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„Extraction of genomic DNA from formalin-fixed and ethanol-fixed, long-term stored museal tissue with regard to SNP analysis in foetuses with Otocephaly-Dysgnathia Complex"

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
%	Percent
A	Adenine/Absorbance
Abbr.	Abbreviation
abs.	Absolute
APS	Adenosine 5´phosphosulfate
ATP	Adenosine triphosphate
bp	Base pairs
C	Cytosine
conc.	Concentration
Ct	Threshold cycle
CTAB	Cetyltrimethylammonium bromide
dF/dT	First derivative of fluorescence with respect to temperature
DNA	Deoxyribonucleic acid
dNDP	Deoxynucleoside diphosphate
dNMP	Deoxynucleoside monophosphate
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
e.g.	Example given
et al.	*et alii/aliae/alia (English: and others)*
EtOH	Ethanol
FF	Formalin-fixed
FFPE	Formalin-fixed paraffin-embedded
fw	Forward
G	Guanine
h	Hour
H_2O	Water
HCC827 Erlo	Human lung cancer cells (erlotinib-resistant)
HRM	High resolution melting

kb	Kilo base
LMW	Low molecular weight
LOD	Limit of detection
mg	Milligram
$MgCl_2$	Magnesium chloride
MGMT	*O-6-Methylguanine-DNA-methyltransferase*
min	Minute
mL	Milliliter
mM	Millimolar
n.a.	Not available
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
nm	Nanometer
ODC	Otocephaly-Dysgnathia Complex
Oligo Calc	Oligonucleotide properties calculator
OTX2	*Orthodenticle homeobox 2*
PAX9	*Paired box 9*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PP_i	Pyrophosphate
prot. K	Proteinase K
PSQ	Pyrosequencing
rpm	Rounds per minute
rs	Reference SNP
RT	Room temperature
rt-PCR	Real-time PCR
rv	Reverse
s	Second
$S_{FFPE\ Kit}$	Standard protocol for DNA extraction using QIAamp DNA FFPE Tissue Kit
$S_{Mini\ Kit}$	Standard protocol for DNA extraction using QIAamp DNA Mini Kit
S_{PC}	Standard protocol for DNA extraction using phenol/chloroform/isoamyl alcohol
SHH	*Sonic hedgehog*
SNP	Single nucleotide polymorphism

SNV	Single nucleotide variant
T	Temperature
T_a	Annealing temperature
Taq	*Thermus aquaticus*
T_m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
V	Variation
ZrO_2	Zirconium oxide
µg	Microgram
µL	Microliter
-[a]	Not amplified
-[b]	Not tested

CONTENT

1 Introduction

The use of formalin as a fixation medium opened up new possibilities in the preservation and collection of organs, body parts, disease-related malformations and morphologically altered tissue, which aided to understand and learn about the human body. It was an easy way of maintaining the morphology of tissue over a long period of time. The collected knowledge led to progresses in medical diagnosis and treatment of diseases. Over time, more and more samples were collected and preserved in formalin and stored for future investigations. Only later it was found, that formalin causes cross-linkages between nucleic acids and proteins inhibiting the tissue from decay and putrefaction but adversely affecting the integrity of DNA. When long-term stored, the DNA is more and more fragmented and altered due to depurination and irreversible cross-links. Nevertheless, formalin-fixed tissue provides a useful resource of sample material, when identifying and analysing diseases on a genetic level. [1,2]

In addition, advancing medical technologies have enormously increased during the last decade and have enabled the search for the cause of not only commonly occurring diseases, such as cancer, but also of rare diseases. Genotyping - the determination of genetic differences between individuals – is becoming a greater and greater field of interest aiming to understand which genes play a crucial role in the development of rare diseases. This knowledge enables molecular diagnosis and new drug design for effective therapies.

1.1 Otocephaly-Dysgnathia Complex

Otocephaly-Dysgnathia Complex (ODC) occurs 1 in 70 000 births only [3] and is therefore considered a rare disease. Since the number of patients is limited, the genetic mechanisms underlying the evolvement of ODC are widely unknown. While the genotype-phenotype correlation of otocephaly patients remains unsolved, the phenotype itself can be characterised by malformations of the face and skull, comprising an abnormal smallness of the tongue (microglossia) and mouth (microstomia), mandibular hypoplasia or agenesis or malposition of the ears [4], making it a fatal disease due to respiratory deficiency. Several studies have investigated candidate genes for the manifestation of ODC and other closely related diseases, such as holoprosencephaly. Beby *et al.* reported that the homeobox gene *OTX2* (*orthodenticle homeobox 2*), which is located on chromosome 14q22.3, plays a major role in the development

of normal brain and sensory organs from early embryogenesis onward. Mutations in the *OTX2* gene can result in failure of proper development of different brain regions, facial skeleton and eyes, resulting in severe facial dysmorphia, which also plays a crucial role in otocephaly [5]. Patat *et al.* also reported the contribution of *OTX2* in the development of ODC and identified two *OTX2* mutations [3]. Chassing *et al.* suggested, that otocephaly is genetically related to anophthalmia (one or both eyes are missing) and identified an *OTX2* mutation, which is associated with the otocephaly phenotype [4]. Nanni *et al.* investigated the role of the *sonic hedgehog* gene (*SHH*) in the development of holoprosencephaly, which shows similar abnormalities of the forebrain and facial features as ODC [6]. Sperber *et al.* reported that PAX9 also plays an important role in the craniofacial development, particularly skeletogenesis, during embryogenesis [7].

However, finding a correlation between the genotype and phenotype caused by specific mutations, so-called single-nucleotide-polymorphisms (SNP), remains difficult and demands a reliable method for the determination of these genetic differences associated with ODC.

In this study, four different SNPs, from which two are located on the *OTX2* gene whereas the other two are located on *SHH* gene and *PAX9* gene were investigated for their role in the phenotype-associated malformations of ODC.

1.2 Pathological-Anatomical Museum

One of the many obstacles when investigating rare diseases is the limited number of patients. In case of ODC, well established medical pregnancy examinations such as prenatal diagnosis and routine ultrasonic examination enable the early detection of disorders and malformations of the embryo or foetus. At the same time the quantity of sample material is reduced, hence, formalin-fixed or ethanol-fixed museal specimens are often the only resource of sample material available, when it comes to investigating rare diseases.

The Fool's Tower in Vienna contains the biggest collection of pathological-anatomical specimens in the world, consisting of more than 50 000 objects, which include not only wax imitations of human body parts showing pathological alterations, but also dry and wet preparations of human body parts, organs and malformations caused by genetic modifications. Wet preparations, for example formalin fixation, take up the biggest part of the collection. The

fixation process, however, is often poorly documented or unknown, which makes biomolecular analysis regarding extraction and amplification of intact DNA, even more challenging. [8]

The Fool's Tower has not always been a museum for the public. It was built in 1784 under the reign of Emperor Joseph II (1741-1790) and was initially meant for mentally ill people. However, the round shape of the building rather suggested it to be a prison than a health institution, specialised in treating psychological disorders. The key interest was to protect the public from lunatics. Over time, the fools tower started to act as its purpose, namely as a health institution. Patients were no longer locked away but received treatment and care. [9]

Since 1971 the Fool's Tower acts as pathological-anatomical museum and is open for the public. Additionally, the collection provides rare sample material for research purposes.

1.3 Formalin-Fixation

Formalin-fixation has been used for decades as a successful way of preserving samples and storing them at room temperature for many years. Formalin is an aqueous solution of formaldehyde, which crosslinks DNA and proteins in the sample, resulting in the maintenance of the morphology of tissues and integrity of nucleic acids. Because the DNA is prevented from sudden severe degradation, formalin-fixed (FF) samples are an indispensable resource for genomic studies and allow retrospective analysis of long-term preserved specimens. [10]

One of the major issues, when analysing formalin-fixed museal samples, is the reversal of these cross-linkages and the extraction of intact DNA with high integrity. Integrity of nucleic acids, however, depends on environmental conditions prior to preservation, duration of fixation and pH of the storing solution. Due to oxidation of formaldehyde in presence of atmospheric oxygen, formic acid is generated, which leads to a decrease in pH followed by depurination of nucleic acids and subsequently to their breakdown. To further complicate matters, DNA integrity is influenced by variations in preservation techniques. Samples can be fixed in formalin, then long-term stored in alcohol or water or fixed and stored in formalin. Depending on the applied method, reactions between formalin and DNA/proteins differ and can be either reversible or irreversible. While cross-links are reversible, fragmentation and sequence alterations are destructive, hence, irreversible. [2]

The exact mechanism of the formation of cross-linkages is still not fully understood. Formaldehyde, which is originally an odourless gas, reacts to methylene hydrate, when dissolved in water. Methylene hydrate then interacts with side chains of proteins and DNA resulting in hydroxymethyl side groups and subsequent methylene bridges. When long-term stored in formalin, more of these cross-linkages can be formed and oxidised to more stable groups, which cannot be reversed easily by washing off the formalin [1]. Because of this difficulty, recovery of DNA with high integrity remains a challenge and has been investigated by several working groups. Shedlock *et al.* claimed to have successfully extracted and amplified DNA fragments of a few hundred base pairs from long-term stored, formalin-fixed tissue [11]. Nagai *et al.* optimised a DNA extraction protocol for the extraction of genomic DNA from long-term preserved human embryos and reported successful PCR amplification of relatively long DNA fragments ranging between 295 bp and 838 bp. [10]

In routine pathological diagnosis, the use of formalin-fixed tissue does not seem to be an issue, when it comes to DNA recovery and amplification. The reason for that lies in the short preservation time and embedment in paraffin. Right after a piece of tissue is removed for later molecular investigation, it is fixed with a buffered or unbuffered formalin solution before embedment in paraffin. This sample material is usually analysed shortly after collection or kept in storage for a few years. It results in less cross-linked, degraded and fragmented DNA than long-term stored formalin-fixed tissue and in a higher recovery of intact genomic DNA. The longer the material is stored, the more fragmented DNA is obtained and the less suitable it is for the investigation of genetic alterations. [2]

1.4 Publications Regarding the Extraction of Genomic DNA from Formalin-Fixed Tissue

Nagai *et al.* (2016) optimised the DNA extraction protocol for the extraction of genomic DNA from long-term preserved human embryos, fixed and stored in formalin or Bouin's solution for 20 – 50 years. They examined the quantity and quality of extracted DNA regarding medium of fixation and reported successful PCR amplification of relatively long DNA fragments (PCR products ranged between 295 bp and 838 bp).

They used various DNA FFPE tissue kits from several suppliers, following the manufacturer's protocol with a few modifications regarding the washing step, lysis step and reparation step.

They used ethanol (EtOH) and phosphate buffered saline (PBS) for washing and further tested the incubation time, which lasted from one hour to two days. Furthermore, the proteinase K concentration for tissue lysis was varied from 4.0 to 40.0 mg/mL. Once more, the period of incubation was investigated ranging from one hour to two days.
The results showed that the washing step is crucial for increasing DNA yield; complete lysis was achieved after overnight incubation, when combined with a proteinase K concentration of 20 mg/mL. Compared to other proteinase K concentrations, 20 mg/mL yielded highest DNA amount and purity. However, quality and quantity of extracted DNA often depended on duration of fixation and preservation medium. The lack of sufficient quality and quantity for genetic analysis made next generation sequencing impossible and demanded for further optimisation of the extraction protocol. [10]

Einaga *et al.* (2017) concluded that heat treatment at 95 °C after lysis, which ought to help reverse formaldehyde-modifications with respect to formalin-fixed tissue resulted in higher quality and quantity of DNA, as well as longer PCR products. Despite the short preservation period of a few days, differences in DNA quality and quantity regarding fixation treatment used were already found. [12]
This confirms the fact, that DNA extraction form formalin-fixed tissue essentially depends on fixation method and preservation time.

Gilbert *et al.* (2007) compared already published protocols focusing on the pre-treatment prior to lysis, lysis conditions and the extraction methods, among other things. They investigated the effect of heat treatment at 98 °C prior to lysis, the impact of incubation time and temperature during lysis and the extraction itself using a silica-based kit versus phenol and chloroform. The results indicate that pre-incubation of tissue at 98 °C yields higher amounts of amplifiable DNA than when this step was omitted. They found a relationship between nucleic acid yield and incubation time but not temperature. They investigated incubation periods ranging between one hour and 96 hours and digestion temperatures between 55 °C and 85 °C. Results suggested that temperatures beyond 65 °C have no increasing effect on DNA quantity but longer incubation times lead to higher DNA yields. However, progressing DNA degradation with longer digestion periods and temperature must be considered.
As for the DNA extraction itself, the silica column-based extraction kit showed to be more promising in regard to PCR amplifiability compared to the phenol-chloroform extraction. [13]

Hykin *et al.* (2015) compared not only published protocols for the extraction of DNA from herpetological specimens, which had been formalin-fixed in 1917 or 1985 and long-term stored in ethanol but tested the applicability of extraction protocols (standard Qiagen kit and phenol-chloroform extraction) regarding DNA yield of different tissue types (liver, leg muscle and tail-tips) of two museal *Anolis carolinensis* specimens.
They found, that phenol-chloroform extraction yielded the highest amount of DNA when using liver tissue as sample material. The Qiagen kit-based extraction method resulted in much lower DNA concentrations. However, this was only obtained for the specimen prepared in 1985. The earlier preserved sample failed to yield sufficient quantities of DNA independent of extraction method and tissue type used. Hykin *et al.* reasoned that before 1970, unbuffered formalin had been commonly used for fixation, causing greater DNA damage than buffered formalin that had been used after 1970. Hence DNA extracted from specimens preserved in unbuffered formalin is usually poor in quantity and quality. [14]

Shedlock *et al.* (1997) found that formalin-fixed tissue yielded higher quantities of genomic DNA than alcohol-fixed specimens, but greater amount of DNA is lost during phenol-chloroform extraction of formalin-fixed tissue than of alcohol-fixed one. They argued that nucleic acids remain cross-linked with proteins.
They used an extraction protocol comprising a washing step (24 h in buffered solution), digestion with proteinase K (10 mg/mL) for 24 h and phenol-chloroform extraction. This allowed them to obtain relatively long DNA fragments. [11]

Paireder *et al.* (2013) tested several (modified) extraction protocols in order to gain access to protein-associated genomic DNA from formalin-fixed heart tissue samples that had been preserved for over 50 years. They further analysed the DNA extracts for PCR amplifiability. DNA extraction consisted of a CTAB standard protocol, which was further modified regarding duration of tissue digestion (1 h - 72 h), lysis temperature (55 °C and 65 °C) and digestion procedure itself, among other things. They also tried a different approach for the extraction of genomic DNA by using spin-column based kits. The QIAamp DNA Mini Kit for universal application and the QIAamp DNA FFPE Tissue Kit, which specially aims at DNA extraction from formalin-fixed and paraffin-embedded (FFPE) tissues.
In case of specimens prepared before 1851, only the QIAamp DNA FFPE Tissue Kit showed to be successful regarding extraction and subsequent amplification of DNA (122 bp and 171 bp

amplicon) from long-term stored tissue. All other variations failed to yield measurable amounts of DNA.
In case of later preserved museal specimens, a variation of the CTAB protocol consisting of the use of additional sodium dodecyl sulfate (SDS) in the CTAB extraction buffer and lysis period of 72 h seemed to be promising regarding DNA yield. [15]

Farrugia *et al.* (2009) compared two types of FFPE human tissue, liver and heart, regarding quantity of extracted DNA. They used a combination of organic and spin-column based extraction methods and found that liver yielded higher amounts of genomic DNA. [16]

Bonin *et al.* (2003) suggested the pre-treatment of DNA prior to PCR amplification. The treatment comprised incubation (1 h at 55 °C) of DNA extracts in a mixture containing 100 µL 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, 2 % Triton X-100 and 200 µM of each dNTP. This step was followed by incubation (20 min, 72 °C) with 1 U *Taq* DNA polymerase. [17]

2 Aims

The aim of this study was to develop and optimise a method for the extraction of intact genomic DNA with high integrity from formalin-fixed and ethanol-fixed long-term stored museal tissue in regard to yield, purity, PCR amplifiability and suitability for single-nucleotide polymorphism (SNP) analysis.

Two long-term preserved foetuses without facial dysmorphia, collected from the Fool's Tower in Vienna, served as controls for the determination of potential Otocephaly-Dysgnathia Complex (ODC)-related SNPs in foetuses with facial dysmorphia. Therefore, several organs, such as liver, lungs, heart, cerebrum, cerebellum and spleen, as well as intestinal organs and bones were to be collected with the assistance of Andreas Glowania, who worked as an otorhinolaryngologist at the Hietzing Hospital, Vienna during this project.

In order to gain access to the cross-linked DNA, following parameters of DNA extraction protocols should be investigated: pre-treatment steps prior to lysis, incubation time during lysis and DNA extraction method. The latter should be investigated using four different extraction methods: an in-house designed and frequently applied CTAB (cetyltrimethylammonium bromide) method, two commercially available DNA extraction kits and a phenol-chloroform extraction, which several papers reported to be most efficient compared to other extraction methods.

All extraction protocols should be optimised regarding yield and purity. DNA purity should be determined by spectrophotometric measurements while DNA yield was additionally assessed with a fluorometric assay. DNA integrity should be analysed using gel-electrophoresis and DNA extracts should further be tested for PCR amplifiability using an in-house designed real-time PCR assay for candidate SNPs. To determine if the correct DNA template is amplified, the DNA sequence should be further analysed by pyrosequencing.

3 Theoretical Background

The following part will explain the theoretical background of applied methods, which were used to carry out the experiments. More precisely, the extraction methods for the retrieval of cross-linked genomic DNA, the application of agarose-gel electrophoresis, the basics of (real-time) PCR combined with high resolution melt analysis (HRM), the design of primers for the amplification of a specific DNA template and finally the principle behind pyrosequencing.

3.1 Deoxyribonucleic Acid (DNA)

Deoxyribonucleic acid (DNA) can be found in all kinds of organisms and carries the whole genetic information that defines one living being. The first to discover the complex, yet simple structure of DNA molecules were James Watson and Francis Crick in 1953, for which they received the Nobel Prize in medicine in 1962. With their discovery, they contributed to the clarification of the structure of the genetic code and subsequently the understanding of how genes are involved in chemical processes within living organisms. [18]

3.1.1 DNA Structure

DNA consists of four different nucleotides, each composed of a nucleobase (adenine (A), cytosine (C), thymine (T) and guanine (G)), the monosaccharide deoxyribose and a phosphate group. The nucleobase is linked to the monosaccharide via N-glycosidic bond. The nucleotides are attached to one another via a phosphodiester bond between 5'-phosphate and the 3'-hydroxyl group of the deoxyribose, forming a polynucleotide strand with a sugar-phosphate backbone. Two of these strands curl around each other, building a helical structure, which is stabilised by hydrogen bonds and stacking interactions between the bases. Base pairing can only occur between purines (A and G) and pyrimidines (C and T); the proper duplex structure is obtained, when A pairs with T, and C pairs with G. [19]

The hydrophilic phosphate backbone is on the outside of the helix, while the paired, hydrophobic bases lie on the inside, which is important for the biological properties of the DNA. Another characteristic of the helical structure are the major and the minor groove, which result from non-diametrical opposed glycosidic bonds of two complementary bases. The grooves function as binding sites for specific proteins e. g. transcription factors. [19]

The two strands of the duplex are complementary, meaning they both function as a template for the reproduction of the genetic code, which is found in each cell of the human body. However, there are differences between genomic and mitochondrial DNA. The latter is found in the mitochondria of eukaryotic cells, occurs in dozens of copies per cell and has a circular shape when compared to nuclear DNA. Nuclear DNA is linear, found in the nucleus of cells and occurs only in one copy per cell but depends on the set of chromosomes. The mitochondrial genome is not inherited from both parents equally as the nuclear genome is but from the mother only. Furthermore, it is much smaller, as it contains 16.569 nucleotides, whereas the nuclear genome is built of 3 billion nucleotides. [20]
These facts are important when it comes to DNA quantification. As mentioned above, nuclear DNA is found in only one copy per cell - depending on the set of chromosomes, consequently, nuclear DNA is suitable for quantitative analysis.

3.1.2 DNA Extraction

The extraction of DNA is the key prerequisite for any type of molecular biological analysis and aims at high concentration of DNA with high integrity and purity. At the same time, the extraction procedure should provide reproducible results, be quick and simple in operation and low in costs. These requirements, however, depend on the biological sample. As in case of formalin-fixed tissue, DNA is often severely degraded and cross-linked, making the extraction more challenging. Additionally, the quantity of sample material available is often limited, especially in case of rare diseases.

A universal protocol for the successful extraction of DNA does not exist. Nevertheless, there are certain steps to be performed, when extracting DNA from tissue. The first step is the sampling, followed by the sample preparation. The risk here lies in the contamination with DNA from the operator himself or from the surrounding environment. The sample preparation comprises several steps prior to cell lysis and can include homogenisation of the biological material, dissolution of the analyte and separation from interfering matrix components, centrifugation, filtration, etc.
In the following step, the sample is submitted to lysis during which the membrane of the cells is broken down and the DNA is released from the nucleus. This can be achieved chemically, e.g. by chaotropic salts, mechanically, e.g. by sonication or applying pressure and enzymatically, e.g. by proteinase K. The use of proteinase K additionally degrades proteins in

the sample, which can be of advantage when extracting DNA only and saves an extra step for the denaturation of proteins.

After lysis, the DNA is isolated using various techniques, for example the addition of cetyltrimethylammonium bromide (CTAB), a cationic detergent that forms a stable complex with nucleic acids. The complex can then be centrifuged and dissolved in an adequate medium. Another possibility of isolating DNA is to use a mixture of phenol and chloroform, which is based on different solubilities of the components of the lysate. While DNA is found in the upper phenolic phase, proteins congregate in the interphase and remaining components are collected in the lower organic phase.

There also exist commercially available spin columns that bind nucleic acids to the stationary phase of the column, while the rest of the components are washed away.

In any case, the DNA can further be purified by precipitation with ethanol and recollection of precipitated DNA in an adequate medium.

3.1.3 Determination of DNA Concentration and Purity

In this master's thesis, two different methods for the determination of the DNA concentration were applied and will be discussed in following section.

3.1.3.1 NanoDrop

NanoDrop is a spectrophotometrically based assessment of the concentration as well as purity of the DNA.

The DNA concentration is determined by measuring the absorbance at 260 nm (absorption maximum of nucleic acids) and inserting the value into the equation given below:

$$c\left[\frac{ng}{\mu l}\right] = A_{260} * D * F$$

c…Concentration [ng/µL]

A…Absorbance at 260 nm

D…Dilution factor

F…Multiplication factor (double stranded DNA = 50)

The assessment of the absorbance follows the Lambert-Beer law, which states the correlation between attenuation of light, when sent through a light absorbing liquid material of a certain path length, concentration and specific molar extinction coefficient. This relation is expressed in following equation:

$$A = \varepsilon * c * l$$

A…Absorbance
ε…Molar extinction coefficient [L*mol^{-1}*cm^{-1}]
c…Concentration [mol*L^{-1}] [21]

The purity is calculated by dividing the value of the absorbance at 260 nm (A_{260}) by the value of the absorbance at 280 nm (A_{280}). A_{280} is the wavelength at which proteins absorb. Additionally, the absorbance at 230 nm (A_{230}) at which organic compounds absorb is determined and the ratio of A_{260} to A_{230} is calculated. The ratio of A_{260}/A_{230} can be used as another parameter for the characterisation of the purity. Nucleic acid solutions are considered as 'pure', if the values of the ratios of A_{260}/A_{280} and A_{260}/A_{230} lie between 1.8 - 2.0 and 2.0 – 2.2, respectively. [22]

3.1.3.2 Qubit®-Fluorometer

A different approach for DNA quantification is the use of a fluorescent dye. This method allows the determination of DNA and RNA concentration by using different fluorescence assays, specifically designed for the detection of the nucleic acid of interest. However, the sample should be treated with RNase or DNase prior to the determination of the nucleic acid concentration to get accurate DNA and RNA yields, respectively. Unbound dye molecules show a low fluorescence light but when bound to DNA or RNA molecules the fluorescence increases. When using the absorbance-based measurement as quantification method, not only DNA but other components that absorb at 260 nm will be included in the calculation of the DNA concentration. Due to this, DNA concentration determined with NanoDrop is often higher than DNA concentration determined with Qubit®-Fluorometer.
DNA quantification using the Qubit®-Fluorometer has got a higher sensitivity and accuracy, thus, allows the determination of lower nucleic acid concentrations.
Therefore, a combination of both above mentioned methods for the determination of the DNA concentration is of advantage.

3.2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is an in-vitro method for the amplification of DNA and was invented by K. Mullis and others in 1985. [23]
It changed the way of DNA analysis dramatically and until now, its development allows an easy and relative fast investigation of genetic information of not only human beings but also of any other species and has become indispensable in today's molecular medicine and biology. Over time, several protocols and technical improvements have been established to further facilitate the workflow of this method without changing the basic principle.

3.2.1 PCR Components

PCR requires several components for the amplification of a target sequence: DNA template of interest, a primer set, which consists of a forward (fw) and reverse (rv) primer and a buffer that contains deoxynucleoside triphosphates (dNTPs), DNA polymerase and $MgCl_2$.

The most commonly used DNA polymerase is *Taq* DNA polymerase, which is synthesised by the thermostable bacteria *Thermus aquaticus*, and is characterised by high robustness and processivity. The *Taq* DNA polymerase has an extension rate of 150 nucleotides per second, which is very high compared to others. When put in relation to other DNA polymerases, a possible downside is the error rate of 1 bp in 103/104 bp per cycle as well as the lack of a 3'-5' exonuclease activity. For activation of the enzyme, the reaction mixture must be heated to 95 °C for 5 minutes. As many polymerases, the *Taq* DNA polymerase elongates the DNA template from 5' to 3' end, if primers are present. [23,24]
Primers are short oligonucleotides consisting of 16-28 bases, depending on the length of the DNA template. [25]
For amplification of a DNA template, a forward and reverse primer are necessary, each annealing to its respective complementary single strand of the denatured DNA template. The DNA polymerase then starts incorporating four different dNTPs until the complementary strand is synthesised: dATPs (deoxyadenosine triphosphates), dCTPs (deoxycytidine triphosphates), dGTPs (deoxyguanosine triphosphates) and dTTPs (deoxythymidine triphosphates). The dNTPs are incorporated from the 3'end of the primers by releasing pyrophosphate (PP_i) and water due to the formation of a covalent bond between the two monosaccharides via a phosphate group. For the proper function of the DNA polymerase, the Mg^{2+} ion concentration is of great importance. Most commercially purchased buffers already contain $MgCl_2$ (1 mM to 2.5 mM),

however, further optimisation of the Mg^{2+} ion concentration is often crucial for increasing amplification efficiency. On the downside, too many Mg^{2+} ions inhibit the enzyme activity of the DNA polymerase, which results in higher Ct values (see Chapter 3.2.3.3) and unspecific PCR products. [24]

3.2.2 PCR Principle

The amplification of a DNA template can be divided into three major steps: denaturation, annealing and elongation, shown in Figure 1. Starting with the denaturation of the double stranded DNA at 95 °C, the helical structure of the nucleic acid gets split up into two single strands. The following annealing step usually requires a temperature between 50 °C and 68 °C depending on the composition of bases of the DNA template. [23]

During this step, forward and reverse primer hybridise with the respective single strands of the denatured DNA template. Afterwards, the heat-stable DNA polymerase synthesises the fully complementary strand by incorporating the dNTPs. For elongation, the temperature is increased to 72 °C, which is the optimal working temperature for the *Taq* DNA polymerase. All three steps represent one cycle. A PCR run normally comprises 30-50 cycles, depending on the amount, purity and integrity of the DNA template. The more cycles are carried out, the higher the probability of unspecific by-products, e.g. primer dimers.

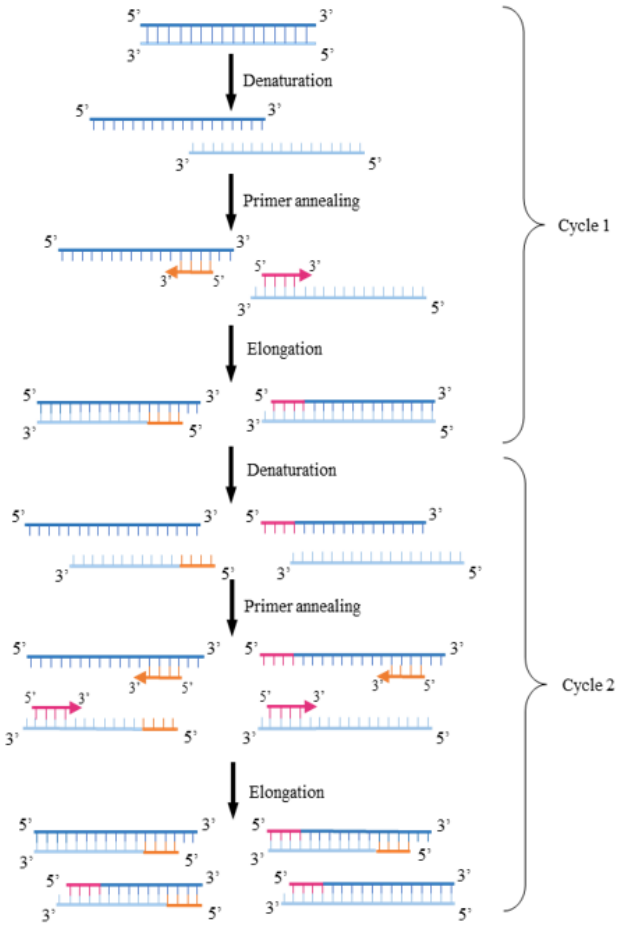


Figure 1: Schematic flow of a PCR.

