in this pyrogram™ is AGGCAG

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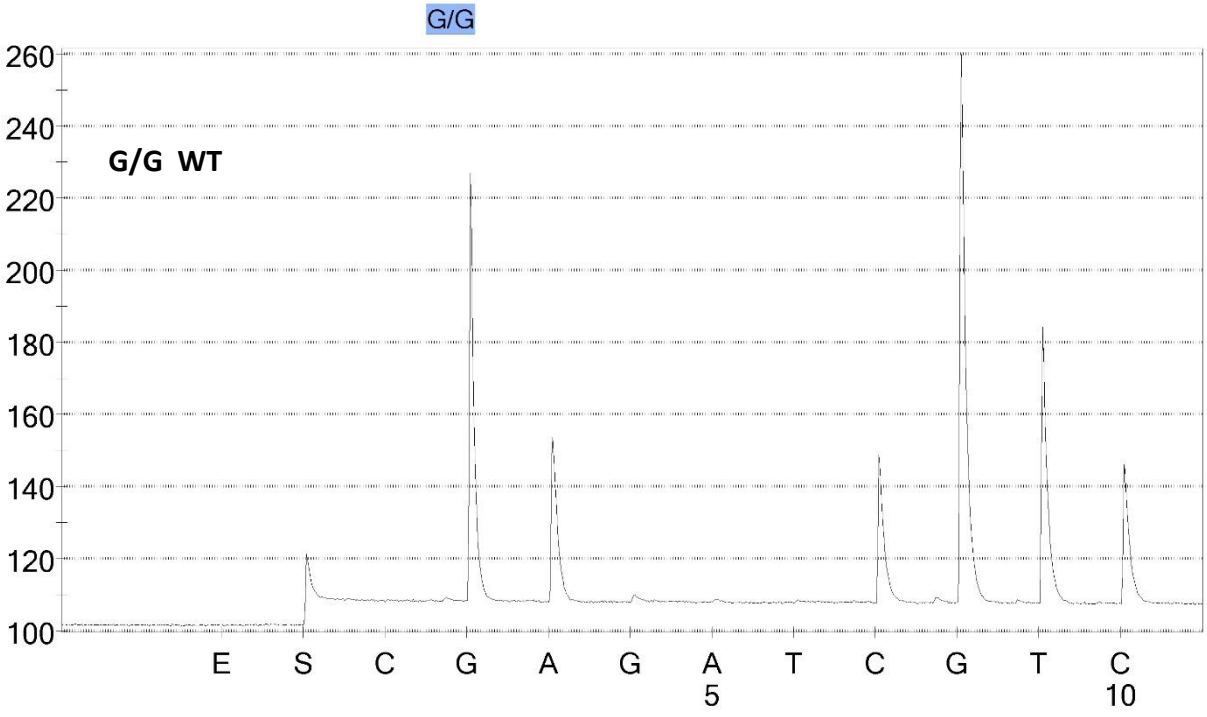
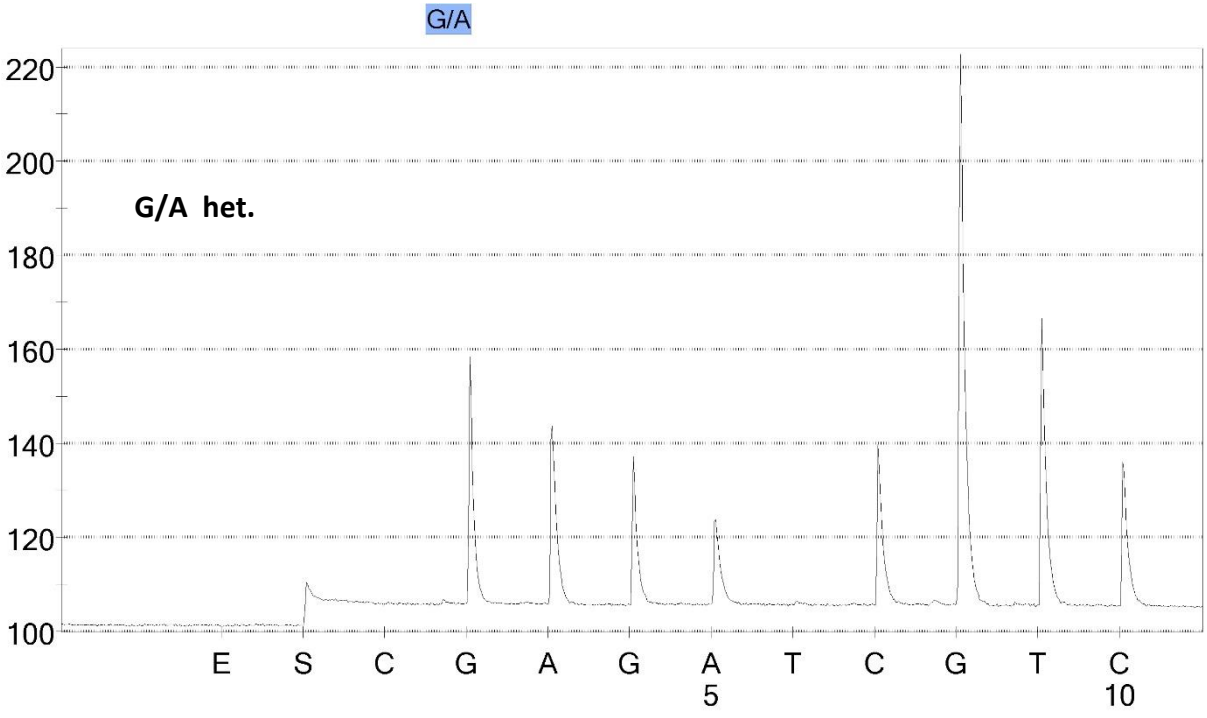
Data analysis

