Charles University in Prague
Faculty of Science
Department of Anthropology and Human Genetics

Thesis

Using the quantitative DNA method as a screening tool for efficient genotyping of samples in forensic DNA laboratory

B.Sc. Ivana Koljenšić

Prague 2011

Supervisor: Mgr. Vlastimil Stenzl

Thesis declaration:

I hereby declare that this thesis covering the topic: “Using the quantitative DNA method as a screening tool for efficient genotyping of samples in forensic DNA laboratory” is my original work written on my own, with the use of the quoted literature**.**

Prague, _____

Signature

Acknowledgments,

Hereby I would like to thank to my supervisor Mgr. Vlastimil Stenzl for valuable advice and comments on my writing and also for the formation of my master's thesis which I hope has met his expectations. Many thanks undoubtedly belong also to colleagues from the Institute of Criminalistics Prague, who helped me to get familiar with the laboratory equipment, supported me and gave me a lot of advice.

Big thanks belong to my parents, siblings and friends for all their support during my studies.

Summary

Quantification of human DNA in forensic samples is an important step during STR profiling because the STR genotyping is sensitive to the quantity of DNA used in the PCR reaction. This study focuses on the importance of quantification in the entire process of genetic analysis. Two real time PCR platforms (Roche LightCycler480 System and ABI 7900 RT PCR) were used to compare two commercial kits in terms of DNA quantification. It was found out that accuracy of absolute quantification values in commercial quantification kits is strongly dependent on the construction of calibration curve. Especially low template DNA samples were used to assess whether Quantifiler™ or Plexor® HY System can determinate a minimum quantification value (**cut off value**) below which STR profiles would consistently fail to be detected. The usage of Plexor® HY System enabled to determine the cut off quantification value more exactly probably due to different molecular background and chemistry used in this kit. Reliability and other issues connected with cut off value are discussed. In order to better understand the relationship between the quantity of DNA and the number of detectable loci series the dilution experiment with standard DNA007 was done. Quantitative and qualitative consequences of input DNA amount in evaluation of DNA profiles performed by different amplification kits are considered.

***Keywords*:** DNA quantification; real-time PCR; low level DNA; validation; cut off quantification value

CONTENT

LIST OF FIGURES

LIST OF TABLES

INDEX OF ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine-5'-triphosphate
cDNA	Complementary DNA
ICP	Institute of Criminalistics Prague
Ct	Threshold Cycle
DNA	Deoxyribonucleic Acid
dH_2O	Ultrapure distilled water without nuclease
dsDNA	Double-stranded Deoxyribonucleic Acid
DTT	Dithiothreitol
FRU	Unit of peak height of genetic profile
FRET	Fluorescence resonance energy transfer
FTA paper	Fast technology for analysis
Iso-C	5'methylisocytosine
Iso-G	Isoguanin
IPC	Internal PCR Control
LT DNA	Low template DNA
mtDNA	Mitochondrial DNA
PCR	Polymerase Chain Reaction
PP_I	Pyrophosphate
RMP	Rotation per minute
Rn	Normalized report signal
RT PCR/qRT PCR	Real Time Polymerase Chain Reaction
SDS software	Sequence Detection system
SINEs	Short interspersed repetitive elements
STR	Short tandem repeat polymorphisms

CHAPTER 1: THESIS OBJECTIVES

Therefore the first goal of my study is to assess the accuracy and reproducibility of RT PCR in different quantification kits, with the focus on the measurement of low quantities DNA, the RT PCR instrument, and different approaches to the construction of calibration curve.

The second goal of my study is to verify whether is possible to set such as absolute quantitative value in whole forensic DNA laboratory workflow on the base of previous findings with all possible implications.

The third goals of my study is performing of series of dilution experiments with standard DNA to find out more detailed relationship between the quantity of input DNA and the number of detectable alleles for different PCR kits. These experiments are included in internal validation study. The absolute value of DNA quantification **(cut off value)** could be a tool for deciding about selection of samples from further workflows. This value must be appropriately accurate to have the predictive strength. Ideally, no samples with DNA concentration under the cut off value should be genotyped.