3.2.3 PCR Analysis

In theory, one DNA strand is enough for the exponential amplification during PCR, which explains the high sensitivity of the method. During each cycle the DNA strands are doubled. For example, after 25 cycles the number of DNA copies is 2^{25} = 33 554 432, resulting in an amplification efficiency of 100 %. In practice, however, this is never achieved because the working efficiency of the enzyme decreases with each cycle due to denaturation. [26]

3.2.3.1 Baseline

Normally, the baseline is set automatically during PCR but can also be manually defined. The baseline phase comprises amplification that is below the detection limit of the instrument and allows the differentiation from background signals, which result from uneven fluorescence

levels in each tube. The baseline phase takes up the first few cycles of a PCR and is characterised by small changes in the fluorescence signal (Figure 2). [26]

3.2.3.2 Curve Progression

During PCR, the progression of the DNA amplification can be divided into four parts (Figure 2). The first part makes up the baseline, which normally consists of cycles 3 to 15 but depends on the template concentration. [23]

During this phase, there is no increase in the fluorescence signal observed, due to low concentration of the PCR product. After several cycles, the fluorescence signal starts to increase exponentially because the DNA template gets doubled during each cycle. This part of the curve takes up the exponential phase followed by a linear phase during which the amplification declines. This means, that the PCR product no longer gets doubled and the progression of the curve ceases into a plateau (Figure 2). The latter can be explained by the degrading efficiency of the DNA polymerase and the synthesis of non-specific products and primer dimers. [26]

3.2.3.3 Threshold and Ct Value

A crucial step for the analysis of an amplification plot is the determination of the threshold line. The threshold line is set in the linear phase and above the background signal of an amplification curve. The point of intersection between the amplification curve and the threshold line is the Ct value, which is defined as the cycle during which the fluorescence signal exceeds the background signals and starts to increase exponentially (Figure 2). The Ct values can be used for the comparison of different amplification plots and the calculation of the amplification efficiency.

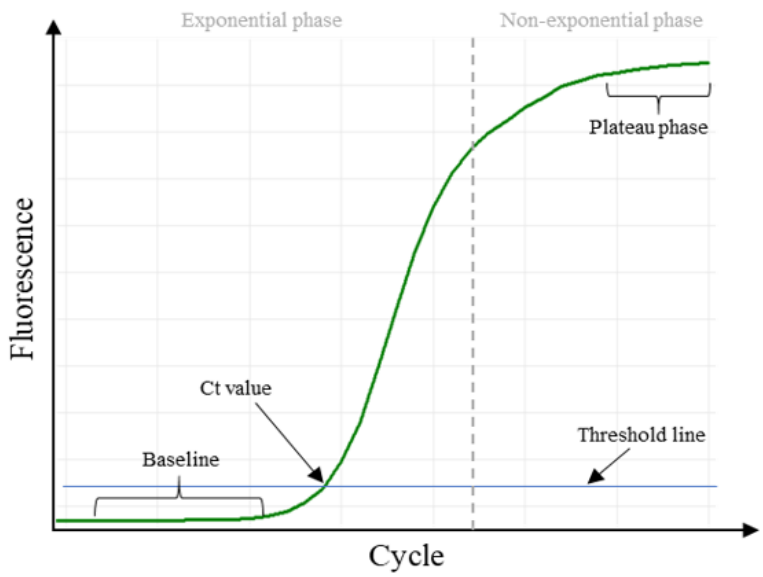


Figure 2: Parameters of an amplification curve.

3.3 Real-Time PCR

A real-time PCR allows the detection of DNA amplification in 'real time' and without the use of gel electrophoresis. For detection of the synthesised DNA, fluorescence markers are used that either intercalate into the double stranded DNA or hybridise with a short section of the DNA template. While hybridisation requires the specific design of a so-called fluorescence-reporter-probe, intercalating dyes can be used for any DNA sequence. Both markers have in common, that the increasing fluorescence signal is proportional to the number of synthesised DNA strands.

The principle of fluorescence-labelled probe systems will not be discussed further because intercalating dyes were used only during the experimental part of this master's thesis.

The mechanism of SYBR Green is illustrated in Figure 3.

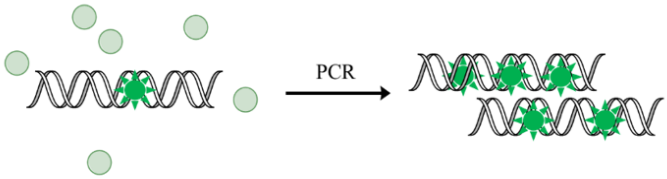


Figure 3: Intercalation of SYBR Green in dsDNA.

The fluorescence signal of unbound dye is very weak and only background signals are detected. As soon as the template DNA is amplified, the dye molecules intercalate into the dsDNA resulting in an increasing fluorescence signal. However, not only template DNA interacts with the dye molecules but unspecific sequences, for example primer dimers, which contributes to the detected fluorescence signal.

The difference between SYBR Green and EvaGreen is that the latter is a saturating dye. It can be used in high amounts without inhibiting the DNA polymerase, which is important for high-resolution melting (HRM) analysis. It allows the discrimination of DNA sequences with similar melting behaviours, e. g. when analysing genotype variants. [27]

3.3.1 High Resolution Melting Analysis

HRM allows the analysis of the melting behaviour of amplified DNA subsequent to the PCR. The amplification plot of a PCR may show correct amplification curves, but since intercalating dyes do not distinguish between template DNA and other double stranded oligonucleotides (e.g. primer dimers, non-specific PCR products), it is crucial to confirm the amplification of the correct dsDNA. This is done by gradually heating the amplified PCR products. Thereby, the helical structure is divided into two single strands, releasing the dye molecules, which can be observed in a decrease of the fluorescence signal. The melting temperature (T_m) is defined as the temperature at which half of the double strands are split up into the single strands.

The shorter a DNA strand, as in the case of primer dimers, the less energy is necessary to dissociate the double strand and the lower the melting temperature (Figure 4).

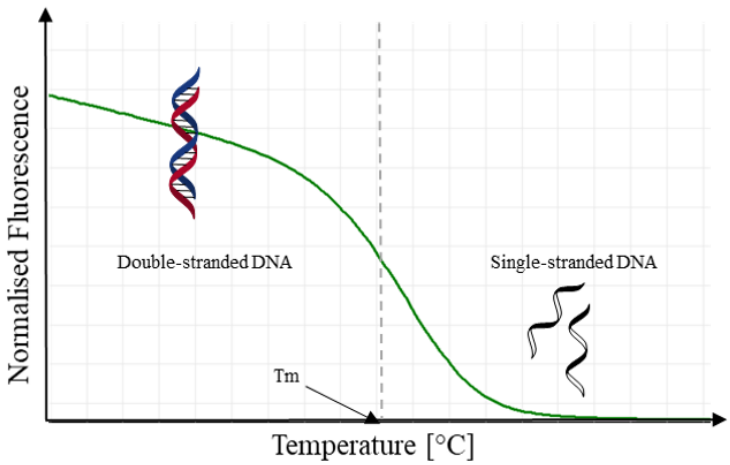


Figure 4: Example of an HRM curve.

The melting temperature depends on the composition of the bases. While for the base pairing of adenine (A) and thymine (T) two hydrogen bonds are involved, guanosine (G) and cytosine (C) interact via three hydrogen bonds (Figure 5). The more hydrogen bonds, the higher the energy required for the breakage.

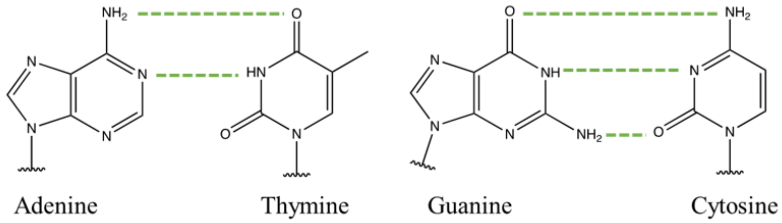


Figure 5: Hydrogen bonds (green) between complementary bases.

The melting plot is obtained by plotting the first derivative of fluorescence with respect to temperature (-dF/dT) against the temperature (Figure 6).

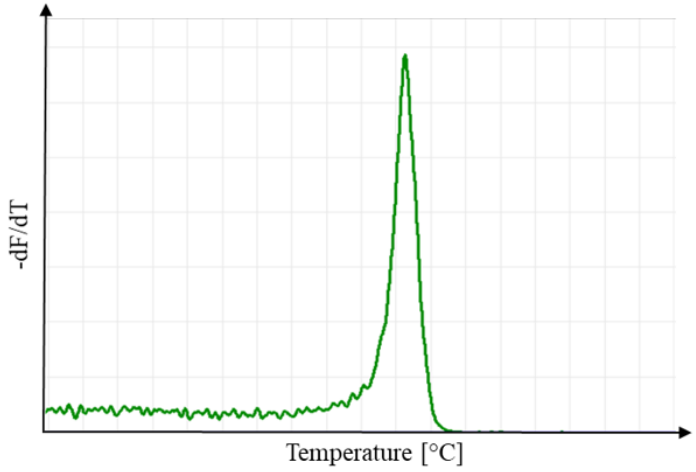


Figure 6: Example for the melting profile of a PCR product.

3.3.2 Genotyping

HRM analysis is a simpler and more cost-effective technique for the determination of different genotypes than other genotype technologies, e.g. sequencing. It allows the detection of sequence variations down to one base pair (e.g. SNP). Since the human organism contains two copies of each gene, the alleles can vary regarding a specific gene locus. During PCR both allelic variants will be amplified, if no allele-specific primers are used, which results in different melting curves. An example is given in Figure 7.

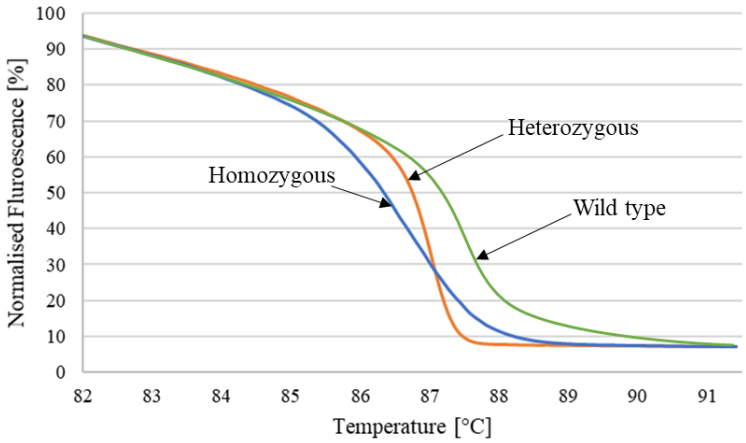


Figure 7: Example of a normalised melt plot for the different genotype variants.

Wild type refers to the genotype variant that most commonly occurs in a defined population (e.g. both alleles contain C/G at a specific gene locus). If a diploid organism is **heterozygous** regarding the same gene locus, one of the two alleles contains a mutation (e.g. one allele contains A/T, the other C/G). If both alleles contain a mutation at the same gene locus, the genotype is called **homozygous** (e.g. both alleles contain A/T).

The genotype can easily be determined by the melting behaviour of the PCR product. Since it takes more energy for the separation of a C/G base pair (wild type) than of an A/T base pair (homozygous), the melting temperature of the PCR product obtained for the wild type genotype variant will be higher than of the homozygous genotype variant.

Because of denaturation and re-annealing of the amplified dsDNA, homoduplexes and heteroduplexes are formed. While homoduplexes are perfectly paired DNA strands, heteroduplexes are characterised by one or more mismatched base pairs due to heterozygosity. As a consequence, the normalised HRM curve of the heterozygous genotype variant shows an intersection point with the normalised HRM curve of the homozygous genotype variant.

3.4 Primer Design

The selection of suitable primers for PCR amplification plays a crucial role for the successful analysis of a DNA template of interest. For the design of primers, it is recommended to follow some general guidelines to ensure specific binding of the primers. The primer design will be discussed in detail in the following section.

3.4.1 Primer Characteristics

The number of nucleotides of a primer sequence can vary between 16 and 28 but depends on the length of the DNA template, which normally ranges between 100 and 250 bp. If the primer sequence is too short, unspecific binding is more likely to occur. On the other hand, if the primer sequence is too long, primer dimer formation and secondary structures will be observed, which lead to a decrease in the efficiency of the PCR. [25]

Furthermore, the forward and the reverse primer should have matching melting temperatures, otherwise the annealing and subsequent amplification will fail. The optimal annealing temperature lies about 5 °C below the melting temperature of the primers. [23]

The latter depends on the nucleotide composition of the primers. In general, the G/C content should range between 35 and 65 %. Exceeding 65 % results in unspecific annealing, while a low G/C content leads to less stable hybridisation. T_m can be calculated by following equation:

$$T_m = 4 * (G + C) + 2 * (A + T)$$

However, the equation does not include the concentration of $MgCl_2$ in the buffer and the concentration of the primers. [24,25]

Another important requirement in the design of a primer set is the complexity of the sequence of the template DNA. The greater the variation of the nucleotide sequence the more probable the correct annealing. The latter is additionally influenced by the base composition of the primer at the 3'- end. At this end, the polymerase starts incorporating dNTPs, hence, the first three nucleotides ought to be complementary to the DNA template, while the specificity at the 5'-end is less important. Furthermore, the sequence of a primer should not contain more than four identical nucleotides in a row; this results in a higher probability for mismatching. [25]

3.5 Agarose Gel Electrophoresis

The use of agarose-gel electrophoresis revolutionised the analysis of DNA in molecular biology. The separation of nucleic acids using an agarose gel allows not only the identification but also purification of DNA after PCR. It successfully removes PCR components, such as short primers, dNTPs, salts and DNA polymerase.

Agarose is a linear polysaccharide, which is found in several forms of seaweed. Compared to polyacrylamide-gels it provides several advantages for the separation and clean-up of PCR

products. The preparation of the gel does neither demand a polymerisation initiator nor catalyst. Simply heating the agarose in buffer and cooling it down to room temperature leads to polymerisation. Furthermore, agarose is non-toxic, which makes its handling much more convenient. Compared to polyacrylamide-gel electrophoresis, agarose-gel electrophoresis requires the preparation of only one gel for the collection and subsequent separation of the DNA. Additionally, purified and separated PCR products can be further analysed by melting the gel and subsequent enzyme digestion. On the downside, agarose-gel electrophoresis is stated to be less efficient when separating DNA of low molecular weight. [28]

3.5.1 Separation Principle

The principle behind DNA separation is simple. The phosphate backbone of the nucleic acids is negatively charged. When applying an electric field, the DNA wanders from the negatively charged cathode to the positive anode. The higher the molecular weight of the DNA fragment, the slower the migration speed. Hence, the shorter the DNA fragment, the faster it can migrate through the gel. The approximate size of the nucleotide strand can be determined by comparing it with a suitable standard, a so-called DNA ladder.

3.5.2 Gel Concentration

The separation of nucleic acids demands a specific density of the gel, which depends on the molecular weight of the PCR products and usually ranges between 0.2 % and 3 %. For example, a 2 % agarose gel (w/v) resolves DNA fragment sizes between 200 and 1500 bp. Lower percentage of agarose in buffer, e.g. 1 % (w/v) separates DNA of 500 – 5000 bp. [28]
This means, if small DNA fragments are analysed, the percentage of agarose (w/v) should be increased.

3.5.3 Visualisation of the DNA

Before applying the DNA sample onto the gel, it is mixed with loading buffer because the sample would otherwise dissolve in the running buffer prior to separation. The density of the loading buffer enables the collection of the sample in the respective gel pocket. Additionally, the blue colour of the loading buffer facilitates the handling of the sample during loading and allows the pursuit of the separation progress. [28]
However, the loading buffer does not interact with the DNA fragments, hence, the detection of the PCR products on the gel must be done otherwise. One popular but rather toxic option is the

use of ethidium bromide, which is an intercalating dye that fluoresces under UV-light. A more environmentally friendly alternative is GelRed, which also intercalates in the DNA and allows the detection under UV-light.

3.6 Pyrosequencing

Gel electrophoresis allows the determination of the approximate fragment size of PCR products. However, when analysing DNA fragments of similar length but different sequence, gel electrophoresis reaches its limits. The verification of the correct PCR product, in other words the distinction between genetic variations regarding mutations or SNPs, can not only be done by high-resolution melting analysis but also by sequencing. In this master's thesis, pyrosequencing was used for the determination of the sequence of amplified DNA.

3.6.1 Principle

Pyrosequencing follows the principle of 'sequencing by synthesis'. This means, that the sequence of the nucleotide strand of interest is determined during its synthesis. This method allows the analysis of DNA sequences comprising 80-100 nucleotides, if the DNA sequence is known and genetic variations at certain positions are investigated. If the DNA sequence is unknown, it can be determined by de-novo-sequencing. [29]

For the successful determination of the DNA sequence, several components are required: DNA template, a sequencing primer, DNA polymerase, dNTPs, adenosine triphosphate (ATP) sulfurylase, adenosine phosphosulfate (APS), luciferase and apyrase.

Prior to sequencing, the DNA fragment of interest is amplified using polymerase chain reaction. Depending on template used for pyrosequencing, the amplified DNA must carry a biotin group at the end of its upper or lower strand. This can be achieved by using a biotinylated reverse or forward primer for PCR amplification. The amplified PCR product is then submitted to pyrosequencing. The biotin group enables the separation of the template DNA during pyrosequencing as follows: Streptavidin-covered magnetic beads interact with the biotin group and form a strong covalent bond. Due to the magnetism of the beads the double strands can be separated after denaturation by applying a magnetic field. It also purifies the template strand from other components of the PCR and allows the annealing of the sequencing primer.

The primer functions as a starting point for the incorporation of dNTPs initiated by the DNA polymerase. During incorporation, pyrophosphate is released due to the formation of a covalent bond between two monosaccharides via a phosphate group. The pyrophosphate is then converted to ATP with the help of ATP sulfurylase and in presence of APS. In the final step, luciferin is oxidised by luciferase and its cofactor ATP to oxyluciferin and light. The light that is proportional to the amount of released pyrophosphate is detected by the PSQ instrument. After the addition of each dNTP, apyrase catalyses the degradation of dNTPs that have not been incorporated to deoxynucleoside diphosphate (dNDP), deoxynucleoside monophosphate (dNMP) and phosphate. [30]

The emitted light signal is shown as a peak in the pyrogram. The signal height correlates with the number of incorporated dNTPs (Figure 8). An overview of the reactions taking place during pyrosequencing is shown in Figure 9.

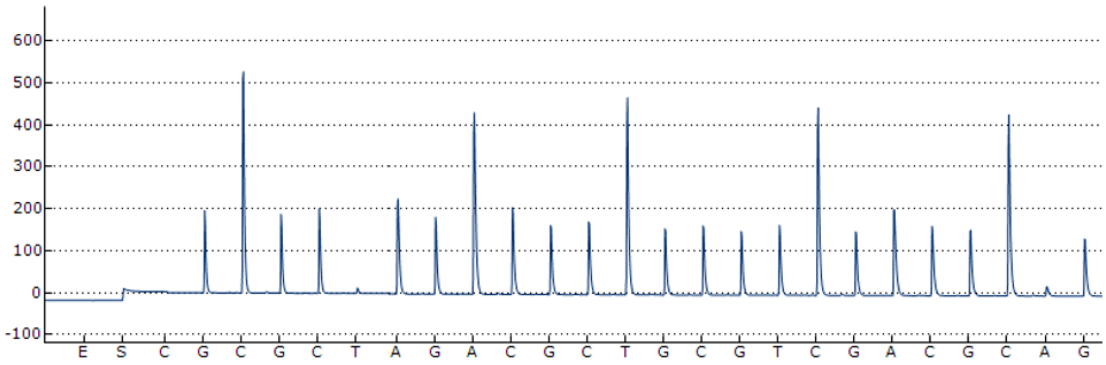


Figure 8: Example of a pyrogram. The y-axis represents the signal intensity, the x-axis the order of dNTPs that are added to the sample; E = enzyme, S = substrate.

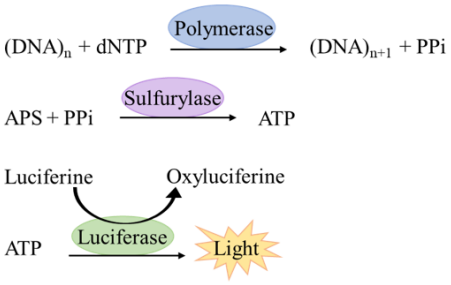


Figure 9: Schematic illustration of pyrosequencing reactions.

Although pyrosequencing allows the determination of DNA sequences, which is crucial for the identification of genetic variations and alterations, the risk of cross-contamination is quite high.

4 Material and Methods

Working with DNA bears the risk of contamination; thus, all work benches were cleaned with DNA Exitus Plus™ IF prior to handling with samples to remove DNA residues. Furthermore, the experimental setup was divided into parts working with and without DNA. The preparation of master mixes took place in a different workstation (laboratory 1) than DNA extraction (laboratory 2) and addition of DNA to the master mix (laboratory 3) to avoid contamination. Handling with amplicons was conducted in laboratory 4. In general, amplicons were kept in a separate fridge, where no other reagents or DNA extracts were stored.

4.1 Sampling

The samples used for the optimisation of DNA extraction protocols consisted of one FF (F3) and one ethanol-fixed (F2) human foetus and were provided by Eduard Winter, collection manager of the Pathological-Anatomical Museum in Vienna. For further investigation of ODC-related SNPs, two formalin-fixed human foetuses (#003, #004) featuring severe facial abnormalities (Figure 10) were also collected from the museum. The project was reviewed and approved by an ethic committee.

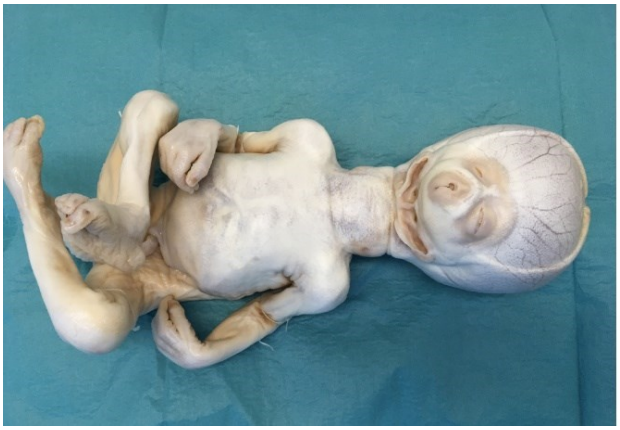


Figure 10: Formalin-fixed foetus #003 showing severe facial abnormalities.

4.1.1 Foetuses Without Facial Dysmorphia

The FF and ethanol-fixed foetuses without facial dysmorphia should serve as a control group for the investigation of ODC-related SNPs and were used for the optimisation of the DNA extraction protocols. Therefore, several tissue types, such as cerebrum, cerebellum, liver, heart,

lung, stomach, spleen, intestine, umbilical cord, muscle and bone were collected under sterile conditions with the help of Dr. Andreas Glowania. Approximately one to two grams of each tissue type (Table 1) were collected in sterile 15 mL Falcon tubes and immediately stored at -20 °C until further use.

Table 1: Overview of specimen name and collected tissue types.

Name		F2	F3
tissue type	cerebrum	x	x
	cerebellum	x	x
	liver	x	x
	heart	x	x
	lung	x	x
	stomach	x	x
	spleen	x	x
	intestine	x	x
	umbilical cord	x	x
	muscle	x	x
	bone	x	x

4.1.2 Foetuses with Severe Facial Dysmorphia

The sampling of the much bigger foetuses with craniofacial abnormalities was conducted with the aid of Dr. Andreas Glowania and Rudolf Schmidt (Intramed) by using a minimal-invasive 3D-based technology for the examination of each foetus. An endoscope was carefully introduced into the foetus in order to determine the morphology and preservation condition of the specimen. Afterwards, approximately one to two grams of various tissue types, such as cerebrum, cerebellum, liver, heart, lung, stomach, spleen, intestine, skin, umbilical cord, muscle and bone were carefully removed with sterile medical tools, transferred to 15 mL Falcon tubes and stored at -20 °C until further use. An overview of the collected tissue types of both foetuses with otocephaly is shown in Table 2.

Table 2: Overview of specimen number and collected tissue types.

Specimen number		#003	#004
tissue type	cerebrum	x	x
	cerebellum	x	
	liver	x	x
	heart	x	x
	lung	x	x
	stomach	x	x
	spleen	x	x
	intestine	x	x
	umbilical cord	x	x
	muscle	x	x
	bone	x	x

4.2 DNA Extraction

For the extraction of genomic DNA from foetal tissue, several extraction procedures were tested regarding yield, purity, DNA integrity and PCR amplifiability. These methods consisted of two commercially available DNA extraction kits, a CTAB-based extraction and the organic phenol-chloroform extraction. The protocols for the column-based kits and phenol-chloroform extraction were further varied, in order to increase DNA quantity and quality. The Chapters 4.2.3 to 4.2.6 give an overview of the applied standard extraction procedures. The variations of these protocols are described in more detail in Table 3, Table 5 and Table 10.

4.2.1 Reagents

QIAamp® DNA Mini Kit (Qiagen)

QIAamp® DNA FFPE Tissue Kit (Qiagen)

QIAquick Spin Columns (Qiagen)

Proteinase K [20 mg/mL] (Qiagen)

RNase A [100 mg/mL] (Qiagen)

PBS buffer

CTAB extraction buffer

- 0.02 M EDTA
- 0.1 M tris(hydroxymethyl)aminomethane (Tris)
- 2 % CTAB (w/v)
- 1.4 M sodium chloride (NaCl)

adjusted to pH 8 with 4 M HCl

Chloroform/isoamyl alcohol (24:1, v/v)

CTAB precipitation solution

- 5 g/L CTAB
- 2.34 g/L NaCl

1.2 M NaCl

10 x RNase buffer

- 3 M NaCl
- 100 mM Tris
- 50 mM EDTA

Adjusted to pH 7.4 with 4 M HCl

- RNase A [10 mg/mL]

Ethanol 70 % (v/v)

Phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v; saturated with 10 mM Tris, pH 8, 1 mM EDTA; Sigma-Aldrich)

Roti®-phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v; pH 7.5 - 8.0; Roth)

SDS 20 % (w/v)

2.26 x TE9 buffer

- 113 mM Tris
- 45.2 mM EDTA

- 22.6 mM NaCl

Reagent DX (Qiagen)

7.5 M NH_4Ac

EB buffer (Qiagen)

4.2.2 Tissue Homogenisation

For the optimisation of tissue digestion, samples were homogenised prior to lysis using either the Fast Prep-24 5G Tissue Homogeniser or a syringe equipped with needles of different sizes and diameters.

In the first case, 25 mg of sample was cut into pieces of about 1 x 1 x 1 mm using a sterile scalpel, transferred to a 2 mL impact resistant tube containing a specific lysing matrix. A lysing matrix consisted of beads of various materials and sizes, e. g. ceramic spheres (matrix D), ceramic beads (matrix M), zirconium oxide beads (matrix I) or stainless-steel beads (matrix S). An overview is given in Appendix A (Table 45).

Then, extraction buffer was added and the sample was homogenised with the tissue disruptor by applying various parameter settings. After completion of tissue homogenisation, the sample was submitted to lysis.

The utilization of a syringe took place during lysis; 25 mg of tissue was cut into pieces as mentioned above, mixed with extraction buffer and proteinase K and lysed. After certain periods of incubation, the lysate was drawn up into a 1 mL syringe using needles of different sizes and diameters. Due to shear forces the tissue particles were torn apart resulting in disruption of the cells.

4.2.3 DNA Extraction Using QIAamp DNA Mini Kit

The extraction of DNA using the QIAamp DNA Mini Kit (Qiagen) was conducted by following the manufacturer's protocol. 25.0 mg of (homogenised) tissue was mixed in a 1.5 mL Eppendorf tube with 180 µL ATL buffer and 20 µL proteinase K [20 mg/mL] by shortly vortexing. The sample was then incubated on a shaking platform at 56 °C, 800 rpm for 2.5 h. After brief centrifugation for the removal of drops from inside the lid, 4 µL of RNase A [100 mg/mL] was added and mixed by pulse-vortexing for 15 s. The mixture was incubated for 2 min and briefly centrifuged before adding 200 µL AL buffer. The solution was pulse-vortexed for 15 s and incubated at 70 °C for 10 min. Next, the sample was centrifuged briefly and mixed with 200

µL abs. ethanol (96-100 %) by pulse-vortexing for 15 s. After short centrifugation, the mixture was applied to the QIAamp Mini spin column (provided with a 2 mL collection tube) and centrifuged at 8000 rpm for 1 min. The filtrate was discarded, and the spin column transferred to a new 2 mL collection tube, before adding 500 µL AW1 buffer. After centrifugation at 8000 rpm for 1 min, the column was placed into a new 2 mL collection tube and 500 µL of AW2 buffer was added. The spin-column was centrifuged at full speed (12000 rpm, 3 min), transferred to a new 2 mL collection tube and centrifuged once more at full speed for 1 min.
For the retrieval of bound DNA, the spin-column was transferred to a 1.5 mL Eppendorf tube, before pipetting 200 µL[1] of AE buffer onto the stationary phase of the column and incubation at room temperature (RT) for 1 min. The DNA was then obtained by centrifugation at 8000 rpm for 1 min.
To make sure all bound DNA was eluted, the last step was repeated two more times. Each eluate was collected in a separate 1.5 mL Eppendorf tube and stored at -20 °C until further use.

4.2.3.1 Modification of the Extraction Protocol

The protocol of the QIAamp DNA Mini Kit described in Chapter 4.2.3 (standard protocol $S_{Mini\ Kit}$) was modified regarding certain parameters. For better understanding, each variation was given an abbreviation. The parameters for each variation are detailed in Table 3.