TNFα 308G>A (Sequence to analyze: **G/A**GGACGGGGTTC)





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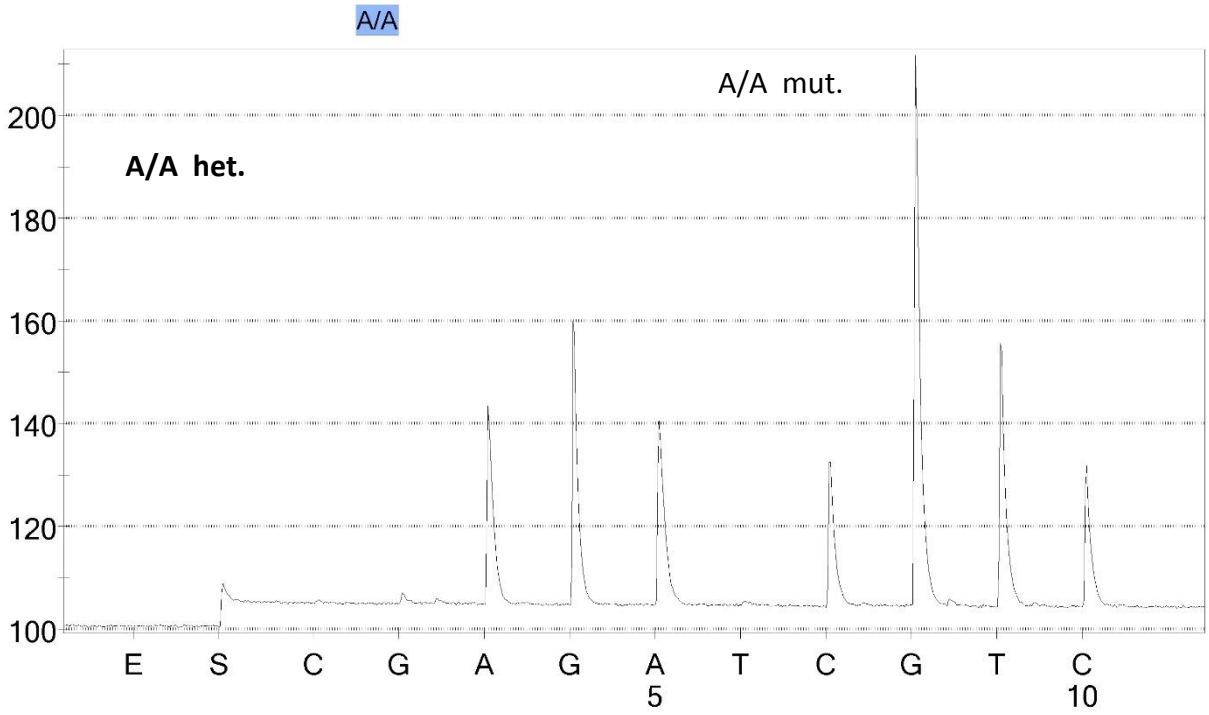