Table 3: Variations of the QIAamp DNA Mini Kit extraction protocol including following parameters: specimen fixation (ethanol-fixed (F2) or formalin fixed (F3)), type of tissue, rehydration prior to lysis, type of homogenisation, lysis duration, changes in the reaction volume, columns and final volume of DNA extract. Each protocol was carried out with two aliquots of the respective type of tissue (F2 and/or F3); exception: V1a and V1b comprised one aliquot of liver tissue (F2).

Variation abbreviation	Foetus/ type of tissue	Rehydration	Homogenisation	Lysis [h]	Reaction volume	Columns	Volume DNA extract [µL]
V1a	F2/liver	-	-	24	180 µL ATL buffer after 4 h lysis	acc. pr.[2]	200
V1b	F2/liver	-	-	24	180 µL ATL buffer, 20 µL prot. K after 4 h lysis	acc. pr.	200
V2a	F2/liver F3/liver	-	syringe	24	s. u. [3]	acc. pr.	50
V2b	F3/liver	-	syringe	48	s. u.	acc. pr.	50

[1] When 200 µL AE buffer was used for DNA extraction, the samples were further purified and enriched in DNA by ethanol precipitation. The protocol can be found in Appendix B.
[2] acc. pr. = according to protocol
[3] s. u. = scaled up to double reaction volume regarding ATL buffer, RNase A, AL buffer and ethanol

V3a	F3/liver	60 °C, 800 rpm, 24 h	syringe	24	s. u.	PCR columns	50
V3b	F3/liver	60 °C, 800 rpm, 24 h	syringe	24	s. u.	acc. pr.	50
V3c	F3/liver	-	syringe	24	s. u.	PCR columns	50
V3d	F3/liver	-	syringe	24	s. u.	acc. pr.	50
V4	F2/liver, cerebrum F3/liver, cerebrum	-	tissue disruptor matrix D	18	s. u.	acc. pr.	50

Variation **V1a** consisted of additional 180 µL ATL buffer, which was added after 4 h of tissue digestion because the tissue had soaked up the liquid completely. For **V1b**, additional 180 µL ATL buffer and 20 µL proteinase K [20 mg/mL] were added after 4 h of lysis.

For **V2a** and **V2b** a 1 mL syringe and needles of following sizes and diameters were used for tissue homogenisation: 1.2 x 38 mm, 0.6 x 30 mm and 0.5 x 25 mm. Furthermore, the volume of ATL buffer, RNase A, AL buffer and ethanol was doubled. Formalin-fixed and ethanol-fixed liver tissue was analysed with variation V2a. For V2b the digestion duration was increased to 48 h using formalin-fixed liver tissue.

A rehydration step was included in variation **V3a** and **V3b** and had been recommended by Moraes-Barros *et al.* [31], who argued, that it was crucial for complete tissue digestion. The step consisted of incubation of tissue in 400 µL AE buffer at given time and temperature. Afterwards, the sample was centrifuged briefly and submitted to the next step.
For comparison, the rehydration step was omitted in protocols **V3c** and **V3d**. In case of V3a and V3c, QIAquick spin columns were used as they bind DNA fragments ranging from 100 bp to 10 kb. [32]
QIAquick spin columns are normally applied to clean up PCR products. Since the extracted DNA from formalin-fixed, long-term preserved tissue is often severely fragmented, the applicability of QIAquick spin columns for DNA binding was investigated.

Liver and cerebrum tissue from formalin-fixed as well as ethanol-fixed foetus were used for variation **V4**. The tissue was homogenised with the Fast Prep-24 5G Tissue Homogeniser using lysing matrix D. The settings are given in Table 4.

Table 4: Settings for homogenisation of tissue for protocol V4 using Fast Prep-24 5G Tissue Homogeniser.

Lysing matrix	Speed [m/s]	Adapter	Time [s]	Quantity	Quantity unit	Repetition
D	6.0	HiPrep	30	1	mL	2

Between the repetitions, the sample was stored on ice to prevent the tissue from overheating. Furthermore, the lysis step was reduced to 18 h due to a more convenient and less time-consuming workflow.

4.2.4 DNA Extraction Using QIAamp DNA FFPE Tissue Kit

DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen) was carried out in compliance with the manufacturer's instructions, omitting the step for the removal of paraffin, since the sample had not been embedded in paraffin. Furthermore, the volume of ATL buffer was doubled.

25.0 mg of liver tissue was cut into 1 x 1 x 1 mm pieces and mixed in a 1.5 mL Eppendorf tube with 360 µL ATL buffer and 20 µL proteinase K [20 mg/mL] by vortexing shortly. The sample was then incubated on a shaking platform at 56 °C, 800 rpm for 24 h. Each two hours, the sample was vortexed thoroughly and centrifuged briefly. For the removal of cross-links induced by formaldehyde, the sample was further incubated at 90 °C for 1 h. After short centrifugation and cooling to RT, 2 µL RNase A [100 mg/mL] was added, incubated at RT for 2 min and mixed with 200 µL AL buffer by vortexing thoroughly. The solution was briefly centrifuged before adding 200 µL of abs. ethanol and vortexing thoroughly. After brief centrifugation, the lysate was transferred to the QIAamp MinElute column (provided with a 2 mL collection tube) and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and the spin column transferred to a new 2 mL collection tube, before adding 500 µL AW1 buffer. After centrifugation at 8000 rpm for 1 min, the column was placed into a new 2 mL collection tube and 500 µL of AW2 buffer was added. The spin-column was centrifuged at 8000 rpm for 1 min, transferred to a new 2 mL collection tube and centrifuged once more at full speed (12000 rpm) for 3 min. For the retrieval of bound DNA, the spin-column was transferred to a 1.5 mL Eppendorf tube, before pipetting 50 µL of ATE buffer onto the stationary phase of the column and incubation at RT for 1 min. The DNA was then obtained by centrifugation at 8000 rpm for 1 min.

To make sure all bound DNA was eluted, the last step was repeated two more times. Each eluate was collected in a separate 1.5 mL Eppendorf tube and stored at -20 °C until further use.

4.2.4.1 Modification of the Extraction Protocol

Following chapter describes the modifications regarding the protocol described in Chapter 4.2.4 (standard protocol $S_{FFPE\ Kit}$). The parameters for each variation are summarised in Table 5.

Table 5: Variations of the QIAamp DNA FFPE Tissue Kit extraction protocol including following parameters: specimen fixation (ethanol-fixed (F2) or formalin fixed (F3)), type of tissue, washing step prior to lysis, type of homogenisation, lysis duration, formaldehyde-modification-reversal step after lysis, changes in the reaction volume, columns and final volume of DNA extract. Each protocol was carried out with two aliquots of the respective type of tissue (F2 and/or F3); exception: V7, V8a and V8b comprised one aliquot of the respective type of tissue (F3).

Variation abbreviation	Foetus/ type of tissue	Washing	Homogenisation	Lysis [h]	Formaldehyde-modification-reversal	Reaction volume	Columns	Volume DNA extract [µL]
V5	F3/liver	-	tissue disruptor matrix A or D	24	90 °C, 800 rpm, 1 h	s. u. + 2 µL carrier RNA for flow through	acc. pr.	50
V6a	F2/liver F3/liver	-	tissue disruptor matrix D	24	90 °C, 800 rpm, 1 h	s. u. + 400 µL prot. K	acc. pr.	50
V6b	F2/liver F3/liver	-	tissue disruptor matrix D	24	-	s. u. + 400 µL prot. K	acc. pr.	50
V7	F3/liver	25 °C, 800 rpm, 1 h (2x)	tissue disruptor either matrix A, A2, D, D1, M, M2, I, S or F (see Appendix A)	24	90 °C, 800 rpm, 1 h (2x)	2x 100 µL prot. K; 0.05 % Reagent DX (v/v)/180 µL ATL buffer	acc. pr.	50
V8a	F3/liver, intestine, cerebrum muscle, heart, bone	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix M2	24	90 °C, 800 rpm, 1 h (2x)	2x 100 µL prot. K; 0.075 % Reagent DX (v/v)/180 µL ATL buffer	acc. pr. and PCR columns	50
V8b	F3/bone	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix S	24	90 °C, 800 rpm, 1 h (2x)	2x 100 µL prot. K; 0.075 % Reagent DX (v/v)/180 µL ATL buffer	acc. pr. and PCR columns	50

For variation **V5**, the tissue was homogenised using the tissue disruptor. Differences in the homogenisation efficiency between lysing matrix A and D as well as homogenisation medium (PBS or ATL buffer) were compared. Therefore, two samples were homogenised in 160 µL PBS using either matrix A or D. Two other samples were homogenised in 360 µL ATL buffer. The first two samples were then mixed with 200 µL ATL buffer and 40 µL proteinase K [20 mg/mL], the other two samples with 40 µL proteinase K [20 mg/mL]. All mixtures were incubated at 56 °C, 800 rpm for 24 h.
The formaldehyde-modifications were reversed after completion of lysis by incubation at 90 °C, 800 rpm for 1h. The mixture was cooled to RT and briefly centrifuged before continuing with the DNA extraction.
Additionally, after applying the sample solution onto the spin columns, the flow through was collected, transferred to a new 1.5 mL Eppendorf tube and heated at 95 °C for 10 min for the inactivation of RNase A. The sample was cooled to RT, mixed with 2 µL carrier RNA [1 µg/µL] and applied to a new spin column prior to DNA extraction. The carrier RNA should enhance binding efficiency of DNA fragments to the spin column.

Formalin-fixed and ethanol-fixed liver tissue were analysed by applying protocols **V6a** and **V6b**. The tissue was homogenised with lysing matrix D using the tissue disruptor. While for V6a a formaldehyde-modification-reversal step was included after tissue lysis, it was omitted in V6b. For both variations, the volume of ATL buffer, RNase A, AL buffer and ethanol was doubled, and the volume of proteinase K was increased from 20 µL to 400 µL.

The washing step for protocol **V7** consisted of incubation of tissue in 80 µL PBS buffer at given time and temperature. Afterwards, the sample was centrifuged at 8000 rpm for 1 min and the supernatant was discarded by pipetting. Another 80 µL PBS was added and the washing step was repeated as before. Subsequent to centrifugation (8000 rpm, 1 min) and discarding the supernatant, the tissue was mixed with 180 µL ATL buffer containing 0.05 % Reagent DX (v/v). The latter was used for inhibiting excessing foam formation during homogenisation. Homogenisation was carried out using various matrices aiming to investigate, which lysing matrix is most suitable for DNA extraction regarding DNA yield. The settings for the homogenisation of the tissue are summarised in Table 6.

Table 6: Settings for the homogenisation of tissue with matrices A, A2, D, D1, M, M2, I, S and F for protocol V7 using Fast Prep-24 5G Tissue Homogeniser.

Lysing matrix	Speed [m/s]	Adapter	Time [s]	Quantity	Quantity unit	Repetition
A for matrix A	6.0	HiPrep	40	1	mL	3
A for matrix A2	6.0	HiPrep	40	1	mL	3
D for matrix D	6.0	HiPrep	40	1	mL	3
D for matrix D1	6.0	HiPrep	40	1	mL	3
M for matrix M	6.0	HiPrep	40	1	mL	3
M for matrix M2	6.0	HiPrep	40	1	mL	3
I for matrix I	6.0	HiPrep	40	1	mL	3
S for matrix S	6.0	HiPrep	40	1	mL	3
F for matrix F	6.0	HiPrep	40	1	mL	3

Prior to lysis, the matrices were removed. However, not all of them could be separated from the liquid medium. The ceramic spheres from matrix D/D1 and the purple garnet from matrix A/A1, matrix I and F remained in the sample solution and were separated by pipetting off the liquid, when the mixtures were applied to the spin columns.

Next, the sample was mixed with 100 µL proteinase K [20 mg/mL] and incubated at 56 °C, 800 rpm for 18 h. Afterwards, the tube was briefly centrifuged and incubated at 90 °C, 800 rpm for 1 h. The mixture was cooled to RT and mixed with another 100 µL proteinase K [20 mg/mL]. Lysis was continued at 56 °C, 800 rpm for 6 h.

The sample was further incubated at 90 °C, 800 rpm for 1 h to reverse formaldehyde-modifications. The mixture was briefly centrifuged and cooled to RT, before continuing with the DNA extraction as instructed by the manufacturer's protocol.

The modified protocols **V8a** and **V8b** were carried out using 100 mg of tissue. For V8a, lysing matrix M2 was used for tissue homogenisation, for V8b, lysing matrix S was considered most suitable for the homogenisation of bones. The settings for the tissue disruptor for V8a and V8b are given in Table 7 and Table 8, respectively.

Compared to V7, the tissue was homogenised in 180 µL ATL buffer, containing 0.075 % Reagent DX (v/v).

Additionally, after applying the sample to the QIAamp MinElute spin column, the flow through was collected and applied to the QIAquick spin column. The extraction was continued for both spin column types as described in Chapter 4.2.4.

Table 7: Settings for the homogenisation of tissue with lysing matrix M2 for protocol V8a using Fast Prep-24 5G Tissue Homogeniser.

Lysing matrix	Type of tissue	Speed [m/s]	Adapter	Time [s]	Quantity	Quantity unit	Repetition
M for matrix M2	liver	6.0	HiPrep	40	1	mL	3
M for matrix M2	intestine	6.0	HiPrep	40	1	mL	3
M for matrix M2	cerebrum	6.0	HiPrep	40	1	mL	3
M for matrix M2	muscle	6.0	HiPrep	40	1	mL	3
M for matrix M2	heart	6.0	HiPrep	40	1	mL	3
M for matrix M2	bone	6.0	HiPrep	1 x 20 followed by 2 x 40	1	mL	3

Table 8: Settings for the homogenisation of bones with lysing matrix S for protocol V8b using Fast Prep-24 5G Tissue Homogeniser.

Lysing matrix	Speed [m/s]	Adapter	Time [s]	Quantity	Quantity unit	Repetition
S	6.0	HiPrep	1 x 20 followed by 3 x 40	1	mL	4

4.2.5 DNA Extraction Using CTAB

CTAB extraction is a standard method for the extraction of DNA from several different sample materials and is frequently used in our working group. For this reason, the procedure was also tried for the extraction of DNA from foetal FF tissue.

25.0 mg of liver tissue was mixed in a 1.5 mL Eppendorf tube with 250 µL CTAB extraction buffer and 20 µL proteinase K [20 mg/mL] by vortexing shortly. The sample was incubated on a shaking platform at 50 °C, 1400 rpm for 24 h. For DNA extraction, 250 µL lysate was added to 300 µL chloroform-isoamyl alcohol mixture provided in a 1.5 mL Eppendorf tube and vortexed for 30 s. After centrifugation at 14600 rpm for 15 min, 165 µL aqueous supernatant from the previous step was mixed with 660 µL CTAB precipitation solution provided in a new 1.5 mL Eppendorf tube by inverting the tube once. The solution was incubated at RT for 1 h, centrifuged afterwards at 14600 rpm for 15 min and the supernatant discarded. Then, 225 µL NaCl-solution and 25 µL 10x RNase buffer was mixed with 2.5 µL RNase A [100 mg/mL] and added to the pellet consisting of DNA-CTAB complexes. The sample was incubated at 56 °C, 1000 rpm for 10 min. It was further centrifuged briefly before adding 250 µL chloroform-isoamyl alcohol mixture. After vortexing for 30 s and centrifugation at 14600 rpm for 15 min,

200 µL of the aqueous solution was added to 200 µL isopropanol provided in a 1.5 mL Eppendorf tube. The solution was incubated at -20 °C for 1 h and centrifuged at 14600 rpm for 15 min, before pipetting off the supernatant and adding 250 µL 70 % ethanol (v/v) to the remaining pellet. Next, the sample was vortexed for 30 s, centrifuged (14600 rpm, 10 min) and the supernatant discarded by pipetting. The DNA pellet was dried at 60 °C for 5 min. After complete drying, 50 µL AE buffer (from QIAamp DNA Mini Kit) was added to the sample, followed by incubation at 70 °C, 1000 rpm for 20 min. It was then cooled to RT, briefly centrifuged and stored at -20 °C until further use.

4.2.6 DNA Extraction Using Phenol-Chloroform-Isoamyl Alcohol

For the organic-based DNA extraction, 25.0 mg of tissue (liver and heart) was transferred to a 2 mL impact resistant tube and washed with 80 µL phosphate buffered saline (PBS) at 25 °C, 800 rpm for 1 h. After centrifuging the sample (8000 rpm, 1 min) and discarding the supernatant, the tissue was washed as before. The mixture was centrifuged (8000 rpm, 1 min) and the supernatant was pipetted off. Then, 15 µL 20 % SDS (w/v), 122.9 µL 2.26x TE9 buffer and 154.8 µL RNase free H_2O were added, followed by the tissue homogenisation using the tissue disruptor (lysing matrix M2). The settings are given in Table 9.

Prior to lysis the matrix was removed. The sample was mixed with 9.13 µL proteinase K [20 mg/mL], vortexed thoroughly and incubated at 56 °C, 800 rpm for 18 h. It was briefly spun down, and further incubated at 90 °C for 1 h. After cooling to RT, 5.69 µL proteinase K [20 mg/mL] was added and the lysis was continued (56 °C, 800 rpm, 6 h).

The lysate was briefly centrifuged and once more heated to 90 °C for 1 h. It was cooled to RT and 700 µL PC[4] (Roti®-phenol-chloroform-isoamyl alcohol; Roth) was added to the sample, vortexed for 15 s and centrifuged at 14000 rpm for 5 min. The upper aqueous phase was transferred to a new 1.5 mL Eppendorf tube, mixed with 700 µL PC by vortexing for 15 s and centrifuged at 14000 rpm for 5 min. Again, the upper aqueous phase was transferred to a new 1.5 mL Eppendorf tube and mixed with 750 µL EtOH/NH_4Ac (8:1, v/v) by inverting the tubes four times. The sample was left at RT for 1 h, before centrifugation at 4 °C, 14000 rpm for 45 min. The supernatant was carefully discarded by pipetting and the remaining pellet washed with 1 mL 70 % EtOH (v/v). After centrifugation (RT, 14000 rpm, 15 min), the supernatant was

[4] The solution had been stored in the fridge at 4°C for about 6 years. Thus, it was very likely, that the phenol had already been (partly) oxidised

again discarded by pipetting and the DNA pellet dried at RT for 20 min. The pellet was resuspended in 50 µL EB buffer by incubation at 25 °C, 800 rpm for 2 h.

Table 9: Settings for the homogenisation of tissue for protocol $S_{FFPE\ Kit}$ using Fast Prep-24 5G Tissue Homogeniser.

Lysing matrix	Speed [m/s]	Adapter	Time [s]	Quantity	Quantity unit	Repetition
M	6.0	HiPrep	40	1	mL	3

4.2.6.1 Modification of the Extraction Protocol

The protocol described in Chapter 4.2.6 (standard protocol S_{PC}) was modified regarding several parameters and is detailed below (Table 10).

Table 10: Variations of the PC extraction protocol including following parameters: specimen fixation (ethanol-fixed (F2) or formalin-fixed (F3)), type of tissue, washing step prior to lysis, type of homogenisation, lysis duration, formaldehyde-modification-reversal step after lysis and final volume of DNA extract. Protocols S_{PC}, V9a, V9b and V11 were carried out with one aliquot of the respective type of tissue (F3), protocols V10 and V12b were carried out with two aliquots of the respective type of tissue (F3) and protocol V12a was carried out with four aliquots of liver tissue (F3).

Variation abbr.	Foetus/ type of tissue	Washing	Homogenisation	Lysis [h]	Formaldehyde-modification-reversal	Volume DNA extract [µL]	Reaction volume	PC- solution
V9a	F3/liver, heart	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix M2	24	90 °C, 800 rpm, 1 h (2x)	50	15 µL SDS 20 % (w/v), 132.5 µL 2.26x TE9, 100 µL prot. K; 57.5 µL prot. K	Roti®-phenol-chloroform-isoamyl alcohol (Roth); stored at 4 °C for 6 years
V9b	F3/liver, heart	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix M2	24	90 °C, 800 rpm, 1 h (2x)	50	15 µL SDS 20 % (w/v), 122.9 µL 2.26x TE9, 154.8 µL RNase free H_2O, 9.13 µL prot. K; 5.69 µL prot. K 15 µL SDS 20 % (w/v), 132.5 µL 2.26x TE9, 100 µL prot. K; 57.5 µL prot. K	phenol-chloroform-isoamyl alcohol 25:24:1 (v/v/v) saturated with 10 mM Tris, pH 8, 1 mM EDTA (Sigma-Aldrich)
V10	F3/liver, heart	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix M2	18	90 °C, 800 rpm, 1 h	50	15 µL SDS 20 % (w/v), 132.5 µL 2.26x TE9, 157.5 µL prot. K	phenol-chloroform-isoamyl alcohol 25:24:1 (v/v/v) saturated with 10 mM Tris, pH 8, 1

								mM EDTA (Sigma-Aldrich)
V11	F3/liver	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix A, A2, D, D1, M, I, F or without homogenisation	18	90 °C, 800 rpm, 1 h	50	15 µL SDS 20 % (w/v), 132.5 µL 2.26x TE9, 157.5 µL prot. K	phenol-chloroform-isoamyl alcohol 25:24:1 (v/v/v) saturated with 10 mM Tris, pH 8, 1 mM EDTA (Sigma-Aldrich)
V12a	F3/liver	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix M2	18	90 °C, 800 rpm, 1 h	50	15 µL SDS 20 % (w/v), 132.5 µL 2.26x TE9, 157.5 µL prot. K/ RNase A treatment	Roti®-phenol-chloroform-isoamyl alcohol (Roth); fresh solution
V12b	F3/liver	25 °C, 800 rpm, 1 h (2x)	tissue disruptor matrix M2	18	90 °C, 800 rpm, 1 h	100	100 mg tissue; scaled up to 4 x reaction volume regarding PBS, lysing buffer, RNase A and PC	Roti®-phenol-chloroform-isoamyl alcohol (Roth); fresh solution

V9a and **V9b** differed from the standard protocol S_{PC} in the lysis buffer composition and proteinase K concentration. The tissue was homogenised in 15 µL SDS (20 %, w/v) + 132.5 µL 2.26x TE9 buffer (tissue homogeniser settings according to Table 9). Then, the volume of proteinase K was increased to 100 µL during the first 18 h lysis, and 57.5 µL during the 6 h lysis. V9a differed from V9b in the PC solution used for DNA extraction. The extraction protocol was continued according to S_{PC}.

For easier handling, a reduced digestion period was investigated in **V10**. The homogenisation was carried out as described in S_{PC}. Afterwards, the sample was mixed with 157.5 µL proteinase K [20 mg/mL], vortexed thoroughly and incubated at 56 °C, 800 rpm for 18 h. Then, the mixture was submitted to the formaldehyde-modification-reversal step (90 °C, 800 rpm, 1 h). The sample was cooled to RT and the extraction was continued with the addition of 700 µL PC as described in S_{PC}.

V11: Homogenisation was carried out using various matrices aiming to investigate, which lysing matrix is most suitable for DNA extraction regarding yield. The settings for the homogenisation using the tissue disruptor are summarised in Table 11.

Table 11: Settings for tissue homogenisation with matrices A, A2, D, D1, M, M2, I, S and F for V11 using Fast Prep-24 5G Tissue Homogeniser.

Lysing matrix	Speed [m/s]	Adapter	Time [s]	Quantity	Quantity unit	Repetition
A for matrix A	6.0	HiPrep	40	1	mL	2
A for matrix A2	6.0	HiPrep	40	1	mL	2
D for matrix D	6.0	HiPrep	40	1	mL	2
D for matrix D1	6.0	HiPrep	40	1	mL	2
M for matrix M	6.0	HiPrep	40	1	mL	2
I for matrix I	6.0	HiPrep	40	1	mL	2
F for matrix F	6.0	HiPrep	40	1	mL	2

Prior to lysis, the matrices were removed. However, not all of them could be separated from the liquid medium. The ceramic spheres from matrix D/D1 and the purple garnet from matrix A/A2 remained in the sample solution.
The sample was mixed with 157.5 µL proteinase K [20 mg/mL], thoroughly vortexed and incubated to 56 °C, 800 rpm for 18 h. Then, the mixture was submitted to the formaldehyde-modification-reversal step (90 °C, 800 rpm, 1 h). After the sample was cooled to RT, the ceramic spheres from lysing matrix D/D1 were removed and the extraction was continued with addition of 700 µL of PC as described in S_{PC}.

V12a: 25 mg of tissue was washed and homogenised as described in S_{PC}. Afterwards, the sample was mixed with 157.5 µL proteinase K [20 mg/mL], thoroughly vortexed and incubated at 56 °C, 800 rpm for 18 h. Then, the mixture was submitted to the formaldehyde-modification-reversal step (90 °C, 800 rpm, 1 h). The sample was cooled to RT, mixed with 2 µL RNase A [100 mg/mL], left at RT for 2 min and incubated at 70 °C for 10 min. The extraction was continued by adding 700 µL of Roti®-phenol-chloroform-isoamyl alcohol as described in S_{PC}. However, the PC solution differed from the one in protocol S_{PC} as it was recently ordered, hence, a fresh solution.

V12b: 100 mg of tissue was washed in 320 µL PBS and homogenised in a mixture of 60 µL SDS (20 %, w/v) and 530 µL 2.26x TE9 buffer as described in S_{PC}. Then, the sample was mixed with 630 µL proteinase K [20 mg/mL], thoroughly vortexed and incubated at 56 °C, 800 rpm for 18 h. The lysate was incubated at 90 °C, 800 rpm for 1 h. The sample was cooled to RT, mixed with 8 µL RNase A [100 mg/mL], left at RT for 2 min and incubated at 70 °C for 10

min. Then, the lysate was split up in equal volumes of about 250 µL and mixed with 700 µL of Roti®-phenol-chloroform-isoamyl alcohol each as described in S_{PC}. The upper aqueous phases were transferred to new 1.5 mL Eppendorf tubes and once more mixed with 700 µL Roti®-phenol-chloroform-isoamyl alcohol according to S_{PC}. The upper aqueous phases were united in a new 2 mL Eppendorf tube, mixed with 1000 µL EtOH/NH_4Ac (8:1) by inverting the tubes four times. The sample was then left at RT for 1 h, before centrifugation at 4 °C, 14000 rpm for 45 min. The supernatant was carefully discarded by pipetting and the remaining pellet washed with 1 mL 70 % EtOH. After centrifugation (RT, 14000 rpm, 15 min), the supernatant was again discarded by pipetting and the DNA pellet dried at RT for 20 min. The pellet was resuspended in 100 µL EB buffer by incubation at 25 °C, 800 rpm for 2 h.

4.3 Determination of Concentration and Purity of Extracted Genomic DNA

For the determination of the quantity of extracted DNA, NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific) and Qubit®-Fluorometer 4.0 (Thermo Fisher Scientific) were utilised as instructed by the respective manufacturer's protocol.

Qubit®: For the determination of the DNA concentration using the Qubit®-Fluorometer, the Qubit® working solution was prepared. This was done by diluting the Qubit® double-stranded DNA (dsDNA) high-sensitivity (HS) reagent 1:200 in Qubit® dsDNA HS buffer. The volume of the working solution was calculated from the number of fractions plus two standards for calibration. The total volume in each assay tube was 200 µL.
With the final volume of 200 µL and n samples[5], 200 x n µL of working solution was prepared by mixing 199 x n µL of Qubit® dsDNA HS buffer with 1 x n µL of Qubit® dsDNA HS reagent.
For the calibration standards, 190 µL of Qubit® working solution plus 10 µL Qubit® standard were added to the appropriate tubes, vortexed for two seconds and incubated at RT for 2 min.
For the determination of the concentration of the DNA extracts, 2-5 µL of DNA extract was pipetted into the remaining assay tubes and mixed with the respective amount of Qubit® working solution to reach a total volume of 200 µL. The tubes were vortexed for two seconds and incubated at RT for 2 min.

[5] n = number of samples plus number of standards

For calibration, the tube containing standard 1 was inserted into the sample chamber, read and exchanged by the tube containing standard 2. Subsequently, the fractions were measured.
The linear detection range for the dsDNA HS Kit lies between 0.2 and 100 ng and is accurate for DNA concentrations ranging between 10 pg/µL and 100 ng/ µL. [33]

Nanodrop:
Prior to concentration measurement, a zero adjustment was carried out, by pipetting 1 µL of the respective elution buffer of the sample onto the pedestal of the instrument. After the blank measurement, an aliquot of 1 µL of the DNA extract was pipetted onto the pedestal and the absorbance at 260 nm was measured. The DNA concentration was calculated by the software following the equation given below. Each sample was measured two times.

$$c\left[\frac{ng}{\mu l}\right] = A_{260} * D * F$$

c…concentration
A…absorbance at 260 nm
D…dilution factor
F…factor for double stranded DNA (F=50)

The purity of each sample was determined by the ratio of A_{260}/A_{280} (contamination with proteins) and A_{260}/A_{230} (contamination with organic molecules, salts, etc.). Values between 1.8 to 2.0 and 2.0 to 2.2, respectively, were considered as DNA extracts with high purity.

4.4 Development and Optimisation of a Real-Time PCR Assay

For the design of a new real-time PCR assay, the gene sequence of interest was downloaded from the National Center for Biotechnology Information (NCBI) database. An appropriate SNP was located and a target region containing the genetic variant was chosen for the development of a primer set.

4.4.1 SNPs

Relevant SNPs were searched on the NCBI web page. An overview of the selected SNPs is given in Table 12.

Table 12: Overview of selected SNPs for PAX9, SHH and OTX2 gene.

Gene of interest	SNP name	Chromosome and location	Variant type	Consequence	Alleles
PAX9	rs121917720	14:36663031	SNV	missense variant	G>A
SHH	rs1057523055	7:155806383	SNV	missense variant	G>A
OTX2	rs786205884	14: 56802313	deletion	frameshift	C/-
	rs786205879	14:56802192	SNV	stop gained	C>G

4.4.2 Primer Design

The design of a primer set was carried out using the PyroMark Assay Design software. First, the downloaded gene sequence was copied to the program. A short nucleotide sequence of about 12 bases containing the SNP of interest was set as target region.

The software then automatically searched for fitting forward and reverse primers. Since long-term preserved, formalin-fixed samples contain low amounts of high-integrity DNA, the DNA template should not exceed 100 bp. This was quite challenging, since for the primer design several requirements needed consideration, as described in Chapter 3.4.1. Therefore, the primer sets suggested by the program were adjusted to fulfil all the criteria by changing the position or length of the primer. If suitable primers had been found, they were tested for their melting temperature [34], for the probability to form secondary structures [35] or primer dimers [36] and for the prediction of the melting behaviour of the defined DNA template [37].

The primer sequences were sent to Sigma-Aldrich, Germany, where the primers were synthesised. The lyophilised primers were dissolved in RNase-free water according to the manufacturer's instructions.

4.4.2.1 Primer Sequences

Table 13 shows a summary of the primer sequences for each established PCR assay, including the accession number of the respective gene sequence, which is found on NCBI.

Table 13: Overview of primer assays regarding associated SNP, accession number, length of the DNA template and DNA sequence of each primer.

	Oligo name	Associated SNP	Accession number	Template length [bp]	DNA sequence 5' → 3'
primer set 1	*PAX9* fw primer *PAX9* rv primer	rs121917720	NC_000014.9	73	ATCCGACCGTGTGACATCA TGTATCGCGCCAGGATCTT
primer set 2	*SHH* fw primer *SHH* rv primer	rs1057523055	NG_007504.2	86	ACATCACCACGTCTGACCG GTAGTACACCCAGTCGAAGC
primer set 3	*OTX2* fw primer 1.1 *OTX2* rv primer 1.1	rs786205884	NG_008204.1	89	GAATTTTCTTTCCCTTCCAAG TTTTGACCTCCATTCTGCTG
primer set 4	*OTX2* fw primer 3.0 *OTX2* rv primer 3.0	rs786205879	NG_008204.1	90	TCAGAGAGTGGAACAAGTGGC CCAGATAGACACAGGAGCACT

4.4.3 Optimisation of the Real-Time PCR Assay

To optimise the amplifiability of a DNA template during the PCR, several preliminary experiments were carried out aiming at low Ct values, high fluorescence signals, no primer dimers and other non-specific products. DNA from a cell line (HCC8277 Erlo; erlotinib-resistant non-small cell lung cancer cell line) and DNA isolated from breast tissue (C8; patient underwent breast reduction mammoplasty at 31 year) that had been used in previous studies served as positive controls (the extraction protocol for C8 can be found in Appendix C). No-Template-Controls (NTC) were used in all experiments to detect potential contamination and primer dimer formation.

The experiments were conducted using the QuantStudio™ 5 (Thermo Fisher Scientific) and the software QuantStudio™ Design & Analysis Software. The PCRs were carried out using the EpiTect HRM PCR Kit (Qiagen). After data analysis the PCR products were stored at -20 °C.

4.4.3.1 Optimisation of the Primer Concentration and Annealing Temperature

First, the reaction mix containing EpiTect HRM PCR Master Mix, RNase-free H_2O, forward and reverse primer and optional $MgCl_2$, was prepared by mixing the components given in Table 14. In order to ensure that the reaction mix was sufficient for the number of samples, the

reaction mix was prepared for two additional samples. The reaction mix was pipetted into 200 µL tubes that had been inserted into a cooled aluminium block. Afterwards, 2 µL of DNA extract from the positive control [0.1 ng/µL] was added to each tube.

Table 14: Example for pipetting scheme for a reaction mix containing fw and rv primer with a final concentration of 0.6 µM.

Component	**Volume per reaction [µL]**	**Volume for 8 +2 samples [µL]**	**Final concentration [µM]**
fw primer [10 µM]	1.2	12	0.6
rv primer [10 µM]	1.2	12	0.6
Epitect HRM PCR Master Mix	10	100	-
$MgCl_2$ (optional)	-	-	-
RNase-free water	5.6	56	-
Total volume reaction mix	18	180	
DNA [X ng/µL]	2		
Total volume	**20**		

The first experiment was performed to optimise the annealing temperature for each primer set and is summarised in Table 15.

Table 15: Conditions for temperature optimisation for all primer sets.

Assay	**PCR run**	**T_a [6]**	**Final primer conc.**
primer set 1 (*PAX9*)	run 1	51 °C - 61 °C	0.2 µM/0.4 µM
	run 2	59 °C - 69 °C	0.4 µM
primer set 2 (*SHH*)	run 1	54 °C - 64 °C	0.2 µM/0.4 µM
primer set 3 (*OTX2* 1.1)	run 1	51 °C - 61 °C	0.2 µM/0.4 µM
	run 2	43 °C - 53 °C	0.4 µM
primer set 4 (*OTX2* 3.0)	run 1	54 °C - 64 °C	0.2 µM/0.4 µM

To determine the adequate concentration of forward and reverse primer for the successful amplification of DNA, the primer concentration was varied covering 0.2 µM, 0.4 µM, 0.6 µM and 0.8 µM.

Another parameter that was investigated was the $MgCl_2$ concentration. Since the activity of the DNA polymerase is influenced by the amount of $MgCl_2$, the $MgCl_2$ concentration was increased

[6] in 2°C steps

using the 25 mM solution supplied with the kit. For each PCR assay, following $MgCl_2$ concentrations were investigated: 0 mM, 1 mM and 2 mM.

However, not only the primer and $MgCl_2$ concentration were varied but the volume of DNA extract. The latter was increased from 2 µL to 4 µL. In order to maintain the volume of 20 µL per reaction, the amount of RNase-free H_2O was decreased. An example is given in Table 16.

Table 16: Example for the pipetting scheme for a reaction containing 4 µL DNA extract.

Component	**Volume per reaction [µL]**
fw primer [10 µM]	1.2
rv primer [10 µM]	1.2
2x Epitect HRM PCR Master Mix	10
$MgCl_2$ [25 mM] (optional)	-
RNase-free water	3.6
Total volume reaction mix	16
DNA [0.1 ng/µL]	4
Total volume	**20**

4.4.4 Temperature Program and Settings

After the tubes were transferred to the PCR device, the program was started. With the QuantStudio™ 5 instrument up to six different annealing temperatures can be analysed simultaneously during each PCR run. The program settings were equal for all assays differing in the annealing temperature only. Table 17 shows the temperature program for the PCR assay with primer set 4.

Table 17: Temperature program for PCR run with primer set 4.

		T [°C]	Duration
hold 1	Initialisation	95	5 min
50 cycles	Denaturation	95	10 s
	Annealing	54 - 64	30 s
	Extension	72	30 s
hold 2	Final elongation	72	10 min
hold 3	Denaturation	95	1 min
hold 4	Hybridisation	40	1 min
HRM		65 - 95	0.05 / s

4.4.5 Pre-Treatment of DNA extracts prior to PCR Analysis

The treatment of DNA extracts with DNA polymerase prior to PCR amplification should seal nicks and restore the DNA sequence. For the reparation of damaged template DNA prior to PCR analysis two different protocols were applied and compared:

Protocol 1: PreCR™ Repair Mix (New England Biolabs)

The reaction protocol and the control reaction protocol were carried out according to the manufacturer's instructions. The repaired template DNA was then analysed with PCR-HRM using the PSQ PCR Master Mix (Table 19, Chapter 4.5.3.1) The PCR conditions can be found in Table 21, Chapter 4.5.3.1.

Protocol 2: Bonin *et al.* (2003)

Protocol 2 consisted of the pre-treatment of DNA extracts prior to PCR amplification, described by Bonin *et al.* (see Chapter 1.4).

For better comparison with protocol 1, the total reaction volume was reduced from 100 µL to 50 µL and the amount of *Taq* DNA polymerase was decreased from 1 U to 0.5 U. The repaired template DNA was then analysed with PCR-HRM using the PSQ PCR Master Mix (Table 19, Chapter 4.5.3.1). The PCR conditions are given in Table 21, Chapter 4.5.3.1.

4.4.6 Application of Developed Assays for Amplification of DNA from Fixed, Long-Term Stored Foetal Tissue

While the optimisation of primer concentration, $MgCl_2$ concentration and annealing temperature of each PCR assay was performed with the QuantStudio™ 5 instrument, the Rotor-Gene® Q (Qiagen) and the software Rotor-Gene® Q Series Software 2.3.1 were used for all further experiments. This is because the thermal cycler has proven to keep temperature more constant over a certain period of time, which is crucial for primer annealing. The applied primer concentration, $MgCl_2$ concentration and annealing temperature for each assay is given in Table 38, Chapter 5.2.5. The PCR settings remained the same as given in Table 17.

Each analysis consisted of two positive controls (HCC827 Erlo and C8) and one no template control with two technical replicates each. DNA extracts were analysed with up to five technical replicates to achieve a representative number of successful PCR reactions. DNA concentration of the samples was rather low, thus, the extracts were used undiluted (see Chapters 5.1.1 to 5.1.4).

4.4.7 Data Analysis

The raw data files were analysed using the Rotor-Gene® Q Series Software 2.3.1. The Ct values of the amplification curves were determined by setting the threshold line. Therefore, the logarithmic view of the amplification plot was chosen and the threshold line was set where the amplification curves ran parallel to one another.

HRM analysis was used for the determination of the melting behaviour and genotype of the PCR product. Furthermore, NTCs were analysed for primer dimer formation.

For genotype determination, the melting curves were normalised between 0 % and 100 % to uniform relative values resulting from different amounts of PCR products per reaction tube. The so called ‘normalisation regions’ were shifted to areas close to the melting region and where the amplification curves ran parallel to one another. The normalised HRM curves, that show the normalised relative fluorescence units (RFU) plotted against the melting temperature, allowed to draw conclusions about the genotype. This was relevant for the investigation of genetic alterations in the case of foetuses with otocephaly.

4.5 Pyrosequencing

4.5.1 Design of a Sequencing Primer

The sequencing primer was designed for the amplicon obtained with primer set 3 (*OTX2*) using the PyroMark Assay Design software and followed the principle of the design of a forward and reverse primer.

The length of the sequencing primer should be shorter than that of the fw or rv primer and should bind closer to the target region to minimise the length that is sequenced. Furthermore, the GC-content should be between 35 and 65 %; binding over a SNP and the formation of secondary structures and primer dimers should be avoided. The sequencing primer suggested by the program was adjusted to fulfil the criteria by changing the position and length of the primer. If a suitable primer had been found, the melting temperature [34], the probability to form secondary structures [35] or primer dimers [36] were determined. The primer sequence was sent to Sigma-Aldrich, Germany, where the primer was synthesised. The lyophilised primer was dissolved in RNase-free water, according to the manufacturer's instructions.

4.5.2 Primer Sequence

The analysed sequence was AGAAT**BR**AAG**W**G**M**TAAGTGCC**K**C**M**A**R**CAACAG**[C]**A ACAACAGCAGAATGGAGGTCAAAA. All variable positions (SNPs) are highlighted in bold characters. The code for each character can be found online [38] or in the manual of the PyroMark Assay Design Software. The design of a sequencing primer for the already existing DNA template for primer set 3 was challenging as only one primer was found to be adequate. However, an A/G SNP was located on the binding site of the sequencing primer (see Figure 11). Therefore, another two primers were generated by exchanging this specific base by an A or G. The primer sequences were sent to Sigma-Aldrich, Germany, where the primers were synthesised. In addition, the reverse primer (primer set 3, see Table 13) containing a biotin group at the 5'- end was ordered. An overview of the sequencing primers is given in Table 18.

…TCAAGTGTTTTTAGACAGAGCCTCCCCAACTTTCTTACAAGTCCAGGAGTTTATATGAGAGTACC
ACATAATAGGTCTTCAGTGGCAGGGGAAATTGTGTGTTTAGCTGATCTGCCCATGTAGGATAGATT
TATAATACGGGAGCCATTCTTGTCCTTAAGGAACTATCAAAACCGAGTTAAAGAATTTTCTTTCCCT
TCCAAGRTATGGTTTAAGAATBRAAGWGMTAAGTGCCKCMARCAACAG[C]AACAACAGCAGAAT
GGAGGTCAAAACAAAGTGAGACCTGCCAAAAAGAAGACATCTCCAGCTCGGGAAGTGAGTTCAGA
GAGTGGAACAAGTGGCCAATTCACTCCCCCCTCTAGCACCTCAGTCCCGACCATTGCCAGCAGCAG
TGCTCCTGTGTCTATCTGGAGCCCAGCTTCCATCTCCCCACTGTCAGATCCCTTGTCC…

Figure 11: Position of the 89 bp DNA template in the gene sequence of OTX2. Binding sites of forward and reverse primer are underlined, binding site of sequencing primer is highlighted in yellow and the SNP of interest is coloured in blue. All SNPs are highlighted in bold characters.

Table 18: Overview of sequencing primers.

Oligo name	DNA sequence 5' → 3'	T_m [°C]
Seq**C**	CCAAG**C**TATGGTTTA[7]	40.6
Seq**G**	CCAAG**G**TATGGTTTA	40.6
Seq**A**	CCAAG**A**TATGGTTTA	37.8

4.5.3 Optimisation of the Real-Time PCR Assay with Biotinylated Reverse Primer and PyroMark PCR Master Mix

The experiments were conducted using the Rotor-Gene® thermal cycler (Qiagen) and the Rotor-Gene® Q Series Software 2.3.1. The PCRs were carried out using the PyroMark PCR Kit (Qiagen).

4.5.3.1 Optimisation of the $MgCl_2$ Concentration

Since the PCR products were further analysed by pyrosequencing, the PCR was performed with a reaction mix different from the one described in Table 14. Instead of the HRM PCR Master Mix, the PSQ PCR Master Mix was applied (Table 19). Furthermore, the $MgCl_2$ concentration was varied (Table 20) in order to ensure optimal working conditions for the DNA polymerase. The reaction mix was pipetted into strip tubes, which had been inserted into a cooled aluminium block. Afterwards, 2 µL of DNA extract from the positive control (C8 or HCC827 Erlo) [0.1 ng/µL] was added to each tube.

[7] This sequence was suggested by the Pyromark Assay Design software

Table 19: Example for pipetting scheme for a reaction mix containing a final $MgCl_2$ concentration of 1.5 mM.

Component	Volume per reaction [µL]	Volume for 8 +2 samples [µL]	Final concentration
fw primer [10 µM]	1.5	15	0.6 µM
biotinylated rv primer [10 µM]	1.5	15	0.6 µM
2x PyroMark PCR Master Mix	12.5	125	-
$MgCl_2$ [25 mM]	0	0	1.5 mM
RNase-free water	3.75	37.5	-
10x Coral Load Concentrate	2.5	25	-
20x EvaGreen Dye	1.25	12.5	-
Total volume reaction mix	23	230	
DNA [0.1 ng/µL]	2		
Total volume	**25**		

Table 20: Required volumes for varying $MgCl_2$ concentrations.

Final $MgCl_2$ concentration in reaction [mM]	Required volume of $MgCl_2$ [25 mM] per reaction [µL]
1.5	0
2.0	0.5
2.5	1

The temperature program for the PCR-HRM run is detailed in Table 21.

Table 21: Temperature program for PCR run with primer set 3 (biotinylated reverse primer).

		T [°C]	Duration
hold 1	Initialisation	95	15 min
50 cycles	Denaturation	95	10 s
	Annealing	53	30 s
	Extension	72	30 s
hold 2	Final elongation	72	10 min
hold 3	Denaturation	95	1 min
hold 4	Hybridisation	40	1 min
HRM		65 - 95	0.05 / s

After the determination of the optimal $MgCl_2$ concentration, the assay was applied to DNA extracts from formalin-fixed, long-term stored foetal tissue.

4.5.4 Pyrosequencing Workflow

The experiments were carried out using PyroMark Q24 Advanced (Qiagen) and PyroMark Q24 Advanced 3.0.1 Software (Qiagen).

First, a run file was set up containing information on the SNP assay, the sequence to analyse, sample names and the volumes of dNTPs, enzyme and substrate required for analysis. The PyroMark Q24 Cartridge (Qiagen) was loaded with dNTPs, enzyme and substrate (according to the set-up file) and inserted into the instrument. Then, the master mix was prepared as follows (Table 22):

Table 22: Pipetting scheme for PSQ master mix.

Component	**Volume per reaction [µL]**
Streptavidin Sepharose HP	1
PyroMark binding buffer	40
Millipore H_2O	24
Total volume	65

65 µL of master mix was pipetted into each well of an iCycler iQ PCR plate (Bio-Rad) followed by 15 µL of PCR products. The plate was sealed with an adhesive film and placed on a shaking platform for 10 min, 14 000 rpm. Meanwhile, 20 µL of 0.375 µM sequencing primer (Table 23) was pipetted into the wells of a PyroMark Q24 plate.

Table 23: Pipetting scheme for the preparation of 0.375 µM sequencing primer.

Component	**Volume per reaction [µL]**
Sequencing primer [10 µM]	0.75
Annealing buffer	19.25
Total volume	20

The iCycler iQ PCR plate and the PyroMark Q24 plate were placed on the PyroMark Q24 Vacuum Workstation. With the help of a vacuum tool, the DNA was immobilised on a membrane, denatured, washed and transferred to the PyroMark Q24 plate in accordance with the user manual. For the annealing of the sequencing primer, the plate was heated for 5 min at 80 °C, immediately placed in the pyrosequencing instrument and the run started.

4.5.4.1 Quantity of Streptavidin Beads and Amplicon & Selection of Sequencing Primer

For the optimisation of the quantity of streptavidin beads, two different master mixes were prepared. One master mix followed the pipetting scheme given in Table 22, while the other one contained 1.5 times the volume of streptavidin beads and binding buffer (Table 24).
In addition, the volume of biotinylated PCR product was increased to 22.5 µL for the second master mix.
Since an A/G SNP was located on the binding site of the sequencing primer, two experimental approaches had to be set up. One consisted of the use of sequencing primer SeqC only, while for the other approach sequencing primers SeqG and SeqA were applied in equimolar amounts. The 0.375 µM sequencing primer solutions were prepared as summarised in Table 23. An overview of the plate setup is given in Table 25.

Table 24: Pipetting scheme for PSQ master mix containing 1.5 times the volume of streptavidin beads and binding buffer.

Component	**Volume per reaction [µL]**
Streptavidin Sepharose HP	1.5
PyroMark binding buffer	60
Millipore H_2O	36
Total volume	97.5

Table 25: Schematic illustration of the plate setup. Each well contains a (mixture of) sequencing primer, master mix (MM; 1 or 1.5 times the volume of streptavidin beads and binding buffer) and PCR product of a positive control. The experiment consisted of two technical replicates per sample.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	SeqC	SeqC	SeqA/SeqG	SeqA/SeqG	SeqC	SeqC	SeqA/SeqG	SeqA/SeqG
	1x MM (65 µL)	1x MM (65 µL)	1x MM (65 µL)	1x MM (65 µL)	1.5x MM (97.5 µL)	1.5x MM (97.5 µL)	1.5x MM (97.5 µL)	1.5x MM (97.5 µL)
A	15 µL PCR product (C8)	15 µL PCR product (HCC827 Erlo)	15 µL PCR product (C8)	15 µL PCR product (HCC827 Erlo)	22.5 µL PCR product (C8)	22.5 µL PCR product (HCC827 Erlo)	22.5 µL PCR product (C8)	22.5 µL PCR product (HCC827 Erlo)
	SeqC	SeqC	SeqA/SeqG	SeqA/SeqG	SeqC	SeqC	SeqA/SeqG	SeqA/SeqG
	1x MM (65 µL)	1x MM (65 µL)	1x MM (65 µL)	1x MM (65 µL)	1.5x MM (97.5 µL)	1.5x MM (97.5 µL)	1.5x MM (97.5 µL)	1.5x MM (97.5 µL)
B	15 µL PCR product (C8)	15 µL PCR product (HCC827 Erlo)	15 µL PCR product (C8)	15 µL PCR product (HCC827 Erlo)	22.5 µL PCR product (C8)	22.5 µL PCR product (HCC827 Erlo)	22.5 µL PCR product (C8)	22.5 µL PCR product (HCC827 Erlo)

5 Results and Discussion

5.1 DNA Extraction

For the extraction of DNA from formalin-fixed and ethanol-fixed long-term preserved foetal tissue three different approaches were investigated, comprising two commercially available DNA extraction kits, a standard method (CTAB) and a phenol-chloroform extraction. The detailed protocols are given in Chapters 4.2.3 to 4.2.6.

5.1.1 QIAamp DNA Mini Kit

The most crucial step in the extraction of genomic DNA from formalin-fixed and long-term stored tissue is the lysis. When using the standard protocol $S_{Mini\ Kit,}$ complete digestion of tissue failed. The DNA concentration did not exceed 0.2 ng/µL and 15 ng/µL for Qubit® measurement and NanoDrop measurement, respectively (Table 26). The reason why higher concentrations were obtained with NanoDrop can be explained by the fact that NanoDrop cannot distinguish between DNA and other components that absorb at 260 nm resulting in a higher DNA concentration.

In order to improve tissue digestion, the volume of the lysis buffer was doubled and the digestion period was increased to 24 h (V1a; Chapter 4.2.3.1). Additional proteinase K, as tested in V1b (Chapter 4.2.3.1), should further enhance the digestion of tissue. However, DNA concentration remained low (Table 26).

The use of a syringe for the homogenisation of tissue, as well as increasing the volumes of ATL buffer, RNase A, AL buffer and ethanol (V2a; Chapter 4.2.3.1) were investigated regarding DNA yield. Furthermore, the incubation time during lysis was prolonged by another 24 h (V2b; Chapter 4.2.3.1), a rehydration step was included (V3a, V3b; Chapter 4.2.3.1) or omitted (V3c, V3d; Chapter 4.2.3.1) and spin columns, usually applied to clean up PCR products of 10-100 bp, were tested (V3a, V3c; Chapter 4.2.3.1). Finally, a tissue disruptor was used for the homogenisation of tissue prior to lysis, as it was more effective than a syringe (V4; Chapter 4.2.3.1).

As shown in Table 26, the DNA concentrations were rather low for each protocol and differed between the spectrophotometric and fluorometric measurement. However, ethanol-fixed liver tissue yielded higher DNA concentrations (0.098 – 0.164 ng/µL, fluorometric measurement) than formalin-fixed liver tissue (0.021 – 0.076 ng/µL, fluorometric measurement), regardless of the protocol applied. Differences in DNA concentration were also observed, when comparing

liver tissue to cerebrum tissue. Liver yielded higher amounts of DNA (~ 55 % higher for foetus 2, ~ 23 % higher for foetus 3; fluorometric measurement) regardless of the method used for the determination of the concentration (V4, Table 26).

The purity determined by A_{260}/A_{230}, was low for all DNA extracts, meaning that there are still contaminants present. Calculated values for A_{260}/A_{280} ranged between 1.57 and 2.86, indicating that the majority of the protein content had been removed. Values above 2.0 are probably related to the severe degradation of the DNA.

In conclusion, none of the modified protocols resulted in a strong increase in DNA concentration compared to the standard protocol.

Table 26: Results for the QIAamp DNA Mini Kit extraction protocols for the first out of three DNA eluates[8].

Extraction method	Foetus/type of tissue	Tissue amount [mg]	DNA concentration according to Qubit®-Fluorometer [ng/µL]	DNA concentration according to NanoDrop [ng/µL]	A_{260}/A_{280}	A_{260}/A_{230}
$S_{Mini\ Kit}$	F2/liver	25.3	0.1230	13.00	1.74	0.54
	F2/liver	25.0	0.1200	14.60	1.71	0.52
V1a	F2/liver	25.3	0.0980	3.05	2.25	0.82
V1b	F2/liver	25.5	0.0992	2.95	2.19	0.98
V2a	F2/liver	24.9	0.1520	13.95	1.68	0.88
	F2/liver	25.0	0.1310	20.10	1.60	0.61
V2b	F3/liver	25.6	0.0240	2.90	2.26	3.03
	F3/liver	25.7	0.0340	5.65	1.81	0.55
V3a	F3/liver	25.2	0.0320	4.35	2.40	0.51
	F3/liver	25.7	0.0536	6.70	1.82	0.35
V3b	F3/liver	25.3	0.0300	4.50	1.98	0.85
	F3/liver	25.4	< LOD	2.45	2.86	2.99
V3c	F3/liver	25.4	0.0756	8.50	1.57	0.30
	F3/liver	25.6	0.0640	7.65	1.91	0.30
V3d	F3/liver	25.5	0.0212	5.45	2.61	0.34
	F3/liver	25.3	0.0224	5.80	2.19	0.38
V4	F2/cerebrum	25.3	0.1060	8.05	1.82	1.00
	F2/liver	25.1	0.1640	23.20	1.62	0.72
	F3/cerebrum	25.0	0.0296	5.95	2.35	1.30
	F3/liver	25.0	0.0364	8.85	2.11	1.59

[8] DNA was eluted from the spin column three times and collected in separate 1.5 ml Eppendorf tubes to ensure that all bound DNA was retrieved (see Chapter 4.2.3).

5.1.2 QIAamp DNA FFPE Tissue Kit

Since the QIAamp DNA Mini Kit did not result in high DNA quantities, the QIAamp DNA FFPE Tissue Kit was tested. However, the standard protocol ($S_{FFPE\ Kit}$) did not yield higher amounts of DNA, therefore several variations of the protocol were investigated regarding DNA yield and purity. A summary of the results is detailed in Table 27.

Table 27: Results for the QIAamp DNA FFPE Tissue Kit extraction protocols for the first out of three DNA eluates[9].

Extraction method	Foetus/ type of tissue	Additional information	Tissue amount [mg]	DNA concentration according to Qubit®-Fluorometer [ng/µL]	DNA concentration according to NanoDrop [ng/µL]	A_{260}/A_{280}	A_{260}/A_{230}
$S_{FFPE\ Kit}$	F2/liver	-	25.4	0.0904	7.85	1.43	0.87
	F2/liver		25.0	0.1190	9.85	1.41	0.78
V5	F3/liver	matrix A; homogenisation in PBS	25.2	0.0444	9.00	1.86	0.70
		flow through	-	1.1900	35.10	2.81	1.84
	F3/liver	matrix A; homogenisation in ATL buffer	25.1	0.1060	13.30	1.47	0.34
		flow through	-	1.2300	46.20	2.61	1.46
	F3/liver	matrix D; homogenisation in PBS	25.5	0.1080	18.00	1.82	1.08
		flow through	-	0.9280	44.40	2.50	1.50
	F3/liver	matrix D; homogenisation in ATL buffer	25.2	0.1410	23.90	1.80	0.86
		flow through	-	1.4100	45.10	2.61	1.53
V6a	F2/liver	matrix D	25.0	0.3010	22.00	1.38	0.47
	F2/liver	matrix D	25.3	0.3180	22.30	1.37	0.52
	F3/liver	matrix D	25.6	0.0940	10.90	1.57	0.41
	F3/liver	matrix D	25.3	0.1020	14.25	1.57	0.42
V6b	F2/liver	matrix D	25.6	0.2790	19.20	1.40	0.54
	F2/liver	matrix D	25.1	0.1920	15.40	1.37	0.50
	F3/liver	matrix D	25.4	0.0316	6.30	1.56	0.29
	F3/liver	matrix D	25.2	0.0296	5.45	1.55	0.37
V7	F3/liver	matrix A	25.3	0.1330	14.00	1.34	0.38
	F3/liver	matrix A2	25.4	0.1520	12.75	1.31	0.47
	F3/liver	matrix D	25.3	0.1700	19.00	1.39	0.53
	F3/liver	matrix D1	25.4	0.1320	10.20	1.47	0.60
	F3/liver	matrix M	25.3	0.1080	8.90	1.50	0.94
	F3/liver	matrix M2	25.5	0.1770	12.75	1.44	0.63
	F3/liver	matrix I	25.5	0.1730	15.30	1.43	0.49
	F3/liver	matrix S	25.2	0.1270	9.85	1.49	0.59

[9] DNA was eluted from the spin column three times and collected in separate 1.5 ml Eppendorf tubes to ensure that all bound DNA was retrieved (see Chapter 4.2.4).

	F3/liver	matrix F	25.6	0.1680	14.05	1.40	0.37
V8a	F3/liver	matrix M2	101.4	0.0836	5.95	1.88	0.41
		flow through	-	0.1620	9.90	1.68	0.50
	F3/intestine	matrix M2	100.1	0.0988	6.70	1.57	0.34
		flow through	-	0.0824	4.60	1.71	0.51
	F3/ cerebrum	matrix M2	100.0	0.0916	5.25	1.48	0.47
		flow through	-	0.0976	5.80	1.70	0.43
	F3/muscle	matrix M2	100.8	0.0736	5.90	1.52	0.24
		flow through	-	0.0916	5.60	1.62	0.35
	F3/heart	matrix M2	101.0	0.1330	10.50	1.39	0.27
		flow through	-	0.0668	3.70	2.25	0.29
	F3/bone	matrix M2	100.5	0.0616	6.20	1.46	1.51
		flow through	-	0.0804	4.70	1.92	-
V8b	F3/bone	matrix S	101.4	0.0840	8.50	1.57	0.90
	F3/bone	flow through	-	0.0840	4.30	2.14	-

First, two different lysing matrices (A and D) and two different homogenisation media (PBS and ATL buffer) were used for the disruption of tissue prior to lysis for V5 (Chapter 4.2.4.1). Furthermore, the volumes of ATL buffer, RNase A, AL buffer and ethanol were doubled for the same protocol.

There was no dramatic difference observable between matrix A and D for the fluorometric-based determination of DNA concentration (V5, Table 27). In general, DNA concentration seemed to increase ($S_{FFPE\ Kit}$ and V6a/V6b, F2, liver tissue; Table 27), when the tissue disruptor was applied. However, due to lack of comparability of the protocols (inclusion or omission of a formaldehyde-modification reversal step ($S_{FFPE\ Kit}$ and V6a or V6b) or upscaling of proteinase K concentration (V6a and V6b)), a clear conclusion about the increase in DNA concentration cannot be drawn.