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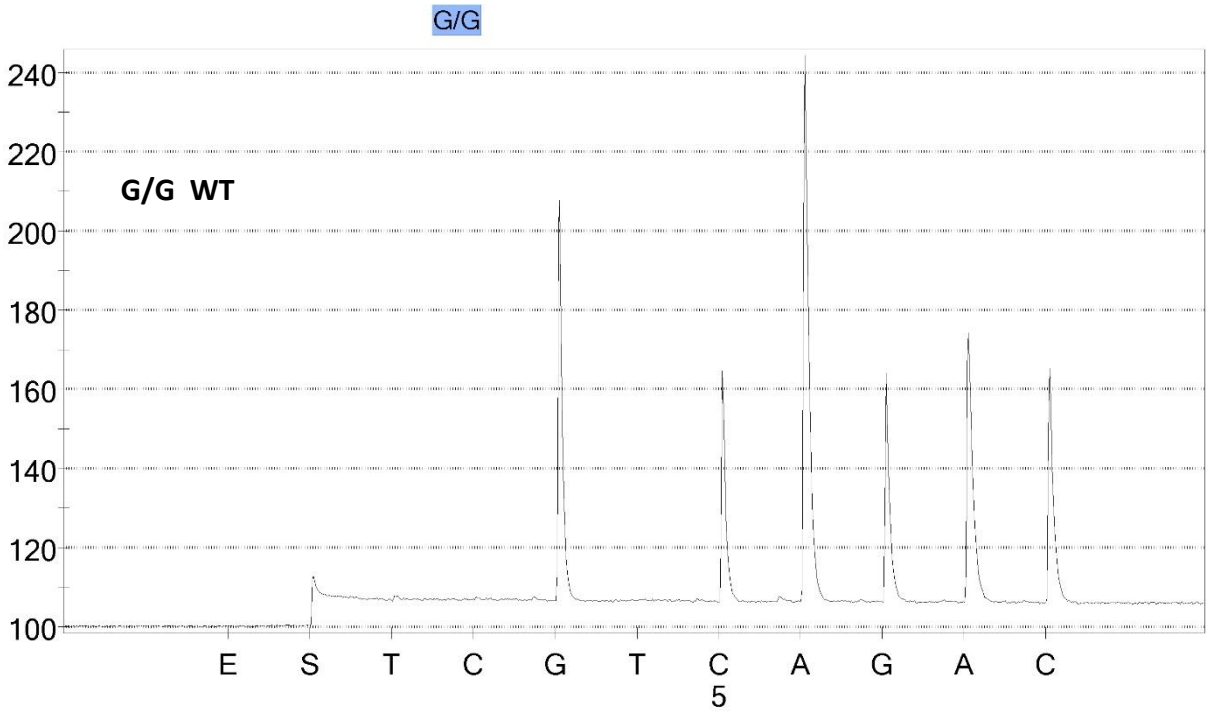
IL-6 -174 C>G (Sequence to analyze: **C/G**GCAAGAC ACAACTAGGG GGAAAAGT)

Hinweis: Revers sequenziert!



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