Lysing matrix D seemed to yield larger amounts of DNA (~ 28 %, fluorometric measurement), compared to matrix A (V7, Table 27). This could be explained by the fact, that matrix A was more difficult to separate from the lysate, thus, more sample liquid was lost during DNA extraction.

However, since this experiment was not based on duplicates, it is impossible to draw a meaningful conclusion whether PBS or ATL buffer is more suitable for tissue homogenisation. For all further experiments regarding the QIAamp DNA FFPE Tissue Kit protocols, the tissue was disrupted in ATL buffer.

To improve binding of DNA fragments to the spin columns, carrier RNA was added to the flow through and the flow through was applied to a new spin column (V5). According to fluorometric and spectrophotometric measurements DNA concentration dramatically increased (~ 8.5 to 27 times for the fluorometric measurement; V5, Table 27). It was likely, that the increment in DNA resulted from carrier RNA. However, the ds HS DNA Kit should specifically determine the amount of double stranded DNA. To reveal whether the ds HS DNA Kit can distinguish between DNA and RNA at such low nucleic acid concentrations, 2 µL carrier RNA was added to 48 µL ATE buffer (final volume matched the volume of DNA extracts). Then, the concentration was determined with Qubit®-Fluorometer. For comparison, ATE buffer without carrier RNA was measured as well. The experiment was carried out with duplicates. The results (Table 28) confirmed, that increasing DNA concentration of the flow throughs was a consequence of the addition of carrier RNA.

Table 28: DNA concentration of ATE buffer containing carrier RNA and ATE buffer without carrier RNA.

	DNA concentration according to Qubit®-Fluorometer [ng/µL]
ATE buffer with carrier RNA	1.38
	1.53
ATE buffer without carrier RNA	< LOD
	< LOD

The effect of additional proteinase K and the impact of the formaldehyde-modification-reversal step were investigated for the ethanol-fixed and formalin-fixed tissue regarding DNA yield and purity (V6a, V6b; Chapter 4.2.4.1). As already concluded for the QIAamp DNA Mini Kit (Chapter 5.1.1), ethanol-fixed tissue yielded higher amounts of DNA than formalin-fixed tissue. When the formaldehyde-modification-reversal step was omitted (V6b), less DNA was obtained. However, increasing the amount of proteinase K from 40 µL (V5) to 400 µL (V6a and V6b, foetus 3) did not lead to an improvement in DNA recovery (Table 27).

Since there are several lysis matrices available for different applications, their impact on homogenisation and consequently DNA yield was investigated (V7; Chapter 4.2.4.1). It can be concluded, that all matrices were equally suitable for tissue disruption (Table 27). However, due to limited number of matrices only single determinations were carried out. Lysing matrix M2 yielded the highest DNA concentration for fluorometric measurement, matrix D for

spectrophotometric measurement. Because NanoDrop is less specific, matrix M2 was used for further experiments.

To increase DNA yield the amount of tissue was scaled up to 100 mg. Furthermore, six different tissue types were compared (V8a; Chapter 4.2.4.1). Despite the fourfold amount of tissue, the DNA concentration did not increase (Table 27). This could be explained by the possibility of the DNA fragments being too short to bind to the spin columns provided with the kit. Therefore, the flow through was collected and applied to QIAquick Spin Columns, which are more suitable for short nucleotide strands. Results showed that the DNA concentration remained quite low regardless the type of spin column or tissue (Table 27).
Bones were additionally tested with lysing matrix S (V8b; Chapter 4.2.4.1), as bones are more promising to yield higher amounts of DNA than tissue because the formalin solution penetrates soft tissue more easily [39]. This results in more degradation and fragmentation of DNA. Lysing matrix S consists of small stainless-steel beads and according to the manufacturer's instructions is suitable for the homogenisation of bones. However, DNA yield did not increase compared to liver tissue homogenised with matrix M2 (Table 27).
In general, DNA yield and purity were unsatisfactory. Therefore, the QIAamp DNA FFPE Tissue Kit was not used further.

5.1.3 CTAB Extraction

The CTAB method had been frequently used in our working group as a successful method for the extraction of DNA. In case of DNA recovery from ethanol-fixed tissue, the method failed to yield satisfying results regarding concentration and purity. The latter was ~ 1.4 and ~ 0.8 for A_{260}/A_{280} and A_{260}/A_{230}, respectively. The concentration was about 0.1 ng/µL (Table 29). Because of that, the CTAB extraction protocol was not further modified.

Table 29: Results for the CTAB extraction protocol.

Foetus/ type of tissue	Tissue amount [mg]	DNA concentration according to Qubit®-Fluorometer [ng/µL]	DNA concentration according to NanoDrop [ng/µL]	A_{260}/A_{280}	A_{260}/A_{230}
F2/liver	25.4	0.0904	7.85	1.430	0.865
F2/liver	25.0	0.1190	9.85	1.405	0.780

5.1.4 Phenol-Chloroform-Isoamyl Alcohol Extraction

The results of the phenol-chloroform-isoamyl alcohol extraction are given in Table 30.
DNA yield ranged between 0.1 and 14 ng/µL for the fluorometric measurement and between 4 – 333 ng/µL for the spectrophotometric measurement (Table 30). Because salts and other contaminants are accumulated in the lower organic phase, the ratio of A_{260}/A_{230} could be notably improved compared to DNA extracts obtained with the spin column-based methods. Proteins, on the other hand, gather in the interphase. When pipetting off the aqueous phase, parts of the interphase might be drawn up as well. As a consequence, the ratio of A_{260}/A_{280} was slightly below the desired range of 1.8 – 2.0. Furthermore, liver tissue yielded ~ 2 to 9 times as much DNA (fluorometric measurement) than heart tissue, regardless of the protocol (S_{PC}, V9a, V9b and V10) applied (Table 30).

Scaling up the amount of proteinase K (V9a and V9b; Chapter 4.2.6.1) and using a fresh PC solution (V9b; Chapter 4.2.6.1) resulted in an increase in the DNA yield of about 20 – 110 % for liver tissue from foetus 3 (fluorometric measurement; Table 30).
Because of the time intensive workflow of the protocols $S_{FFPE\ Kit}$, V9a and V9b, the lysis time was reduced to 18 h, the addition of proteinase K was performed in one single step and the second formaldehyde-modification-reversal step was omitted. As expected, DNA concentration decreased when compared to V9b (Table 30). However, DNA concentration varied between duplicates because of differences in the volume of the collected aqueous phases. Variations in the DNA concentration also depended on the homogeneity of tissue, as it differed in its e.g. lipid or protein content. The smaller the piece, the more difficult it was to obtain tissue samples of equal homogeneity. Because the DNA concentration of the respective tissue type did not vary significantly between V9b and V10, a digestion period of 18 h was maintained for further optimisation of the extraction protocol.
Protocol V11 (Chapter 4.2.6.1) showed, that a reduced lysis time is sufficient to yield satisfying amounts of DNA (Table 30). Differences in the DNA concentration for each lysing matrix were marginal and a clear conclusion cannot be drawn, since the experimental setup did not consist of duplicates. Surprisingly, when omitting the homogenisation step (no matrix; Table 30), DNA extraction yielded the same DNA quantity as samples disrupted with certain matrices.
To exclude the possibility of RNA still being present in the DNA extract, an RNase A treatment was included prior to PC extraction (V12a; Chapter 4.2.6.1). Additionally, a fresh Roti®-phenol-chloroform-isoamyl alcohol solution was tested.

Results indicate that RNase A treatment prior to DNA extraction had no effect on DNA concentration. Here again, differences in the DNA concentrations of the duplicates can be explained by the fact, that the volume of the collected aqueous phases varied between the samples.

To obtain higher DNA concentrations after extraction, the amount of tissue was increased to 100 mg (V12b; Chapter 4.2.6.1). However, despite the fourfold amount of tissue, the DNA concentration did not increase. A DNA pellet was only visible in the sample that contained DNA from liver tissue, while the DNA pellet in the other sample (heart tissue) was probably lost during the pipetting steps. This explains the low concentration of 0.25 ng/µL for the fluorometric measurement.

Table 30: Results for the PC extraction protocols.

Extraction method	Foetus/ type of tissue	Additional information	Tissue amount [mg]	DNA concentration according to Qubit®-Fluorometer [ng/µL]	DNA concentration according to NanoDrop [ng/µL]	A_{260}/A_{280}	A_{260}/A_{230}
S_{PC}	F3/liver	matrix M2	25.4	4.96	77.80	1.65	2.34
	F3/heart	matrix M2	25.4	2.20	31.10	1.68	2.23
V9a	F3/liver	matrix M2	25.3	6.00	130.80	1.65	2.16
	F3/heart	matrix M2	25.3	1.90	49.70	1.64	2.12
V9b	F3/liver	matrix M2; low prot. K conc.	25.5	6.63	118.20	1.65	2.19
	F3/liver	matrix M2; high prot. K conc.	25.5	10.40	257.95	1.67	2.28
	F3/heart	matrix M2; low prot. K conc.	25.2	0.92	24.85	1.60	1.93
	F3/heart	matrix M2; high prot. K conc.	25.3	3.73	80.05	1.62	2.14
V10	F3/liver	matrix M2	25.3	7.33	139.05	1.64	2.30
	F3/liver	matrix M2	25.7	5.58	104.65	1.63	2.30
	F3/heart	matrix M2	25.1	0.14	4.60	1.88	1.73
	F3/heart	matrix M2	25.3	1.28	25.00	1.69	2.27
V11	F3/liver	matrix A	25.6	12.30	254.25	1.65	1.99
	F3/liver	matrix A2	25.5	11.10	193.15	1.65	2.16
	F3/liver	matrix D	25.5	14.70	333.25	1.66	2.15
	F3/liver	matrix D1	25.4	11.70	287.35	1.65	2.25
	F3/liver	matrix M	25.4	11.80	265.95	1.66	2.20
	F3/liver	matrix I	25.5	12.80	254.15	1.66	2.16
	F3/liver	matrix F	25.2	8.23	124.60	1.65	2.12
	F3/liver	no matrix	25.6	13.80	276.70	1.66	2.23

V12a	F3/liver	matrix M2; no RNase A	25.8	7.99	163.00	1.63	1.95
	F3/liver	matrix M2; no RNase A	25.5	4.43	79.35	1.63	1.86
	F3/liver	matrix M2; RNase A	25.6	5.01	150.25	1.58	2.10
	F3/liver	matrix M2; RNase A	25.3	6.08	174.25	1.58	2.19
V12b	F3/liver	100 mg tissue; matrix M2;	101.2	3.94	131.05	1.51	1.43
	F3/liver	100 mg tissue; matrix M2;	101.0	1.49	81.70	1.49	1.30
	F3/liver	100 mg tissue; matrix M2;	100.7	0.25	24.25	1.43	1.05
	F3/liver	100 mg tissue; matrix M2;	100.9	0.25	24.60	1.42	0.97

In literature, PC extraction is often described as a more promising approach for the isolation of DNA from formalin-fixed or ethanol-fixed specimens than commercial kits. This was confirmed for all variations of the standard extraction protocol.

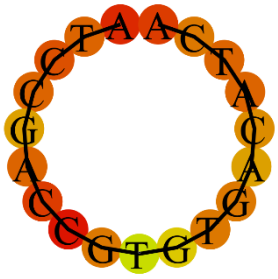
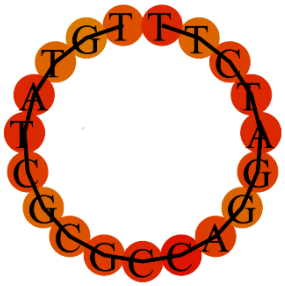
In conclusion, phenol-chloroform-isoamyl alcohol extraction yielded highest DNA concentration (0.1 – 14 ng/µL for the fluorometric measurement and 4 – 333 ng/µL for the spectrophotometric measurement) and purity (1.5 – 1.8 for A_{260}/A_{280} and 1.4 – 2.8 for A_{260}/A_{230}) compared to the commercial DNA extraction kits (QIAamp DNA Mini Kit: < 0.2 ng/µL for Qubit® measurement and < 15 ng/µL for NanoDrop measurement, 1.6 – 2.8 for A_{260}/A_{280} and 0.3 – 3.0 for A_{260}/A_{230}; QIAamp DNA FFPE Tissue Kit: < 0.3 ng/µL for Qubit® measurement and < 24 ng/µL for NanoDrop measurement, 1.3 – 2.8 for A_{260}/A_{280} and 0.3 – 3.0 for A_{260}/A_{230}) and CTAB method (~ 0.1 ng/µL for the fluorometric measurement and ~ 8 ng/µL for the spectrophotometric measurement, ~ 1.4 and ~ 0.8 for A_{260}/A_{280} and A_{260}/A_{230}, respectively). Because of lack of information about fixation medium and conservation period, little conclusions can be drawn about the integrity of the DNA. The commercial DNA extraction kits have proven to be unsuitable for the DNA extraction from long-term stored FF specimens. We assume that most of the DNA fragments did not bind to the spin column because they were too short. The CTAB method was found to be inappropriate for the DNA extraction as well and was not pursued further.

5.2 Development and Optimisation of Four Real-Time PCR Assays

5.2.1 Primer Set 1 (PAX9)

The characteristics of primer set 1 are summarised in Table 31. All criteria, described in Chapter 3.4.1, were fulfilled. Both primers consist of 19 bases, have matching melting temperatures and show no secondary structures according to OligoCalc and RNAfold web server, respectively. The amplicon length is 73 bp.

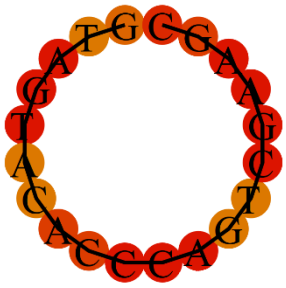
Table 31: Characteristics of primer set 1 (PAX9).

	Forward primer (*PAX9*)	**Reverse primer (*PAX9*)**
Sequence 5' → 3'	ATCCGACCGTGTGACATCA	TGTATCGCGCCAGGATCTT
T_m [°C] according to OligoCalc	57.5	57.5
Secondary structures at 50 °C according to RNAfold web server		

5.2.2 Primer Set 2 (SHH)

The characteristics of primer set 2 are summarised in Table 32. All criteria, described in Chapter 3.4.1, were fulfilled. The forward primer and reverse primer consist of 19 and 20 bases, respectively. Similar melting temperatures and no secondary structures according to OligoCalc and RNAfold web server, were observed. The amplicon length is 86 bp.

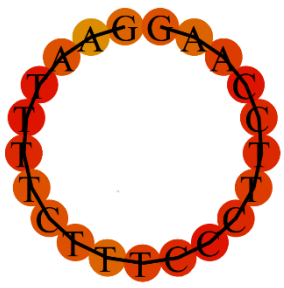
Table 32: Characteristics of primer set 2 (SHH).

	Forward primer (*SHH*)	**Reverse primer (*SHH*)**
Sequence 5' → 3'	ACATCACCACGTCTGACCG	GTAGTACACCCAGTCGAAGC
T_m [°C] according to OligoCalc	59.5	60.5
Secondary structures at 50 °C according to RNAfold web server		

5.2.3 Primer Set 3 (OTX2 1.1)

Table 33 shows the characteristics of primer set 3. All criteria, described in Chapter 3.4.1, were fulfilled. The forward primer comprises 21 bases, the reverse primer 20 bases. According to OligoCalc and RNAfold web server, both primers have similar melting temperatures and show no secondary structures, respectively. The amplicon length is 89 bp.

Table 33: Characteristics of primer set 3 (OTX2).

	Forward primer (*OTX2*)	**Reverse primer (*OTX2*)**
Sequence 5' → 3'	GAATTTTCTTTCCCTTCCAAG	TTTTGACCTCCATTCTGCTG
T_m [°C] according to OligoCalc	55.4	56.4
Secondary structures at 50 °C according to RNAfold web server		

5.2.4 *Primer Set 4 (OTX 3.0)*

The characteristics of primer set 4 are detailed in Table 34. All criteria, described in Chapter 3.4.1, were fulfilled. Both primers consist of 21 bases, have matching melting temperatures and show no secondary structures according to OligoCalc and RNAfold web server, respectively. The amplicon length is 90 bp.

Table 34: Characteristics of primer set 4 (OTX2).

	Forward primer (*OTX2*)	**Reverse primer (*OTX2*)**
Sequence 5' → 3'	TCAGAGAGTGGAACAAGTGGC	CCAGATAGACACAGGAGCACT
T_m [°C] according to OligoCalc	61.2	61.2
Secondary structures at 50 °C according to RNAfold web server		

5.2.5 *Optimisation of Annealing Temperature, Primer Concentration and $MgCl_2$ Concentration*

Low Ct values and primer-dimer formation were taken into account for the determination of the optimal annealing temperature and primer concentration.

The annealing temperature was varied for each primer set (Table 15, Chapter 4.4.3.1) using a concentration of 0.4 µM of forward and reverse primer. Figure 12 to Figure 15 show the amplification plots, Table 35 the Ct values obtained with the respective primer set.

Primer set 1:

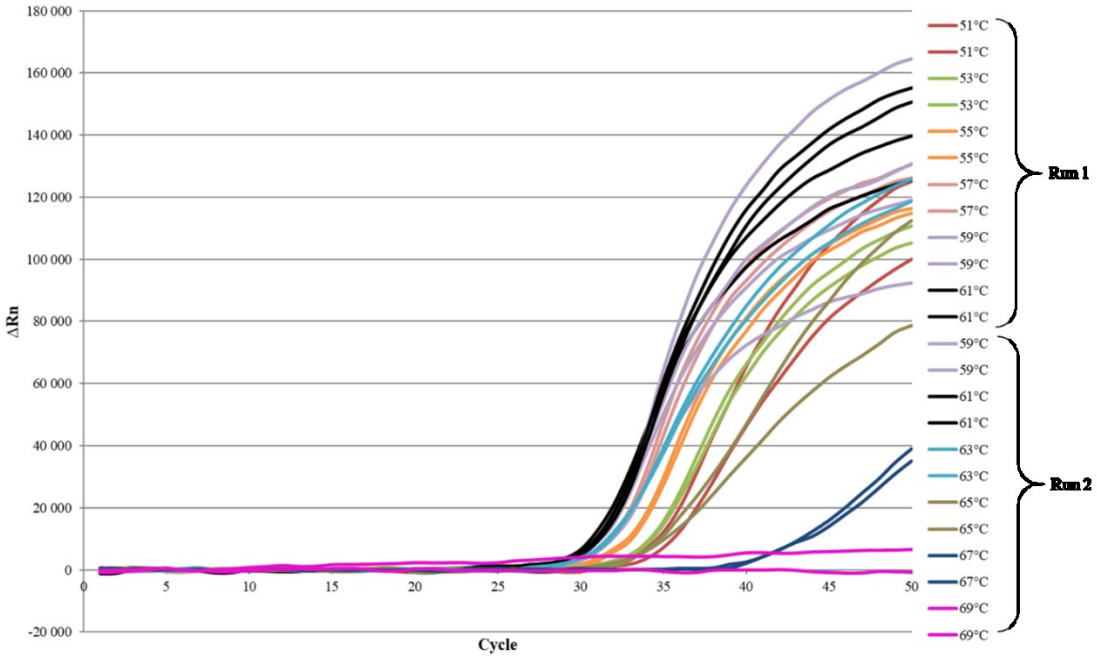


Figure 12: Amplification plot obtained with HCC827 Erlo and primer set 1 (0.4 µM) for PAX9 gene. The experiment consisted of two technical replicates per sample (indicated by the same colour).

Primer set 2:

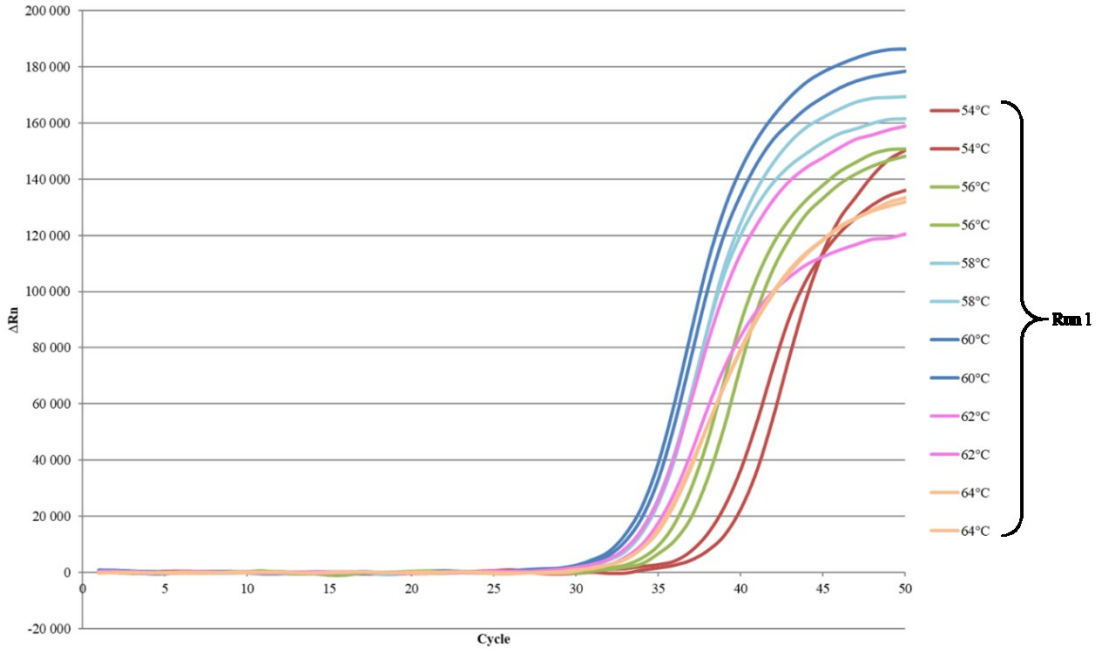


Figure 13: Amplification plot obtained with HCC827 Erlo and primer set 2 (0.4 µM) for SHH gene. The experiment consisted of two technical replicates per sample (indicated by the same colour).

Primer set 3:

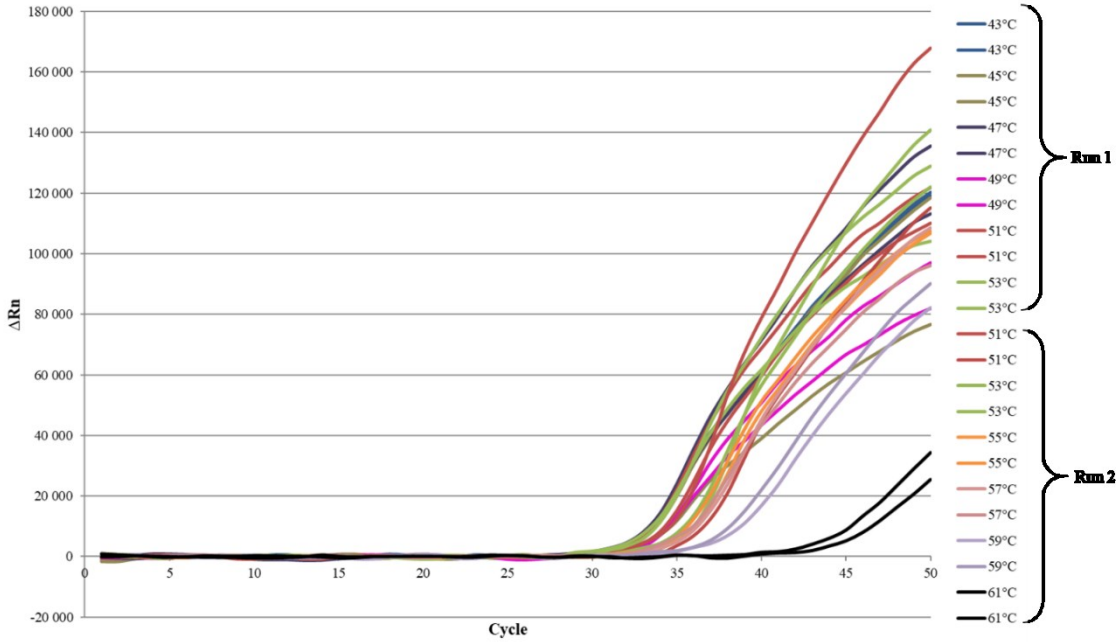


Figure 14: Amplification plot obtained with HCC827 Erlo and primer set 3 (0.4 µM) for OTX2 gene. The experiment consisted of two technical replicates per sample (indicated by the same colour).

Primer set 4:

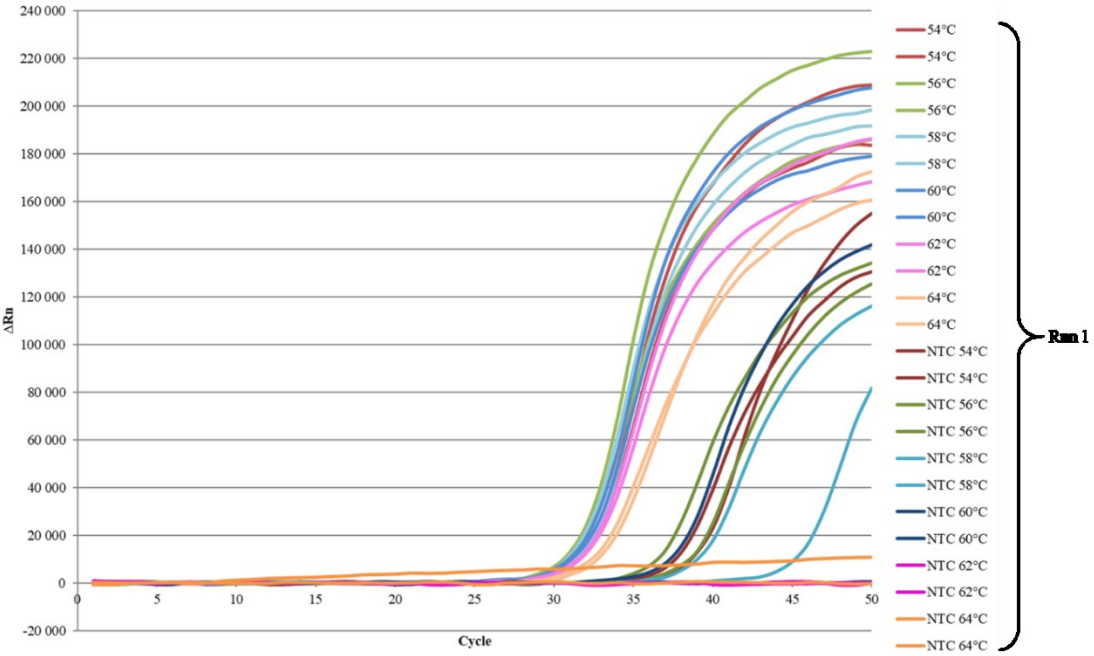


Figure 15: Amplification plot obtained with HCC827 Erlo and primer set 4 (0.4 µM) for OTX2 gene. The experiment consisted of two technical replicates per sample (indicated by the same colour).

Table 35: Ct values obtained with HCC827 Erlo and primer sets 1, 2, 3 and 4. Values for the No-Template-Controls are highlighted in grey. Given Ct value represents the mean of two technical replicates. -[a]: not amplified.

	Primer set 1		Primer set 2		Primer set 3		Primer set 4	
	Temperature [°C]	Ct value	Temperature [°C]	Ct value	Temperature [°C]	Ct value	Temperature [°C]	Ct value
Run 1	51	33.37	54	38.27	51	34.54	54	31.56/38.19
	53	32,54	56	35.70	53	34.68	56	30.81/37.54
	55	31.19	58	33.63	55	34.85	58	31.15/42.15
	57	30.11	60	32.95	57	34.86	60	31.11/37.35
	59	29.93	62	33.92	59	37.16	62	31.65/-[a]
	61	29.22	64	34.54	61	44.35	64	32.60/-[a]
Run 2	59	29.51			43	32.20		
	61	29.48			45	32.92		
	63	30.02			47	32.18		
	65	33.07			49	33.21		
	67	40.75			51	32.66		
	69	-[a]			53	32.31		

With primer set 1, lowest Ct values were obtained when the annealing temperature was set to 59 °C or 61 °C. Because this was the case for run 1 and 2, an annealing temperature of 60 °C was used for all future PCRs with primer set 1. The same temperature was found to be most suitable for primer set 2. With primer set 3, the lowest Ct value was obtained when the annealing temperature was set to 47 °C (Ct value = 32.18, run 2). However, the lower the T_a, the more probable is the formation of non-specific products. Therefore, 53 °C (Ct value = 32.31, run 2) was chosen as T_a for primer set 3. Using primer set 4, primer dimer formation was observed for annealing temperatures up to 60 °C. Consequently, 62 °C was selected as the optimal annealing temperature for primer set 4.

The optimal primer concentration was determined by using forward and reverse primer at 0.2 µM, 0.4 µM, 0.6 µM or 0.8 µM.

Figure 16 depicts the amplification curves obtained with primer set 4 (*OTX2*). Similar Ct values were achieved, when the concentrations of forward and reverse primer were 0.6 µM and 0.8 µM (Table 36). Therefore, a primer concentration of 0.6 µM was used for all further experiments.

The same conclusion was drawn for primer sets 1, 2 and 3. The obtained Ct values are given in Appendix D.

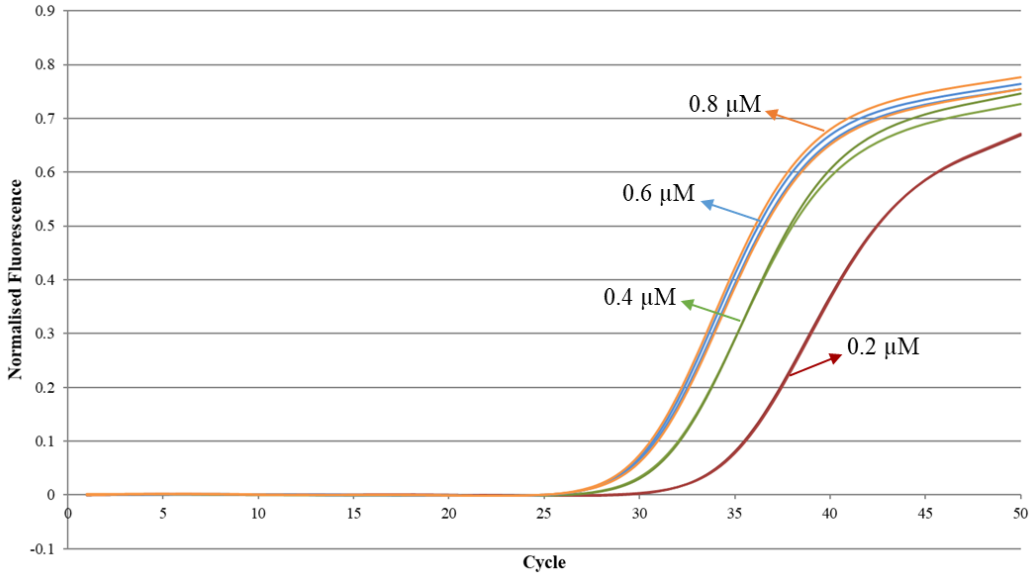


Figure 16: Amplification plot obtained with positive control HCC827 Erlo and different primer concentrations (0.2 µM, 0.4 mM, 0.6 µM, 0.8 µM) of primer set 4 (OTX2). The experiment consisted of two technical replicates per sample (indicated by the same colour).

Table 36: Ct values obtained with HCC827 Erlo and primer set 4 (OTX2) at different concentrations. The experiment consisted of two technical replicates per sample. Given Ct value represents the mean of two technical replicates.

Primer (fw + rv) concentration [µM]	Ct value
0.2	32.59
0.4	29.36
0.6	28.17
0.8	28.15

Commercial PCR buffers often contain $MgCl_2$ at a certain concentration. Nevertheless, the $MgCl_2$ concentration must be optimised for maximal amplification efficiency. Hence, three different conditions were tested with primer set 1 and 2 consisting of 0 mM, 1 mM or 2 mM additional $MgCl_2$ in the PCR buffer. Table 37 shows that without the addition of $MgCl_2$ lower Ct values were obtained.

Table 37: Ct values obtained with positive control HCC827 Erlo and primer set 2 (SHH). The experiment consisted of two technical replicates per sample. Given Ct value represents the mean of two technical replicates.

$MgCl_2$ concentration	**Ct value**
0 mM	28.23
1 mM	29.14
2 mM	29.85

The melting plot in Figure 17 confirms, that additional $MgCl_2$ (1mM or 2 mM) does not improve but worsen DNA replication, possibly by inhibiting the DNA polymerase. The addition of $MgCl_2$ changed the melting behaviour of the amplicons. Higher $MgCl_2$ concentrations caused a shift of the melting curves to higher temperatures.

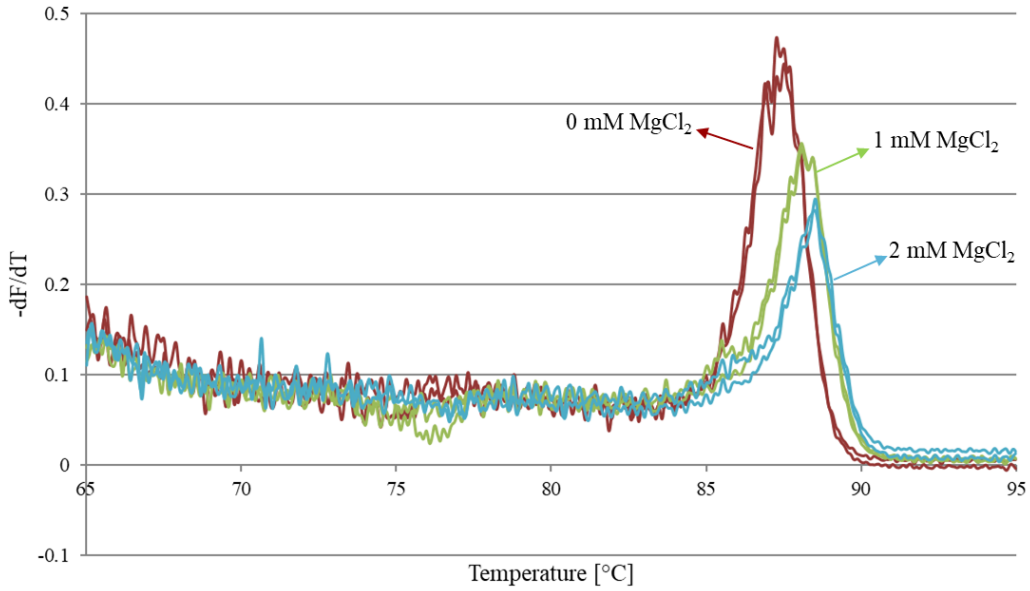


Figure 17: Melting plot obtained with positive control HCC827 Erlo and primer set 2 (SHH). The experiment consisted of two technical replicates per sample (indicated by the same colour).

Because this was observed for both primer sets (1 and 2), the optimisation of the $MgCl_2$ concentration was omitted for primer set 3 and 4. It was presumed that results match those of primer set 1 and 2.

All future PCRs for the amplification of DNA from extracts were carried out under following conditions (Table 38).

Table 38: PCR conditions for the respective primer set.

Primer set (*gene*)	T_a [°C]	Primer (fw + rv) concentration	Additional $MgCl_2$ concentration
Primer set 1 (*PAX9*)	60	0.6 µM	0 mM
Primer set 2 (*SHH*)	60	0.6 µM	0 mM
Primer set 3 (*OTX2*)	53	0.6 µM	0 mM
Primer set 4 (*OTX2*)	62	0.6 µM	0 mM

5.3 Application of Developed Assays for Amplification of DNA From Formalin-Fixed, Long-Term Stored Foetal Tissue

Although DNA concentrations were quite low for the commercial spin column-based methods and CTAB method, DNA extracts (first eluates) were further analysed regarding PCR amplifiability.

5.3.1 PCR Amplification of DNA Extracts Obtained with Protocols Based on the QIAamp DNA Mini Kit

All DNA extracts obtained with protocols $S_{Mini\ Kit}$, V1-V4 (see Table 26, Chapter 5.1.1) were analysed with PCR-HRM. Figure 18 shows the amplification curves obtained with primer sets 1, 2, 3, 4 and DNA extracts obtained with protocols $S_{Mini\ Kit}$, V1, V2 and V4. Additionally, an already established PCR assay (primer set X) for the *MGMT* promoter region was applied to test if low number of PCR products resulted from the so far not validated methods or low integrity of DNA due to formalin fixation or DNA extraction.

Each primer set was tested in an individual PCR run due to different annealing temperatures of the primers. Because the amplification curves obtained with primer set 2 did not reach the plateau within 50 cycles (indicated by the black dashed line), the PCR run was prolonged by 10 cycles.

Not all extraction protocols have proven to yield sufficient DNA for PCR and HRM analysis. DNA isolated with protocols V3a, V3b, V3c and V3d could not be amplified. In contrast, protocol V2b (double volume of ATL buffer, RNase A, AL buffer and ethanol; 48 h lysis) yielded DNA that was successfully analysed with two different PCR assays (primer set 2 and

4). DNA extracted from liver and cerebrum from foetuses without facial dysmorphia (F2 and F3) using protocol V4 (tissue was homogenised with the Fast Prep-24 5G Tissue Homogeniser using lysing matrix D) differed not only in DNA concentration (see Table 26, Chapter 5.1.1) but in suitability for PCR and HRM analysis. Only DNA isolated from liver tissue from foetus 2 showed an increase in the fluorescence signal.

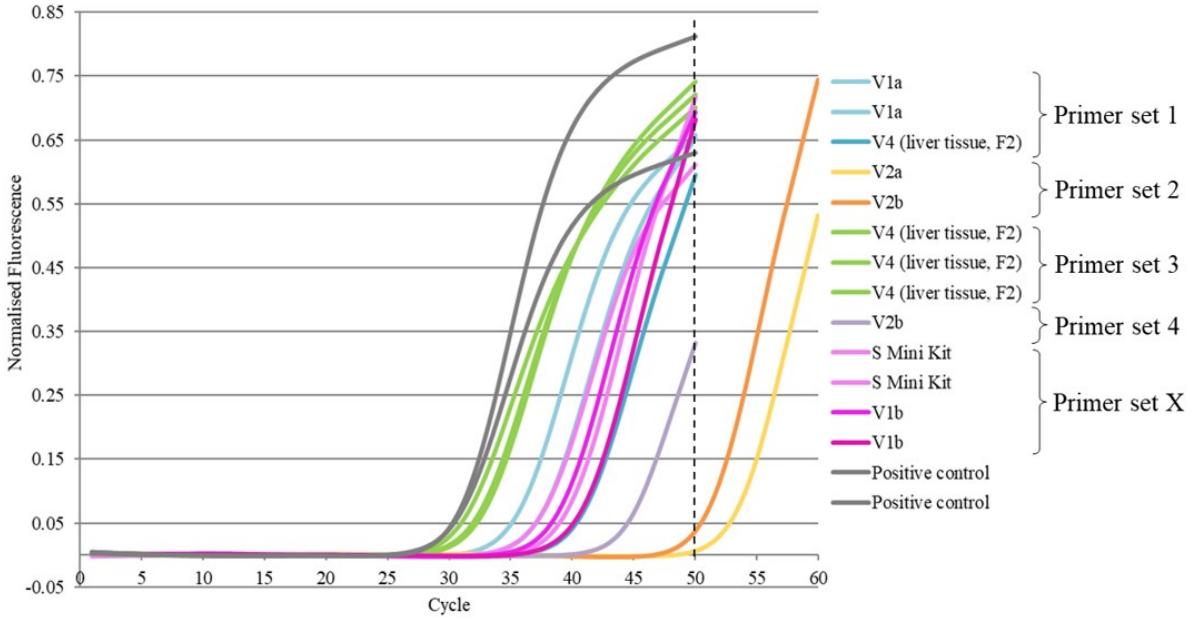


Figure 18: Amplification plot obtained with primer sets 1 (PAX9), 2 (SHH), 3 (OTX2), 4 (OTX2), X (MGMT) and DNA extracts obtained with protocols $S_{Mini\ Kit}$ (F2; liver tissue), V1a (F2; liver tissue), V1b (F2; liver tissue), V2a (F2; liver tissue), V2b (F3; liver tissue) and V4 (F2; liver tissue). PCR runs performed within 50 cycles are indicated by the black dashed line. HCC827 Erlo and C8 were used as positive controls. PCRs with primer sets 1, 2, and 4 were carried out using two technical replicates per sample; PCR with primer set 3 was carried out with three technical replicates per sample.

The corresponding melting plot (Figure 19) shows, that the melting profiles of the amplicons match the positive controls. However, in most cases the atypical broad shape of the melting curves made it difficult to determine the exact melting temperature. The low fluorescence signals may be due to the shortness of the amplicons. The shorter the sequence, the less dye incorporates.

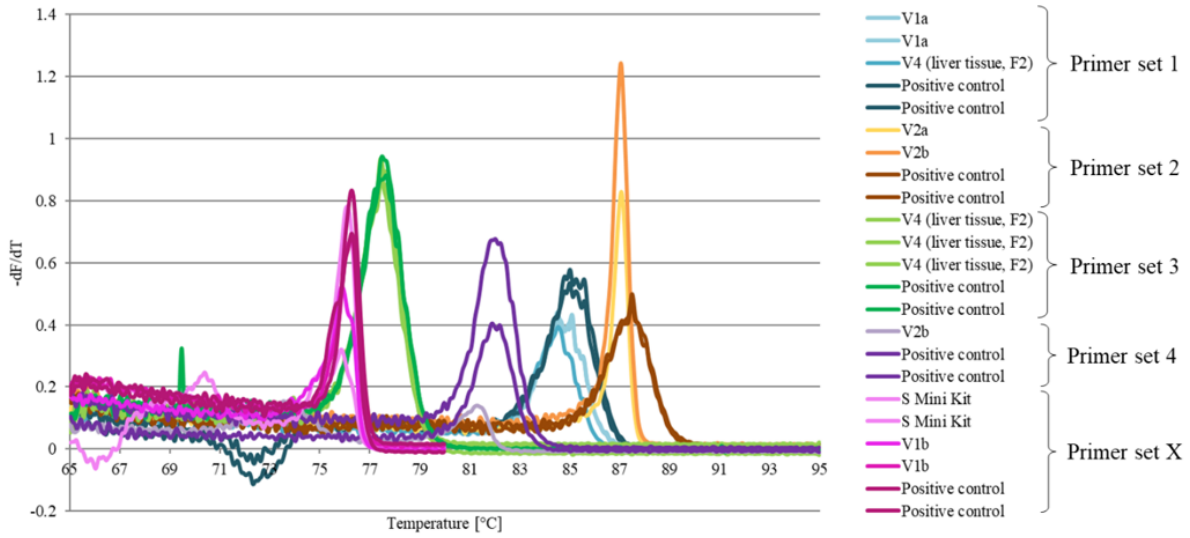


Figure 19: Melting plot obtained with primer sets 1 (PAX9), 2 (SHH), 3 (OTX2), 4 (OTX2), X (MGMT) and DNA extracts obtained with protocols $S_{Mini\ Kit}$ (F2; liver tissue), V1a (F2; liver tissue), V1b (F2; liver tissue), V2a (F2; liver tissue), V2b (F3; liver tissue) and V4 (F2; liver tissue). HCC827 Erlo and C8 were used as positive controls. PCRs with primer sets 1, 2 and 4 were carried out using two technical replicates per sample; PCR with primer set 3 was carried out with three technical replicates per sample.

5.3.1.1 Genotype Determination

For the determination of the genotype of foetus 2 and 3, the normalised HRM curves of the four developed PCR assays and DNA extracts obtained with protocols V1a, V2a, V2b and V4 were chosen (Figure 20).

The normalised melt plot on the top left and bottom left show that the HRM curves of the tissue samples and the positive controls overlap, respectively. The shape of the melting curves in the top right melting plot implies a heterozygous genotype variant in both tissue samples (*SHH* gene; see Table 12, Chapter 4.4.1), while the melting curves in the bottom right suggest a homozygous genotype variant in the tissue sample (*OTX2* gene; see Table 12, Chapter 4.4.1). However, due to the broad shape of the melting curves (Figure 19), a clear conclusion about the genotype cannot be drawn.

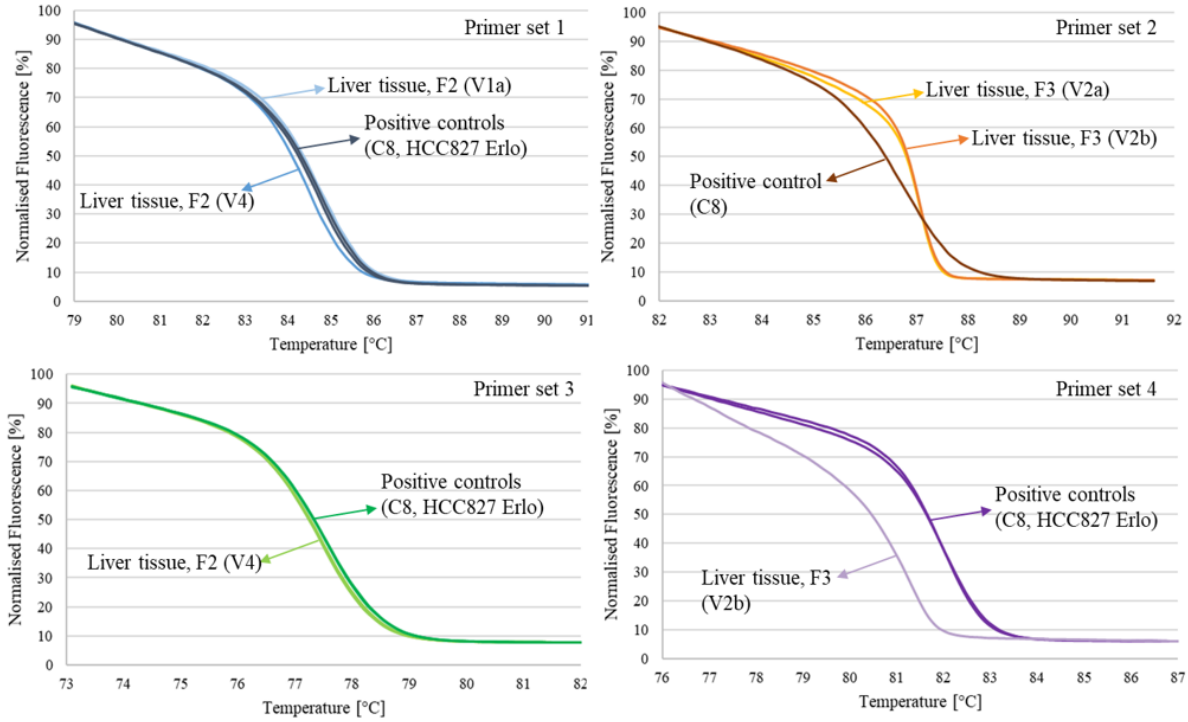


Figure 20: Normalised HRM curves of the PCR products obtained with protocols based on the QIAamp DNA Mini Kit and primer sets 1 (PAX9), 2 (SHH), 3 (OTX2) and 4 (OTX2).

5.3.2 PCR Amplification of DNA Extracts Obtained with Protocols Based on the QIAamp DNA FFPE Tissue Kit

DNA isolates obtained with the QIAamp DNA FFPE Tissue Kit ($S_{FFPE\ Kit}$, V5-V8; see Table 27, Chapter 5.1.2) were also analysed with PCR-HRM using primer sets 1, 2, 3, 4 and X. Only few extraction protocols yielded DNA suitable for PCR amplification. Figure 21 shows the amplification curves obtained with primer sets 1, 2, 3, 4, X and DNA extracts obtained with protocols V5, V6b, V7, V8a or V8b.

As already observed for DNA extracts obtained with QIAamp DNA Mini Kit, DNA isolated with QIAamp DNA FFPE Tissue Kit was amplified randomly.

Protocols V5 and V8a yielded DNA that was successfully analysed with two different PCR assays (V5 with primer sets 1 and 2, V8 with primer sets 2 and X), respectively. Scaling up the amount of tissue from 25 mg to 100 mg (V8a) did not increase DNA concentration (Table 27) but number of samples amplified during PCR. Liver tissue and bones yielded more amplifiable DNA than tissue from intestine and cerebrum.

Furthermore, the application of lysing matrix D (V5, Table 5) and D1 (V7, Table 5) for the homogenisation and subsequent extraction and amplification of DNA seemed to work best compared to other matrices (A, A2, M, M2, I, F).

DNA extracted from liver tissue from foetus 2 and 3 (foetuses without facial dysmorphia) using protocol V6b (the formaldehyde-modification-reversal step after tissue lysis was omitted, the volume of ATL buffer, RNase A, AL buffer and ethanol was doubled and the volume of proteinase K was increased from 20 µL to 400 µL) differed not only in DNA concentration (Table 27) but in suitability for PCR and HRM analysis. Only DNA isolated from liver tissue from foetus 2 showed an increase in the fluorescence signal.

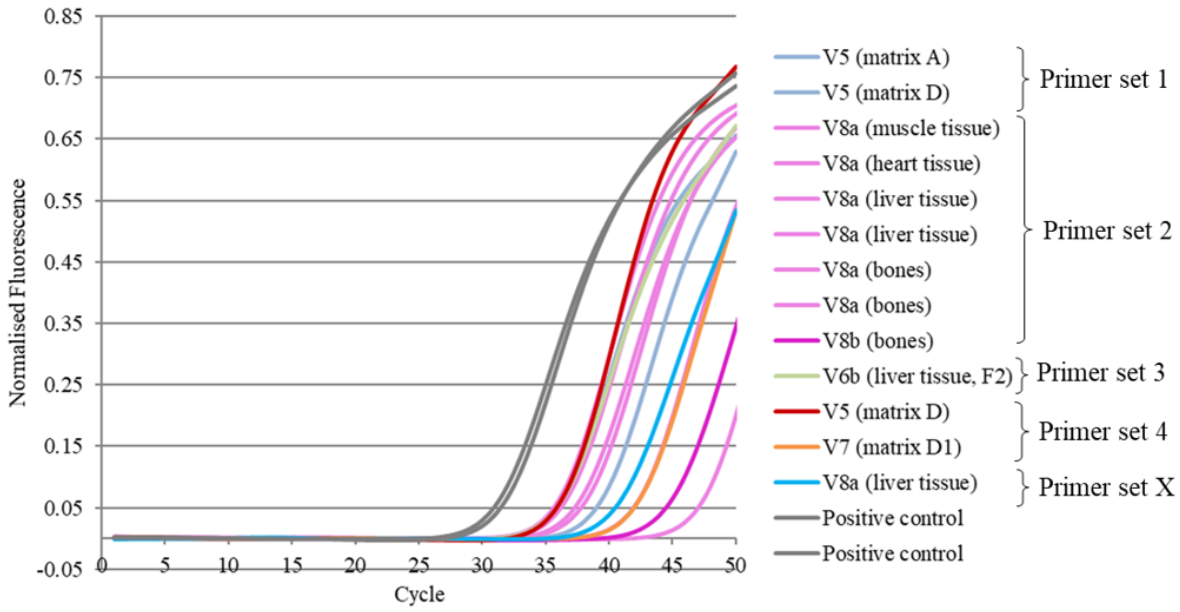


Figure 21: Amplification plot obtained with primer sets 1 (PAX9), 2 (SHH), 3 (OTX2), 4 (OTX2), X (MGMT) and DNA extracts obtained with protocols V5 (F3; matrix A, liver tissue homogenised in PBS; matrix D, liver tissue homogenised in ATL buffer), V6b (F2; liver tissue), V7 (F3; matrix D and D1; liver tissue), V8a (F3; muscle, heart, liver tissue and bones) and V8b (F3; bones). HCC827 Erlo and C8 were used as positive controls. PCRs with primer sets 1, 4 (V5) and X were carried out using two technical replicates per sample; PCRs with primer set 2, 3 and 4 (V7) were carried out with five, three and four technical replicates per sample, respectively.

Figure 22 depicts the corresponding melting plot obtained with primer sets 1, 2, 3, 4 and X. As already observed for extracts obtained with protocols based on the QIAamp DNA Mini Kit (Figure 19), the melting curves showed an atypical broad shape resulting from low number of fluorescence molecules due to the shortness of the amplicon. Consequently, the determination of the genotype variant was omitted.

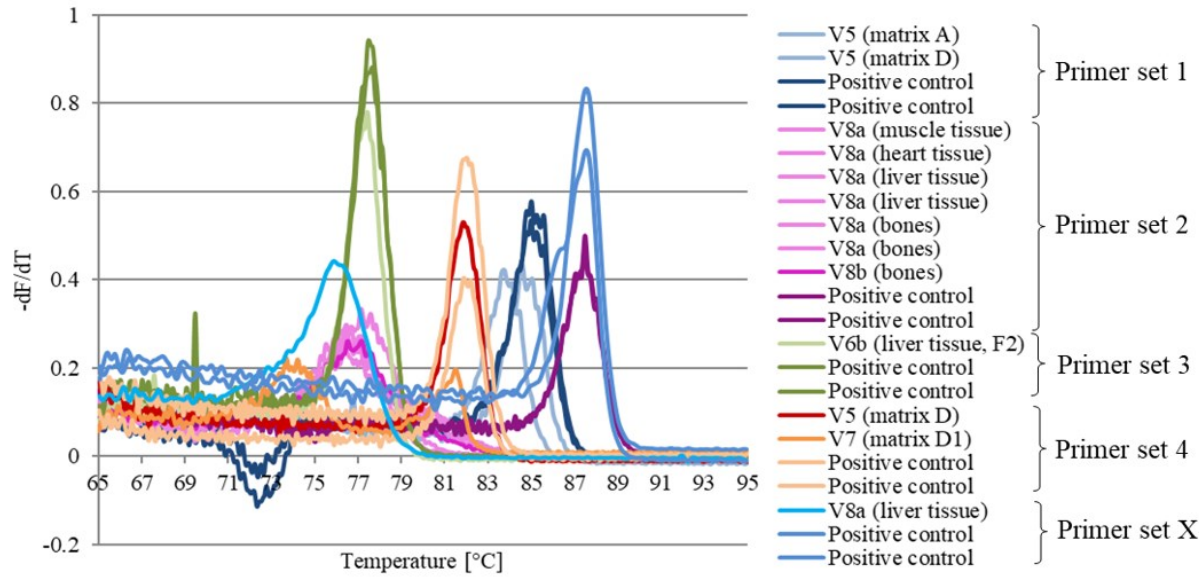


Figure 22: Melting plot obtained with primer sets 1 (PAX9), 2 (SHH), 3 (OTX2), 4 (OTX2), X (MGMT) and DNA extracts obtained with protocols V5 (F3; matrix A, liver tissue homogenised in PBS; matrix D, liver tissue homogenised in ATL buffer), V6b (F2; liver tissue), V7 (F3; matrix D and D1; liver tissue), V8a (F3; muscle, heart, liver tissue and bones) and V8b (F3; bones). HCC827 Erlo and C8 were used as positive controls. PCRs with primer sets 1, 4 (V5) and X were carried out using two technical replicates per sample; PCRs with primer set 2, 3 and 4 (V7) were carried out with five, three and four technical replicates per sample, respectively.

5.3.3 PCR Amplification of DNA Extracts Obtained with CTAB Protocol

DNA extracts obtained with CTAB protocol (see Chapter 5.1.3) were analysed with PCR-HRM using primer sets 1, 2, 3, 4 and X.

Figure 23 shows the amplification plot (left) and melting plot (right). As already observed for the spin-column based extraction methods, the CTAB extraction did not yield high DNA concentrations, thus, PCR amplification failed in most cases. Only with primer set 1 (*PAX9*) and 3 (*OTX2*) PCR products were obtained.

As already observed in Figure 19 and Figure 22, the melting curves showed an atypical broad shape resulting from low number of fluorescence molecules due to the shortness of the amplicon. Consequently, the determination of the genotype variant was omitted.

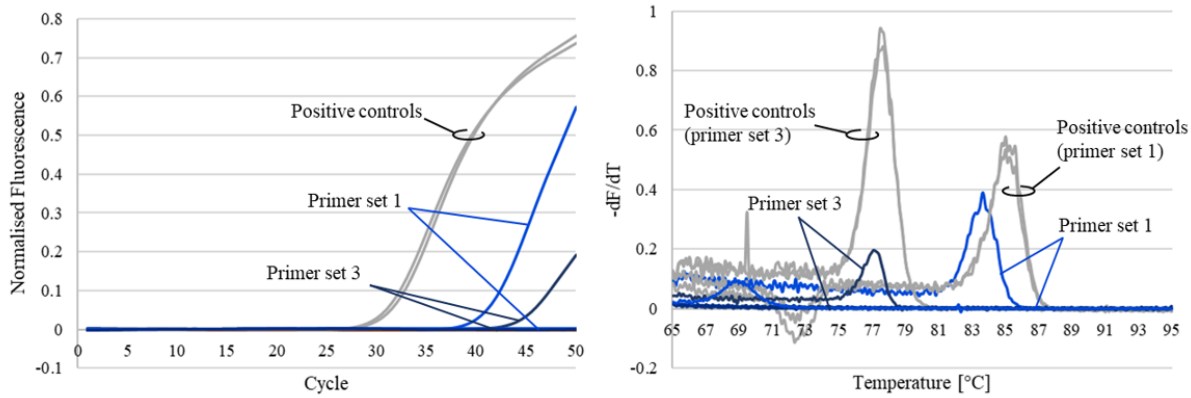


Figure 23: Amplification plot obtained with primer sets 1 (PAX9) and 3 (OTX2) and DNA extracts obtained with CTAB protocol. The experiment was carried out in duplicates.

To further improve DNA amplification, the volume of DNA extract per reaction was increased from 2 µL to 4 µL. The experiment was carried out using both positive controls (C8 and HCC827 Erlo) and primer set 1 and 2. The obtained Ct values are shown in Table 39.

Table 39: Ct values of the amplification curves obtained with primer sets 1 (PAX9) and 2 (SHH) and 2 µL /4 µL DNA extract from positive controls HCC827 Erlo and C8. The experiment consisted of two technical replicates per sample. Given Ct value represents the mean of two technical replicates.

Primer set	Sample	Volume DNA extract [µL]	Ct value
Primer set 1	HCC827 Erlo	2	26.28
	HCC827 Erlo	4	25.54
	C8	2	27.24
	C8	4	26.46
Primer set 2	HCC827 Erlo	2	28.49
	HCC827 Erlo	4	27.69
	C8	2	29.90
	C8	4	28.87

It can be concluded, that lower Ct values were obtained, when increasing the volume of DNA extract per reaction to 4 µL. The experiment was repeated using DNA from extracts obtained with various DNA extraction protocols. However, only DNA extracts that had been successfully amplified in previous experiments (Figure 18, Figure 21 and Figure 23) were analysed with PCR-HRM using primer sets 1 and 2. The Ct values are given in Table 40.

It can be concluded, that doubling the amount of DNA (4 µL DNA extract) per PCR reaction does not significantly improve PCR amplification. On the contrary, random DNA amplification was observed.

Table 40: Ct values obtained with primer sets 1 (PAX9) and 2 (SHH) and DNA extracts (2 µL and 4 µL) obtained with protocols V1a, V2a, V2b, V4, V5 and CTAB standard protocol, that had been amplified in previous experiments (Chapters 5.3.1, 5.3.2 or 5.3.3). The experiment consisted of two technical replicates per sample. Given Ct value represents the mean of two technical replicates. -[a]: not amplified; -[b]: not tested.

Extraction method	Primer set	Volume DNA extract [µL]	Ct value	Ct value
V1a	1	2	-[a]/-[a]	32.09/34.43
		4	43.25/-[a]	-[b]
CTAB	1	2	35.26/-[a]	37.95/-[a]
		4	32.93/-[a]	-[b]
V5	1	2	-[a]/-[a]	36.04/-[a]
		4	-[a]/-[a]	-[b]
V5	1	2	-[a]/-[a]	32.81/-[a]
		4	36.89/-[a]	-[b]
V4	1	2	43.16/-[a]	37.43/-[a]
		4	-[a]/-[a]	-[b]
V2a	2	2	44.88/-[a]	50.64/-[a]
		4	-[a]/-[a]	-[b]
V2b	2	2	-[a]/-[a]	48.42/-[a]
		4	42.14/-[a]	-[b]

5.3.4 PCR Amplification of DNA Extracts Obtained with Protocols Based on Phenol-Chloroform-Isoamyl Alcohol Extraction

DNA extracts obtained with protocols S_{PC}, V9 - V12 (see Table 30, Chapter 5.1.4) were analysed with PCR-HRM using primer sets 2, 3, 4 and X. Figure 24 shows the obtained amplification plot. In general, DNA extracts obtained with PC-based protocols yielded more satisfactory results regarding DNA concentration and PCR amplification compared to DNA extracted with the spin-column based methods.

Protocols S_{PC} (9.13 µL proteinase K after tissue homogenisation and 5.69 µL proteinase K after formaldehyde-modification reversal step) and V9a (volume of proteinase K was increased to 100 µL and 57.5 µL after tissue homogenisation and formaldehyde-modification reversal step, respectively) yielded DNA that was successfully analysed with three different PCR assays (S_{PC} with primer sets 2, 3 and 4; V9a with primer sets 2, 4 and X), respectively. Furthermore, DNA from heart and liver tissue (F3) were equally suitable for PCR amplification.

The application of lysing matrix F (V11) for the homogenisation and subsequent extraction and amplification of DNA seemed to work best, compared to other matrices (A, A2, D, D1, M2, I).

However, scaling up the amount of tissue from 25 mg to 100 mg (V12b) did neither increase DNA concentration (Table 30) nor did it improve PCR amplification.

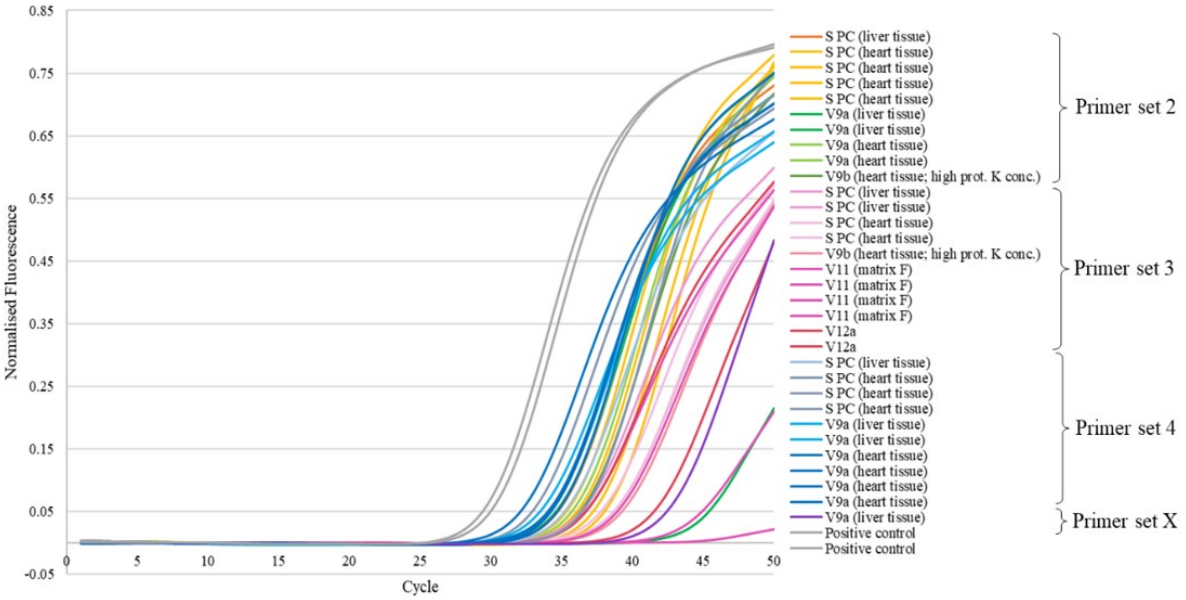


Figure 24: Amplification plot obtained with primer sets 2 (SHH), 3 (OTX2), 4 (OTX2) and X (MGMT) and DNA extracts obtained with protocol S_{PC} (F3; liver and heart tissue), V9a (F3; liver and heart tissue), V9b (F3; heart tissue; high prot. K conc.), V11 (F3; matrix F; liver tissue) and V12a (F3; liver tissue). HCC827 Erlo and C8 were used as positive controls. PCRs with primer sets 2, 3 and 4 were carried out with five technical replicates per sample; PCR using primer set X was carried out in duplicates.

The corresponding melting plot (Figure 25) shows that in most cases the melting behaviour of the PCR products matches that of the respective positive controls. However, PCR products of lower melting temperatures were observed for two PCR assays (comprising primer set 3 and 4). As already observed in Figure 19, Figure 22 and Figure 23 the melting curves showed an atypical broad shape resulting from low number of intercalated fluorescence molecules due to the shortness of the amplicon. Consequently, the determination of the genotype variant was omitted.

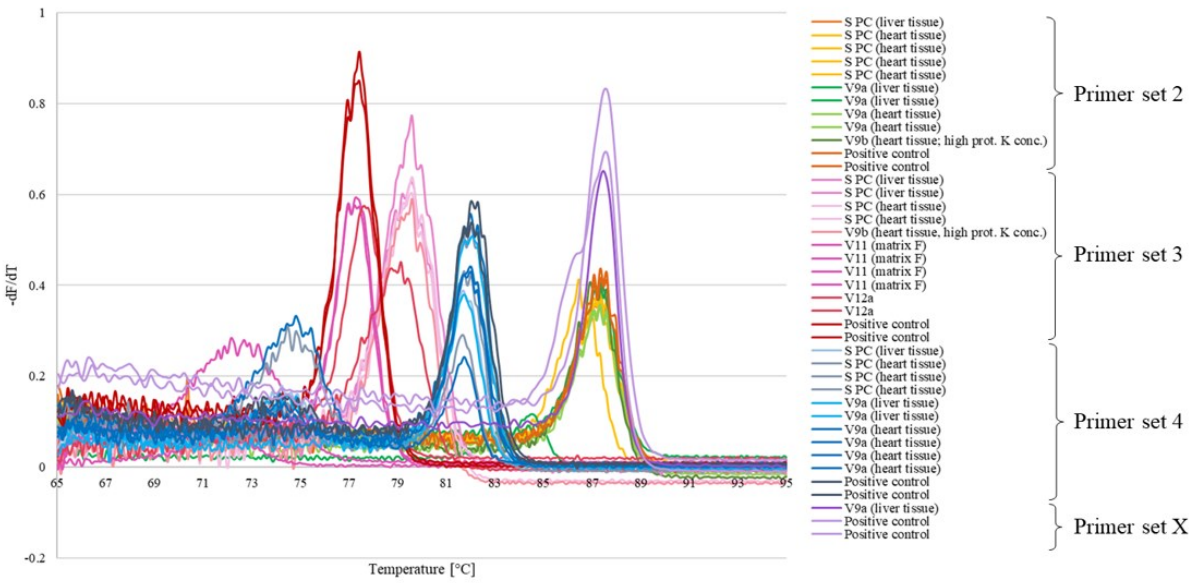


Figure 25: Melting plot obtained with primer sets 2 (SHH), 3 (OTX2), 4 (OTX2) and X (MGMT) and DNA extracts obtained with protocol S_{PC} (F3; liver and heart tissue), V9a (F3; liver and heart tissue), V9b (F3; heart tissue; high prot. K conc.), V11 (F3; matrix F; liver tissue) and V12a (F3; liver tissue). HCC827 Erlo and C8 were used as positive controls. PCRs with primer sets 2, 3 and 4 were carried out with five technical replicates per sample; PCR using primer set X was carried out with duplicates.

5.3.5 Determination of the Integrity of PCR Products Using Agarose-Gel Electrophoresis

To investigate if the lack of reproducible DNA amplification results from poor DNA integrity, an agarose-gel electrophoresis was performed using PCR products (PCR assay with primer set 2) from extracts obtained with various DNA extraction protocols (Figure 26). The length of most PCR products ranged between 50 – 75 bp and was, therefore, lower than the desired length of 86 bp (primer set 2). Furthermore, amplicons of 200 bp or longer were obtained for four DNA extracts obtained with protocols $S_{Mini\ Kit}$ (F2; liver tissue), V2a (F2; liver tissue), V2b (F3; liver tissue) and V5 (F3; matrix A and D; liver tissue; Figure 26), resulting from non-specific products.

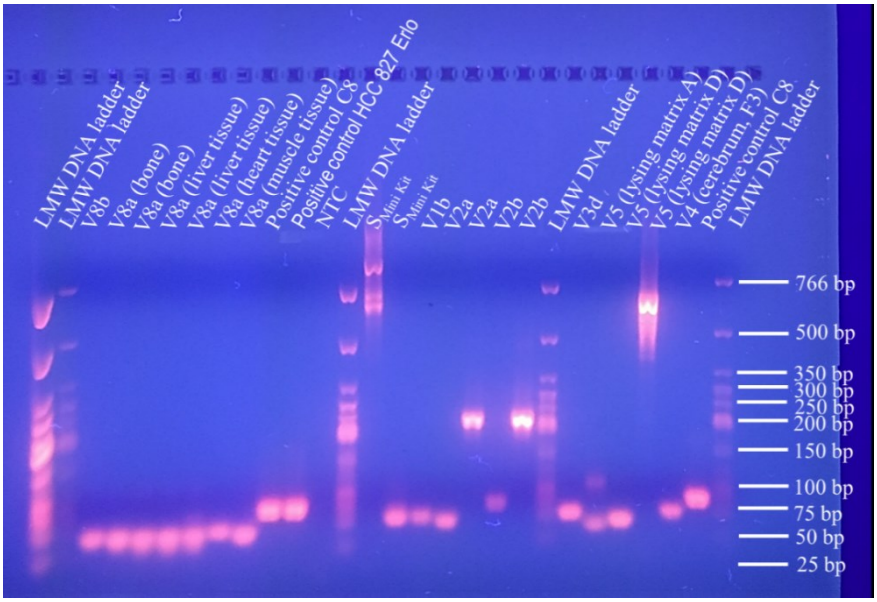


Figure 26: Agarose-gel (2 %) under UV light. Low molecular weight (LMW) DNA ladder was diluted (1:2, 1:4 or 1:8) to determine which dilution was most suitable for optimal visualisation of the reference bands. Amplified DNA from extracts obtained with protocols $S_{Mini\ Kit}$ (F2; liver tissue), V1b (F2; liver tissue), V2a (F2; liver tissue), V2b (F3; liver tissue), V3d (F3; liver tissue), V4 (F3; cerebrum tissue), V5 (F3; matrix A and D; liver tissue), V8a (F3; liver, heart and muscle tissue and bone) and V8b (F3; bone) were applied onto the gel. C8 and HCC827 Erlo served as positive controls.

To check if DNA extracts contained double-stranded nucleotides of high integrity, i.e. DNA fragments longer than 100 bp, an agarose-gel electrophoresis with DNA extracts was performed prior to PCR-HRM analysis.

DNA isolated with protocols $S_{FFPE\ Kit}$, CTAB standard protocol, V4, V5 and V8a showed no bands on the gel (Figure 27). An explanation would be the low DNA concentration of the extracts (Table 26, Table 27, Table 29 and Table 30). Nevertheless, the smeared band from the DNA extract obtained with protocol V9b indicates, that DNA fragments longer than 100 bp had successfully been extracted. Hence, PCR-HRM analysis with developed PCR assays should have been possible. However, DNA could be too degraded or the proportion of long DNA fragments could be too low to be amplified in each technical replicate.

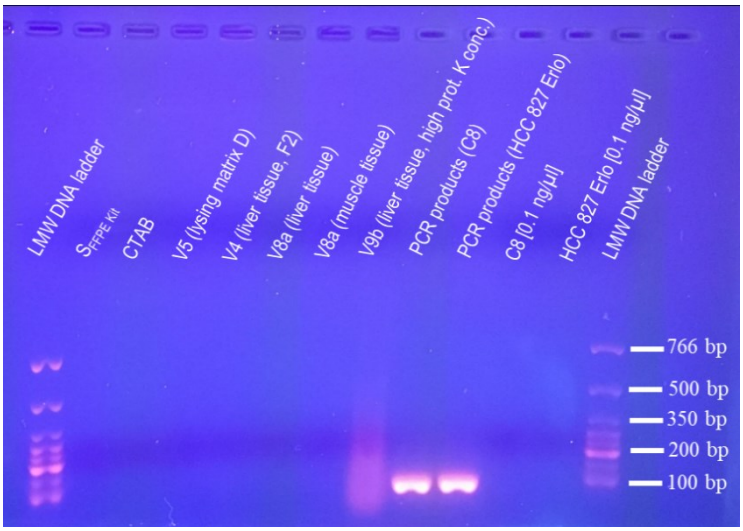


Figure 27: Agarose-gel (1 %) under UV-light. DNA extracts obtained with protocols SFFPE Kit (F2; liver tissue), CTAB standard protocol (F2; liver tissue), V5 (F3; liver tissue; matrix D), V4 (F2; liver tissue), V8a (F3; liver and muscle tissue) and V9b (F3; liver tissue; high prot. K conc.) were applied onto the gel. DNA extracts [0.1 ng/µL] and PCR products of C8 and HCC827 Erlo served as positive controls.

Furthermore, the impact of different lysing matrices on DNA integrity was investigated by performing an agarose-gel electrophoresis with DNA extracts obtained with protocol V11 (Figure 28). The use of lysing matrices seemed to have no impact on DNA integrity when compared to the sample that had not been homogenised with the tissue disruptor. Nevertheless, the tissue homogeniser was used, as the tissue could be completely lysed resulting in a clear, brownish solution.

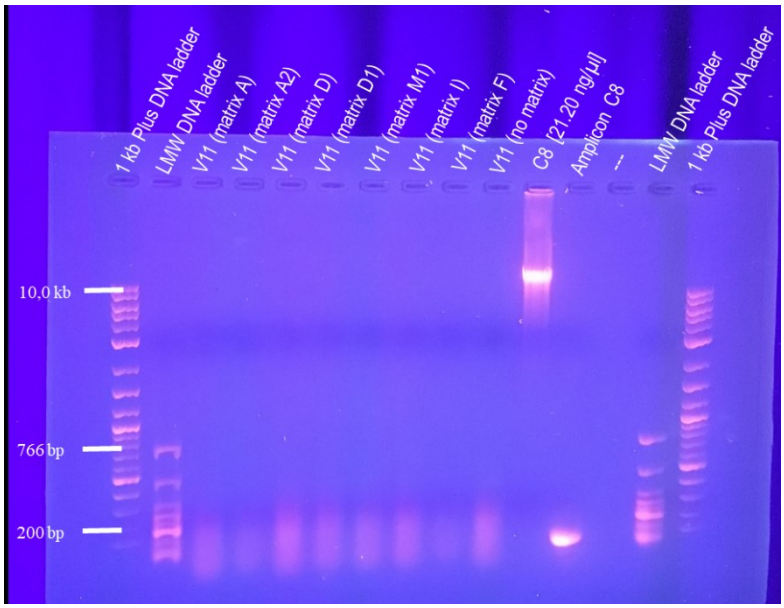


Figure 28: Agarose-gel (1 %) under UV-light. DNA extracts obtained with protocol V11 (F3; liver tissue; lysing matrices A, A2, D, D1, M1, I or F or without homogenisation) were applied onto the gel. DNA extract and PCR products of C8 served as positive controls.

5.3.6 Test for DNA Polymerase Inhibition by Components Other Than DNA Fragments

Another possible explanation for the lack of amplifiability of DNA extracts could be the inhibition of the DNA polymerase by components other than DNA fragments in the extract. Therefore, 2 µL of the positive control HCC827 Erlo were left at RT until completely evaporated and mixed with 2 µL DNA extract (Table 41). Then, a PCR under standard conditions (primer set 2) was performed. The coinciding Ct values in Table 41 suggest that components other than DNA fragments in the extracts did not inhibit the DNA polymerase.

Table 41: Ct values of the amplification curves obtained with 2 µL of the positive control HCC827 Erlo + 2 µL DNA extract obtained with protocols V9b or V10 and 2 µL of positive control (HCC827 Erlo) without the addition of 2 µL DNA extract. The experiment consisted of two technical replicates per sample. Given Ct value represents the mean of two technical replicates.

DNA extract	Ct value
V9b (liver tissue, low prot. K conc.)	28.29
V9b (heart tissue, low prot. K conc.)	28.90
V9b (liver tissue, high prot. K conc.)	28.78
V9b (heart tissue, high prot. K conc.)	28.45
V10 (liver tissue)	29.03
V10 (liver tissue)	28.61
V10 (heart tissue)	28.46
V10 (heart tissue)	28.16
positive control	28.63

5.3.7 Post-Treatment of PCR Products

A different approach to improve amplifiability comprised purification of PCR products by using the QIAquick PCR Purification Kit (Qiagen). The procedure was carried out according to the manufacturer's instructions. The purified DNA was then analysed with PCR-HRM. The PCR conditions are given in Table 21, Chapter 4.5.3.1.

According to Figure 29 (left), all five technical replicates of each sample were amplified. However, the melting curves of the PCR products differed from those obtained for the positive controls as they showed lower melting temperatures of about 73 °C, indicating that the amplified DNA fragments were much shorter (Figure 29; right).

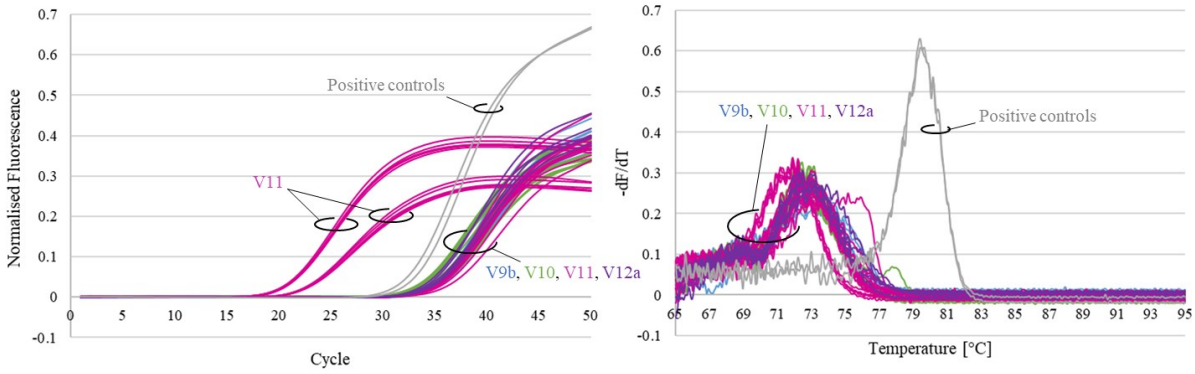


Figure 29: Amplification plot (left) and melting plot (right) obtained with primer set 3 (OTX2) and purified DNA isolated with protocols V9b, V10, V11 and V12a. HCC827 Erlo and C8 served as positive controls. PCR was carried out with five technical replicates per sample.

5.3.8 Pre-Treatment of DNA Extracts prior to PCR Analysis

The lack of amplifiability of template DNA could be further explained by the occurrence of random single strand breaks due to DNA degradation [17]. The treatment of DNA extracts with DNA polymerase prior to PCR amplification should seal nicks and restore the DNA sequence. For the reparation of damaged template DNA prior to PCR analysis two different protocols (protocol 1: application of PreCR™ Repair Mix; protocol 2: described by Bonin *et al.*; see Chapter 4.4.5) were applied and compared.

Results (Figure 30) indicate that neither protocol 1 nor protocol 2 yielded the 89 bp amplicon (primer set 3). It seems that DNA was too degraded, thus, the reparation of single strand breaks was unsuccessful.

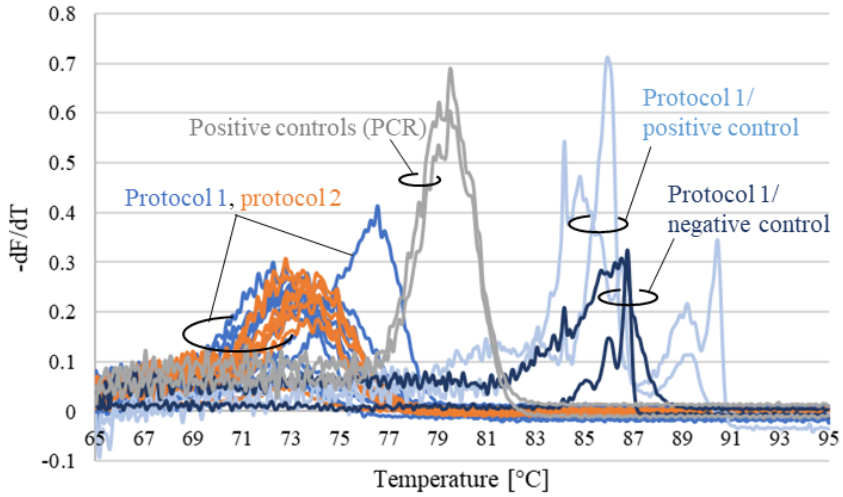


Figure 30: Melt plot obtained with primer set 3 (OTX2) and DNA extracts treated with protocols 1 and 2 prior to PCR analysis. DNA isolated with protocols V2b, V3a, V3b, V3c, V8a, V8b, V11 and V12a were used. HCC827 Erlo and C8 served as positive controls. PCR was carried out with two technical replicates per sample.

5.4 Application of Optimised Methods to Formalin-Fixed, Long-Term Stored Foetuses with Otocephaly-Dysgnathia Complex

Because further optimisation of DNA extraction protocols would have gone beyond the scope of this master's thesis, protocol V12a (with RNase treatment) was applied to the extraction of DNA from liver and heart tissue collected from two foetuses (#003 and #004) with ODC. Differences in the colour and morphology of each tissue type were observed for each specimen (Figure 31) indicating that DNA yield would vary between foetus #003 and #004. This was confirmed after determination of the concentration of DNA extracts (Table 42). While liver and heart tissue from foetus #003 yielded 4-5 ng/µL DNA (fluorometric measurement), DNA isolation from foetus #004 failed completely. The purity determined by A_{260}/A_{230}, was low for all DNA extracts, meaning that there were still contaminants present. Calculated values for A_{260}/A_{280} ranged between 0.9 and 1.8 for DNA extracts from foetus #004 and 2.0 and 2.2 for DNA extracts from foetus #003, indicating that the majority of the protein content had been removed from DNA extracts from foetus #003 only.

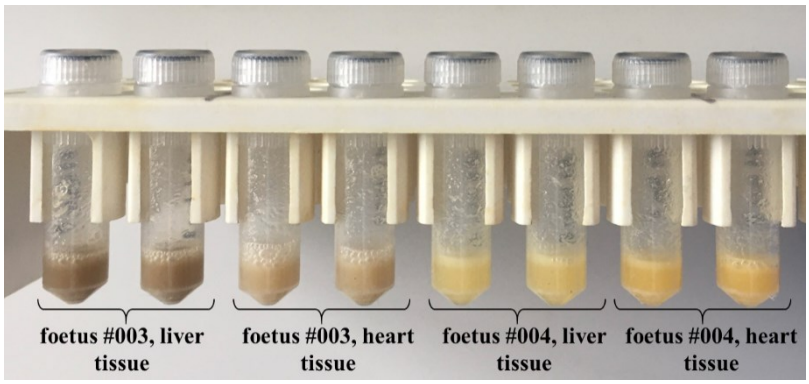


Figure 31: Homogenised liver and heart tissue from foetus #003 and #004. Two aliquots per tissue type from foetus 2 and 3.

Table 42: Results of phenol-chloroform-isoamyl alcohol extraction of tissue from foetuses with ODC.

Extraction method	Foetus/ type of tissue	Tissue amount [mg]	DNA concentration according to Qubit®-Fluorometer [ng/µL]	DNA concentration according to NanoDrop [ng/µL]	A_{260}/A_{280}	A_{260}/A_{230}
V12a	#003/liver	25.7	4.46	82.45	1.52	2.10
	#003/liver	25.4	4.98	92.85	1.52	2.04
	#003/heart	25.5	4.06	71.45	1.50	2.12
	#003/heart	25.5	4.05	73.95	1.50	2.15
	#004/liver	25.4	< LOD	1.30	1.43	1.26
	#004/liver	25.7	< LOD	1.50	1.86	0.91
	#004/heart	25.4	< LOD	1.05	3.32	1.83
	#004/heart	25.8	< LOD	0.30	0.17	0.95

Nevertheless, DNA extracts were analysed with PCR-HRM using the PSQ PCR Master Mix according to Table 19, Chapter 4.5.3.1. The PCR conditions are given in Table 21, Chapter 4.5.3.1.

The obtained amplification plot in Figure 32 (left) shows that the DNA extracted from heart of foetus #003 could not be amplified at all. For DNA isolated from liver tissue (foetus #003 and #004) and heart tissue (foetus #004), only one out of five technical replicates resulted in an increase in the fluorescence signal. Additionally, the PCR product of interest could not be generated, as the melting behaviour of the amplicons differed from that of the positive controls (Figure 32, right).

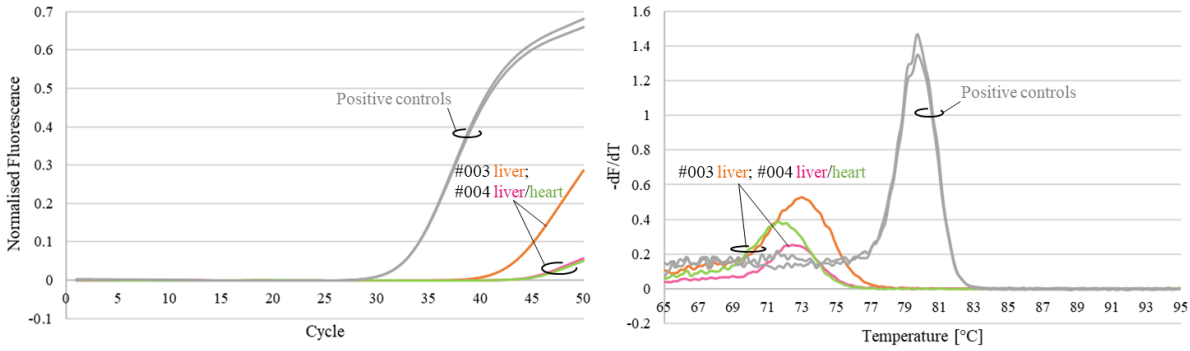


Figure 32: Amplification plot (left) and melting plot (right) obtained with primer set 3 (OTX2) and DNA extracts from liver and heart tissue from foetuses #003 and #004. The experiment was carried out with five technical replicates per sample.

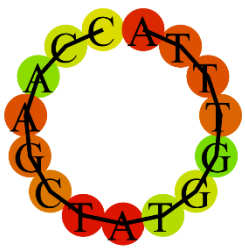
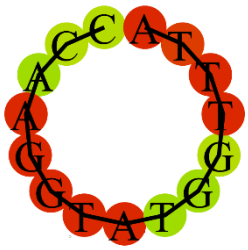
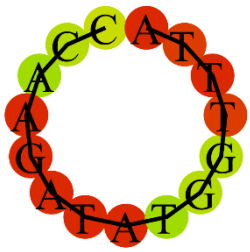
5.5 Pyrosequencing

To investigate if the DNA sequence of generated amplicons matched the sequence of template DNA, selected PCR products were submitted to pyrosequencing. Therefore, a sequencing assay had to be established and optimised regarding choice of sequencing primer, $MgCl_2$ concentration and amount of streptavidin beads and biotinylated PCR product.

5.5.1 Design of Sequencing Primer

Due to an A/G SNP located on the binding site of the sequencing primer (Figure 11, Chapter 4.5.2), three different sequencing primers had to be designed, differing in only one base (Table 43, highlighted in green). The characteristics of the sequencing primers are summarised in Table 43. Each primer consists of 15 bases and shows no secondary structures according to RNAfold web server.

Table 43: Characteristics of sequencing primers. Differences in the sequence are highlighted in green.

	SeqC	SeqG	SeqA
Sequence 5' → 3'	CCAAGCTATGGTTTA	CCAAGGTATGGTTTA	CCAAGATATGGTTTA
T_m [°C] according to OligoCalc	40.6	40.6	37.8
Secondary structures at 50 °C according to RNAfold web server			

5.5.2 *Optimisation of the $MgCl_2$ Concentration*

According to the Ct values detailed in Table 44 the DNA polymerase worked most efficiently at a final $MgCl_2$ concentration of 1.5 mM. All future PCRs for the amplification of DNA from extracts were carried out with a reaction mix containing 1.5 mM $MgCl_2$.

Table 44: Ct values of the amplification curves obtained with positive controls C8 and HCC827 Erlo and primer set 3 (biotinylated rv primer). The experiment consisted of two technical replicates per sample. Given Ct value represents the mean of two technical replicates.

Positive control	Additional $MgCl_2$ concentration [mM]	Ct value
C8	1.5	29.35
HCC827 Erlo		29.48
C8	2.0	30.26
HCC827 Erlo		30.27
C8	2.5	30.47
HCC827 Erlo		30.16

5.5.3 *Quantity of Streptavidin Beads & Amplicon and Selection of Sequencing Primer*

For the optimisation of the quantity of streptavidin beads, two different master mixes were prepared. One master mix followed the pipetting scheme given in Table 22, while the other one contained 1.5 times the volume of streptavidin beads and binding buffer (Table 24).

In addition, the volume of biotinylated PCR product was increased to 22.5 µL for the second master mix.

Since an A/G SNP was located on the binding site of the sequencing primer, two experimental approaches had to be set up. One consisted of the use of sequencing primer SeqC only, while for the other approach sequencing primers SeqG and SeqA were applied in equimolar amounts (Table 25).

When primer SeqC was used, the obtained pyrograms showed that the genotype determination failed, indicated by red highlighted values. This was the case regardless of the master mix used. A representative pyrogram is shown in Figure 33. The analysed sequence was AGAAT**BR**AAG**W**G**M**TAAGTGCC**K**C**M**A**R**CAACAG**[C]**AACAACAGCAGAATGGAG GTCAAAA. All SNPs are highlighted in bold characters, the SNP of interest is additionally coloured in blue. The sequence comprised 60 bp and 8 SNPs.

A combination of primers SeqA and SeqG was suitable for the determination of the DNA sequence and worked with both master mixes. Values highlighted in blue were considered as accurate, while yellow highlighted values needed to be inspected more closely. In all cases, the SNP of interest (frameshift variant, [C/-]) was identified. At this position a peak is visible, which results from the incorporation of a C. A representative pyrogram is shown in Figure 34. The analysed sequence was AGAAT**BR**AAG**W**G**M**TAAGTGCC**K**C**M**A**R**CAACAG**[C]**AAC AACAGCAGAATGGAGGTCAAAA and consisted of 60 bp. All 8 SNPs are highlighted in bold characters, the SNP of interest is additionally coloured in blue.

Due to this outcome, a mixture of primers SeqA and SeqG was used for all further pyrosequencing experiments. Since the signals obtained for the samples with the second master mix were too high, the upscaling of beads and amplicons were omitted.

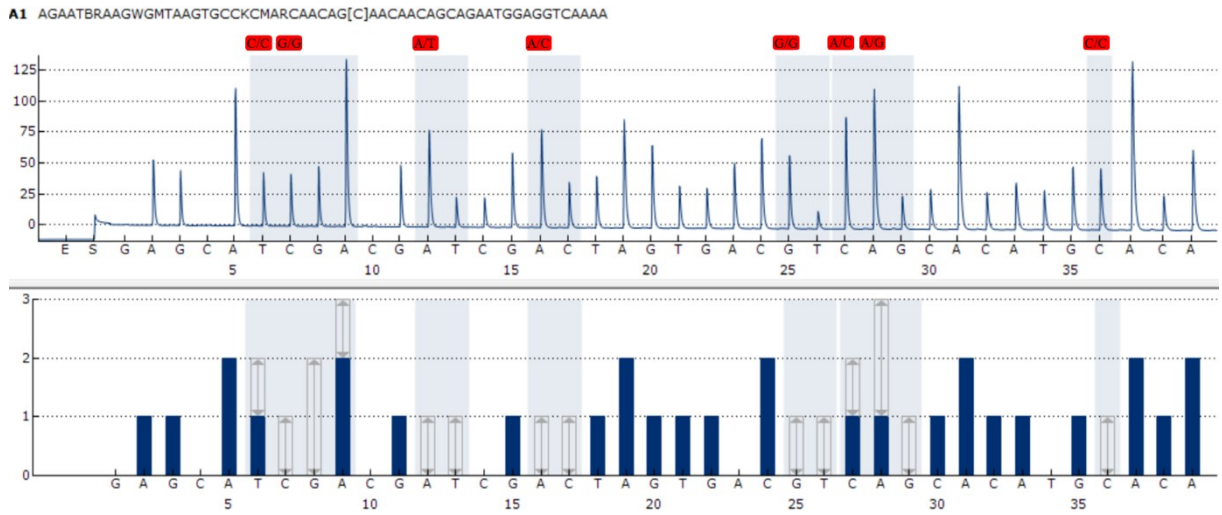


Figure 33: Representative pyrogram for positive control C8 in 1 x MM (master mix) and sequencing primer C (upper image) in comparison to the histogram (image below) showing the expected peak pattern. Variable positions are highlighted in grey.

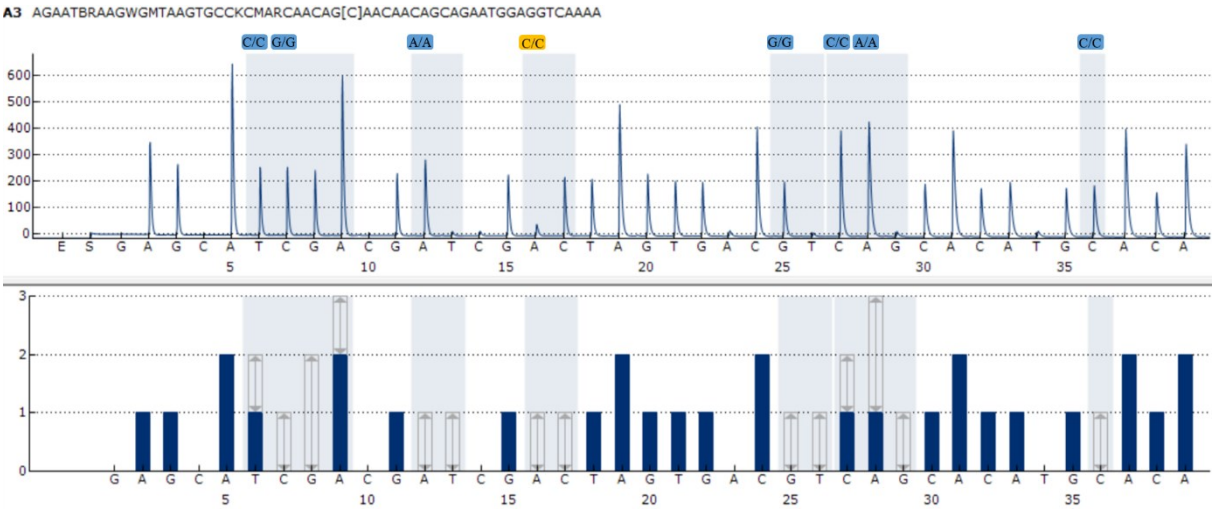


Figure 34: Representative pyrogram for positive control C8 in 1 x MM (master mix) and sequencing primers A and G (upper image) in comparison to the histogram (image below) showing the expected peak pattern. Variable positions are highlighted in grey. SNP of interest is located at the last variable position.

5.5.4 Application of Established Pyrosequencing Assay for Determination of DNA Sequence of PCR Products from DNA Extracts

Five selected PCR products that had shown the correct melting behaviour (T_m = 80 °C) after PCR-HRM analysis were submitted to pyrosequencing using the optimised sequencing conditions described in Chapter 5.5.3. Furthermore, 15 PCR products that had shown a different melting behaviour (T_m = 72 °C - 74 °C) were also analysed by PSQ. The unknown sequence of the unspecific products was determined by de-novo-sequencing. Therefore, the dispensing

order of the dNTPs was set manually. The four dNTPs were added to the reaction mix in following order: CAGT.

Figure 35 demonstrates that the determination of the DNA sequence of the unspecific PCR products failed. This could be explained by the fact that the sequencing primer was unable to bind to the DNA sequence. It confirmed that the 89 bp amplicon had not been generated during PCR. Hence, the sequence of interest (AGAAT**BR**AAG**W**G**M**TAAGTGCC**K**C**M**A**R**CAACA G**[C]**AACAACAGCAGAATGGAGGTCAAAA; SNPs are highlighted in bold characters; the SNP of interest is additionally coloured in blue) could not be determined by de-novo-sequencing.

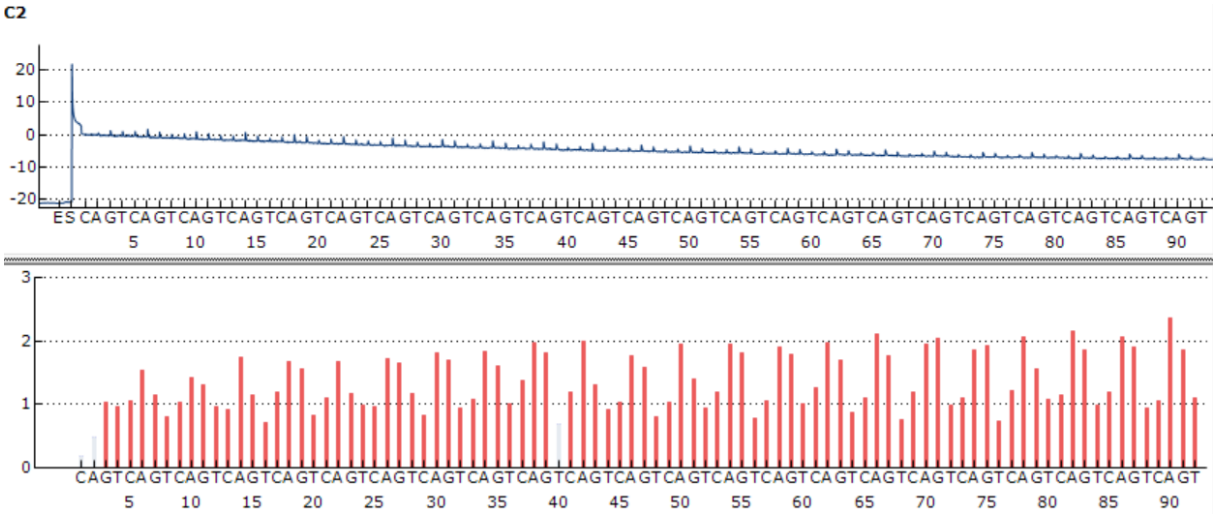


Figure 35: Representative pyrogram for unspecific PCR product obtained with DNA extract from liver tissue (F3) with protocol V8a (upper image) in comparison to dispensation order (image below).

Nevertheless, PSQ analysis of PCR products that had shown the correct melting behaviour after HRM analysis was successful, as the DNA sequence matched the sequence of the positive control, hence, the sequence of interest. In addition, all genotype variants were determined. An example is given in Figure 36.

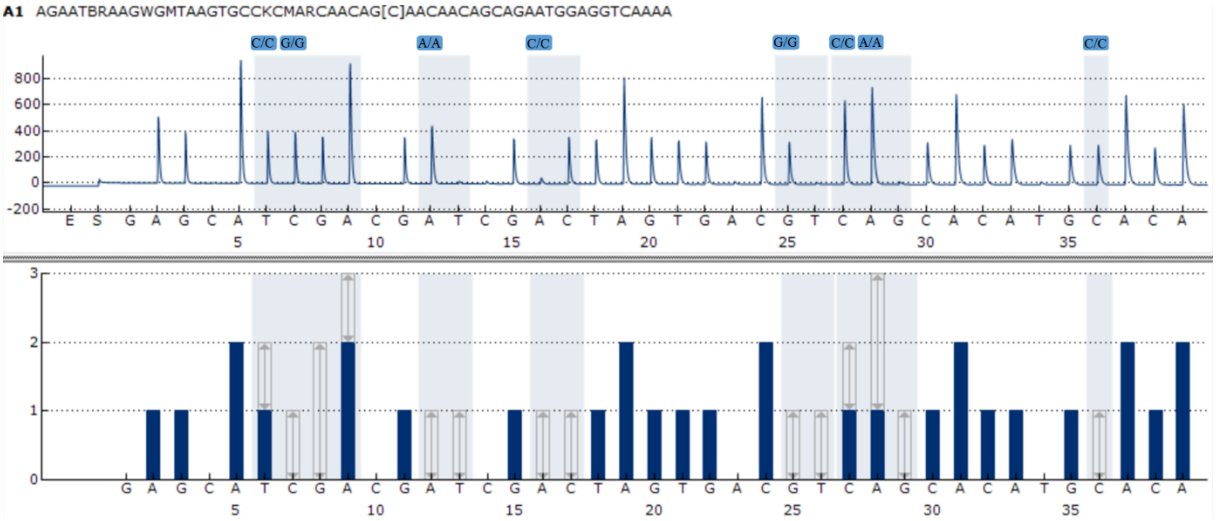


Figure 36: Representative pyrogram for correct PCR product obtained with DNA extract from liver tissue (F3; low prot. K conc.) with protocol S_{PC} (upper image) in comparison to the histogram (image below) showing the expected peak pattern. Variable positions are highlighted in grey. SNP of interest is located at the last variable position.

6 Conclusion

The use of formalin as a fixation medium opened up new possibilities for the collection and the long-term storage of valuable specimens. It was an easy way of maintaining the morphology of tissue over a long period of time. Today, these collected specimens are an indispensable resource for genomic studies regarding analysis of rare diseases, e.g. Otocephaly-Dysgnathia Complex (ODC). However, formalin fixation causes cross-linkages of nucleic acids, which makes DNA extraction difficult. DNA often lacks high integrity due to degradation and fragmentation caused by the fixative solution.

Previous studies have reported the successful extraction of intact, genomic DNA from formalin- or ethanol-fixed, long term stored tissue.

In the present master's thesis, several DNA extraction methods were investigated, in order to obtain genomic DNA with high integrity regarding yield, purity, PCR amplifiability and suitability for SNP analysis. The optimised DNA extraction protocol should further be applied to formalin-fixed tissue from foetuses with ODC.

For DNA isolation, two commercially available DNA extraction kits, a CTAB extraction and phenol-chloroform extraction were applied. The protocol for the DNA extraction kits and the phenol-chloroform extraction were further optimised regarding pre-treatment prior to lysis and lysis duration.

The phenol-chloroform extraction, which had been described in literature as one of the most appropriate techniques for isolation of DNA from formalin-fixed, long-term stored tissue has proven to be best suitable for DNA extraction compared to the CTAB method and commercial DNA extraction kits. The latter two yielded low amount of DNA of poor integrity, which resulted in random PCR amplification. Highest DNA quantities were obtained for the organic solvent-based extraction method. Protocols V11 and V12a worked best regarding DNA yield. It consisted of the rehydration of tissue in PBS for 2 h, the homogenisation using the Fast Prep-24 5G Tissue Homogeniser and a 18 h lysis in a mixture of TE9 buffer, proteinase K and 20 % SDS (w/v). A heating step (1 h, 90 °C) for the reversal of formaldehyde-modifications was included prior to phenol-chloroform extraction.

For PCR-HRM analysis four different assays were developed and optimised regarding primer concentration, annealing temperature and $MgCl_2$ concentration. Two assays consisted of primers targeting DNA segments in the *OTX2* gene, while the other two assays comprised primers targeting DNA sequences in the *SHH* and *PAX9* gene, respectively. All four developed

PCR assays were suitable for the amplification of DNA from extracts. However, due to poor DNA integrity, random amplification was observed and, in most cases, even failed. As a consequence, SNP analysis was impossible for many DNA samples. Amplified DNA was further analysed by pyrosequencing. It allowed the determination of the amplicon sequence and therefore the identification of relevant SNPs.
For the determination of the genotype of foetuses with ODC, the protocol V12a was applied to liver and heart tissue from two foetuses with facial dysmorphia. DNA yield varied between the two foetuses. While liver and heart tissue from foetus #003 yielded 4-5 ng/µL DNA, DNA isolation from foetus #004 failed completely. Subsequent PCR-HRM analysis revealed that the 89 bp DNA template (*OTX2* gene) could not be amplified. Consequently, the determination of the SNP of interest failed.

Future experiments should focus on optimising DNA extraction protocols further, as it is crucial for increasing DNA yield. The application of a hot-alkali extraction protocol [13,40] for the isolation of DNA could be investigated regarding yield, purity, PCR amplifiability and SNP analysis. Furthermore, the development of a new PCR assay could improve PCR amplification and subsequent SNP analysis.

7 List of Utensils

7.1 Chemicals and Kits

1 kb Plus DNA ladder	New England Biolabs
Agarose	Sigma-Aldrich
Ammonium acetate (NH_4Ac)	Sigma-Aldrich
Carrier RNA	Qiagen
Cetyltrimethylammonium bromide (CTAB)	Sigma-Aldrich
DNA Exitus Plus™ IF	AppliChem
EB buffer	Qiagen
EDTA	VWR International
EpiTect HRM PCR Kit	Qiagen
Ethanol (EtOH) abs.	Merck
EvaGreen dye	Biotium
GelRed nucleic acid stain	Sigma-Aldrich
Low molecular weight DNA ladder	New England Biolabs
Millipore H_2O	
NaCl	Jena Bioscience
Nucleic acid sample loading buffer	Bio-Rad Laboratories
Phenol-chloroform-isoamyl alcohol (25:24:1)	Sigma-Aldrich
Phosphate buffered saline (PBS)	Sigma-Aldrich
PreCR™ Repair Mix	New England Biolabs
Primer	Sigma-Aldrich
Proteinase K solution [20 mg/mL]	Qiagen
PyroMark denaturation solution	Qiagen
PyroMark PCR Kit	Qiagen
PyroMark Q24 Advanced CpG Reagents	Qiagen
PyroMark wash buffer, 10x	Qiagen
QIAamp® DNA Mini Kit	Qiagen
QIAamp® DNA FFPE Tissue Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific
Reagent DX	Qiagen

RNase A solution [100 mg/mL]	Qiagen
RNase-free water	Qiagen
Roti®-phenol-chloroform-isoamyl alcohol (25:24:1)	Roth
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Streptavidin sepharose high performance	GE Healthcare
Taq DNA polymerase	Qiagen
Tris/HCl	Jena Bioscience
Triton X-100	Sigma-Aldrich

7.2 Consumables

Centrifuge tubes (15 mL, sterile)	Thermo Fisher Scientific
Centrifuge tubes (50 mL, sterile)	Sarstedt, Labcon
Impact resistant tubes (2.0 mL)	Sarstedt
Lysing matrices	MP Biomedicals
Reaction Tubes (0.65 mL, sterile)	VWR International
Reaction Tubes (1.5 mL, sterile)	VWR International
Reaction Tubes (2.0 mL, sterile)	Eppendorf
Reaction Tubes (5.0 mL, sterile)	Eppendorf
iQ 96-well PCR plate	Bio-Rad Laboratories
Microseal 'B' adhesive seals	Bio-Rad Laboratories
PCR tubes (0.2 mL, sterile)	Bio-Rad Laboratories
Petri dishes, 60 x 15 mm	Eppendorf
Pipette tips (filter tips BRAND® Tip-Box)	VWR International, Sigma-Aldrich
PyroMark Q24 cartridge	Qiagen
PyroMark Q24 24-well plate	Qiagen
Qubit Assay Tubes	Thermo Fisher Scientific
Needles	B. Braun
Strip tubes and caps (0.1 mL)	Qiagen
Scalpel	B. Braun
Syringe	B. Braun

7.3 Equipment

Analytical balance TE214S	Sartorius
Centrifuge 4K10	Sigma
Centrifuge 5424	Eppendorf
Centrifuge Galaxy Mini	VWR International
Centrifuge Biofuge 28 RS (Rotor 3740)	Heraeus Sepatech
Centrifuge mini spin	Eppendorf
Fluorometer Qubit 4.0	Thermo Fisher Scientific
PCR System QuantStudio™ 5	Thermo Fisher Scientific
Pipettes	Eppendorf, BioRad
PyroMark Q24 advanced instrument	Qiagen
PyroMark Q24 vacuum workstation	Qiagen
Spectrophotometer NanoDrop® 2000c	Thermo Fisher Scientific
Thermocycler iCycler	BioRad
Thermocycler Rotor-Gene®	Qiagen
Tissue Disruptor Fast Prep-24 5G	MP Biomedicals
Thermomixer comfort (24 x 1.5 ml)	Eppendorf
Vortexmixer genie 2	Scientific Industries
Vortexmixer classical advanced	VELP Scientifica
Vortexmixer VV3	VWR International
UV Transilluminator UVT-20 M	Herolab

7.4 Web Servers & Databases

National Center for Biotechnology Information (NCBI)

https://www.ncbi.nlm.nih.gov

OligoAnalyzer 3.1

http://eu.idtdna.com/calc/analyzer

OligoCalc: Oligonucleotide Properties Calculator

http://biotools.nubic.northwestern.edu/OligoCalc.html

RNAfold WebServer

http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

uMelt Batch 2.0

https://dna-utah.org/umelt/umb.php

7.5 Software

Citavi 6.4	Swiss Academic Software
Microsoft 365 ProPlus (Word, Excel, Powerpoint)	Microsoft
NanoDrop 2000/2000c Software	Thermo Fisher Scientific
PyroMark Assay Design 2.0	Qiagen
PyroMark Q24 Advanced 3.0.1	Qiagen
Rotor-Gene® Q Series Software 2.3.1	Qiagen

8 List of Figures

9 List of Tables

10 References

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Appendix A

Table 45: Different types of matrices and their applications, according to MP Biomedicals. [41]

Type of lysing matrix		Lysing matrix composition	Examples for sample type
Matrix A		garnet matrix and one ¼" ceramic sphere	lung, heart, skin, vascular tissue, bone, liver, brain, intestine, muscle, microorganism, plant tissue
Matrix A2		acc. to matrix A with an additional ¼" ceramic sphere	n.a.
Matrix D		1.4 mm ceramic beads	lung, heart, skin, vascular tissue, liver, brain, intestine, muscle, insects and worms
Matrix D1		acc. to matrix D with an additional ¼" ceramic sphere	n.a.
Matrix M		one ¼" ceramic bead	bone, infected tissue, plant seeds and needles
Matrix M2		two ¼" ceramic beads	n.a.
Matrix I		2 mm yellow ZrO_2 beads and 4 mm black ceramic beads	bacterial and fungal spores, environmental samples, plant seeds, wood, ticks and flies

Matrix S		six 1/8” stainless-steel beads	lung, heart, bone, liver, brain, intestine, muscle, ear, hair, nail, plant seeds, roots and needles
Matrix F		1.6 mm aluminium oxide particles	plant tissue, bacteria, yeast, mold, fungal and bacterial spores

Appendix B

DNA purification via precipitation (FAQ ID-305, Qiagen; improved)

20 µL of 3 M Na-Acetate (pH 5) and 400 µL of ice-cold 100 % ethanol were added to the DNA extract and vortexed thoroughly. The mixture was stored at -20 °C for one hour for DNA precipitation. Then, the sample was centrifuged at 4 °C, 15 000 rpm for 20 min, before carefully pipetting off the ethanol. 200 µL 70 % ethanol was added to the precipitated DNA, vortexed for 30 s and centrifuged at 4 °C, 15 000 rpm for 10 min. Again, the ethanol was pipetted off and the sample was incubated at RT for 1 h (with the lid open) allowing the pellet to dry. The remaining DNA was resuspended in 20 µL EB buffer by shaking gently for 4 h 40 min.

Appendix C

DNA extraction from breast tissue (control tissue C8) using the QIAamp DNA Mini Kit

In a 1.5 mL Eppendorf tube 25.4 mg of tissue was mixed with 180 µL ATL buffer and 20 µL proteinase K [20 mg/mL] and incubated at 56 °C, 800 rpm for 4 h. After brief centrifugation, 4 µL of RNase A [100 mg/mL] was added and mixed by pulse-vortexing for 15 s. The mixture was incubated for 2 min, briefly centrifuged, before adding 200 µL AL buffer. The solution was pulse-vortexed for 15 s and incubated at 70 °C for 10 min. Next, the sample was centrifuged briefly and mixed with 200 µL abs. ethanol (96-100 %) by pulse-vortexing for 15 s. After short

centrifugation the mixture was applied to the QIAamp Mini spin column (provided with a 2 mL collection tube) and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and the spin column transferred to a new 2 mL collection tube, before adding 500 µL AW1 buffer. After centrifugation at 8000 rpm for 1 min, the column was placed into a new 2 mL collection tube and 500 µL of AW2 buffer was added. The spin-column was centrifuged at full speed (12000 rpm, 3 min.), transferred to a new 2 mL collection tube and centrifuged once more at full speed for 1 min.

For the retrieval of bound DNA, the spin-column was then transferred to a 1.5 mL Eppendorf tube, before pipetting 100 µL of AE buffer onto the stationary phase of the column and incubation at room temperature (RT) for 1 min.

The DNA was then obtained by centrifugation at 8000 rpm for 1 min. The DNA extract was stored at -20 °C until further use.

DNA concentration determined with Qubit®-Fluorometer and NanoDrop®-Spectrophotometer

Table 46: DNA concentration of positive control C8.

DNA concentration according to Qubit®-Fluorometer [ng/µL]	DNA concentration according to NanoDrop [ng/µL]	A_{260}/A_{280}	A_{260}/A_{230}
21.20	24.05	1.80	2.30

Appendix D

Table 47: Ct values obtained with primer sets 1-3 and positive control HCC827 Erlo at different primer concentrations. The experiments consisted of duplicates. Given Ct value represents the mean of two technical replicates.

Primer (fw + rv) concentration [µM]	Primer set 1	Primer set 2	Primer set 3
0.2	31.89	35.38	35.38
0.4	28.38	30.53	30.91
0.6	27.30	29.38	29.39
0.8	27.55	28.93	29.60

Abstract

Otocephaly-Dysgnathia Complex (ODC) occurs 1 in 70 000 births only and is therefore considered a rare disease. Since the number of patients is limited, the genetic mechanisms underlying the evolvement of ODC are widely unknown. For decades fixation has been used for maintaining the integrity of tissues. Therefore, fixed and long-term preserved museum specimens are an indispensable resource for genomic studies.

In this study, several methods for DNA extraction from formalin- or ethanol-fixed foetal tissues were compared. The extracted DNA was characterised with regard to yield, purity, PCR amplifiability and suitability for single nucleotide polymorphism (SNP) analysis.

In order to gain access to the cross-linked DNA, following parameters of DNA extraction protocols were investigated: DNA extraction method (commercial kits, CTAB extraction and phenol-chloroform-isoamyl alcohol extraction), pre-treatment of the samples prior to lysis and lysis duration. DNA purity was determined by spectrophotometric measurements while DNA yield was additionally assessed with a fluorometric assay. Regions containing candidate SNPs were amplified and analysed by real-time PCR. The obtained PCR products were further analysed by pyrosequencing.

In general, DNA concentrations were rather low. Both commercial kits and the CTAB extraction method failed to yield sufficient DNA for PCR amplification. The highest DNA yield was obtained from formalin-fixed tissue using the organic solvent-based approach. Tissue rehydration, the use of high proteinase K concentrations in combination with overnight lysis and a formaldehyde-modification-reversal step improved DNA extraction. DNA isolated from tissue from foetuses with ODC could not be amplified. Consequently, the determination of the genotype failed. However, the few amplified DNA extracts from the healthy foetuses could successfully be sequenced and the SNP of interest determined.

In conclusion, time of fixation, fixative solution and preservation time influence yield and purity of extractable genomic DNA. Further investigations regarding the optimisation of the DNA extraction protocol are necessary in order to obtain reproducible results.

Zusammenfassung

Mit einer Häufigkeit von 1 in 70 000 Geburten zählt Otozephalie zu den seltenen Erkrankungen. Aufgrund der begrenzten Anzahl an Patienten sind die genetischen Faktoren, die zur Entwicklung von Otozephalie führen, bis heute noch wenig erforscht. Seit Jahrzehnten werden Fixierungsmethoden zur Erhaltung der Integrität von Geweben angewendet. Diese fixierten und lange Zeit konservierten Präparate repräsentieren eine unverzichtbare Ressource für genomische Untersuchungen.

Die Masterarbeit hatte zum Ziel, verschiedene Methoden zur Extraktion von genomischer DNA aus Formalin- oder Ethanol-fixiertem, fetalem Gewebe hinsichtlich Ausbeute, Reinheit, PCR Amplifizierbarkeit und Eignung zu Analyse von Einzelnukleotid-Polymorphismen (SNPs) zu vergleichen.

Um die quervernetzte DNA bestmöglich zu extrahieren, wurden folgende Parameter der DNA Extraktionsprotokolle untersucht: DNA Extraktionsmethode (kommerzielle Kits, CTAB Extraktion und Phenol-Chloroform-Isoamylalkohol Extraktion), Behandlung der Proben vor dem Gewebeverdau und Dauer der Lyse. Die Konzentration und Reinheit der erhaltenen DNA wurden mittels Spektrophotometer bestimmt, wobei die Konzentration zusätzlich noch fluorimetrisch ermittelt wurde. Genabschnitte, die potenzielle SNPs von Interesse enthielten, wurden amplifiziert und mittels real-time PCR analysiert. Die erhaltenen PCR Produkte wurden des Weiteren noch sequenziert.

Die Ergebnisse zeigten, dass die DNA Konzentrationen im Allgemeinen niedrig waren. DNA, welche mit kommerziellen Kits oder der CTAB Extraktion isoliert wurde, konnte nur unzureichend amplifiziert werden. Die höchsten DNA Konzentrationen wurden bei der Phenol-Chloroform-Isoamylalkohol Extraktion erzielt. Im Allgemeinen konnte die DNA Ausbeute durch die Rehydratisierung des Gewebes vor der Lyse, den Einsatz von hohen Proteinase K Konzentrationen in Kombination mit Lyse über Nacht und einem Erhitzungsschritt für die Umkehr von Formaldehyd-Modifikationen verbessert werden. Dennoch konnten die DNA Extrakte nicht reproduzierbar amplifiziert werden, was die weitere Optimierung der Extraktionsprotokolle komplizierte. Auch die DNA, welche aus dem Gewebe der Föten mit ODC isoliert wurde, konnte nicht amplifiziert werden, weshalb in weitere Folge die Bestimmung des Genotyps nicht möglich war. Dennoch konnten bei den wenigen amplifizierten DNA Extrakten von den gesunden Föten die DNA Sequenz und somit der SNP von Interesse bestimmt werden.