CHAPTER 2: INTRODUCTION

2.1. Methods of forensic genetics

The genetic analysis of the forensic biological material is carried out in several steps. The first step is the collection of the biological material from the crime scene using tools such as swabs, FTA paper etc. The second step is the isolation of DNA from this sample using commonly used isolation methods (commercial isolation kits). The third step is the quantification of the isolated DNA, a method of measuring DNA. The value obtained by means of this method is essential for the subsequent steps of the genetic analysis. The quantification can be measured by using the quantity and concentration of the isolate which is amplified, to set the best possible genetic profile. The fourth step is the analysis of the DNA itself. The quantity of DNA isolated from the biological material is relatively small and it is not easy to analyze it genetically. Therefore, the method uses the multiplication of DNA molecules in vitro, the so-called polymerase chain reaction (PCR, Polymerase Chain Reaction) (Mullis, 1990; Saiki et al., 1985). A suitable pair of primers is chosen; such primers should correspond to the specific fragments in DNA so as to reach the right multiplication during the PCR reaction, in our case, fragments containing one particular STR locus. The method of individual identification is currently a short tandem repeat analysis (STR polymorphisms, short tandem repeat polymorphisms), so-called microsatellites. It is a repetitive sequence with a motif of several nucleotides, which are abundant throughout the genome. The number of motifs repetition is very individually influenced by specific characteristics of the forensic genetics analyses, which are transmitted from parents to a child and which are not related to one another. The forensic genetics uses motifs consist of four, sometimes five nucleotides.

Reliable quantification of the human DNA in a forensic genetic sample makes it possible to adjust the concentration of the template DNA used for STR analysis in order to obtain optimal PCR reaction and STR typing results with as small amount of DNA as possible (Nielsen et al., 2008).

2.2. Real- Time PCR

2.2.1. Basic features of real time PCR

Real-time polymerase chain reaction, also called quantitative real-time polymerase chain reaction (RT-PCR/ qPCR), is a laboratory technique based on the PCR, which is used to

amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. Real-time quantitative PCR is a method that includes both the amplification and the analysis. The principle of real-time PCR is a rapid and accurate recording of PCR products immediately after their formation, in each cycle of PCR (Van Guilder et al. 2008).

2.2.2. Quantification models

Quantification analysis in RT PCR can be subdivided into two basic types, absolute and relative quantification (Fig.1). Each type uses the experimentally determined Ct (threshold cycle) value differently.

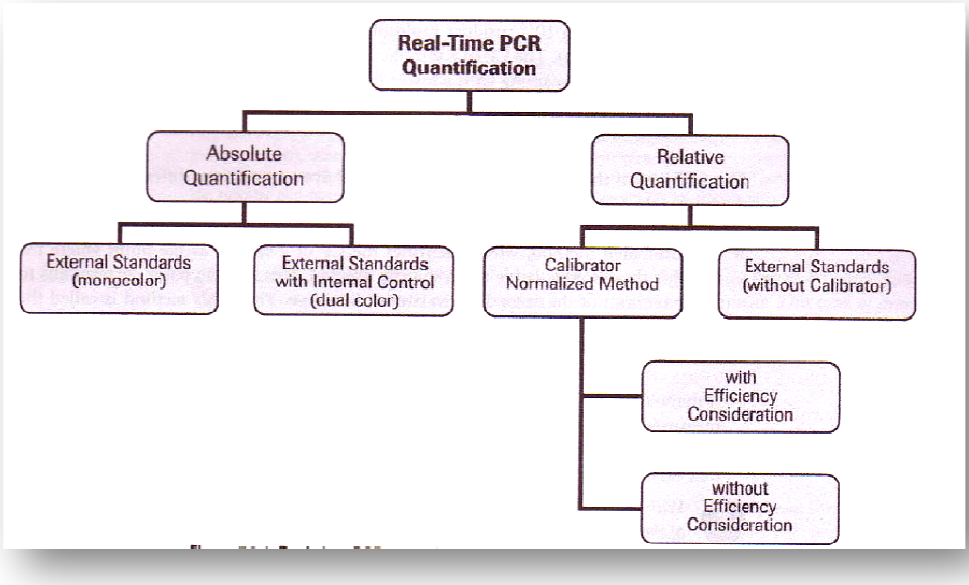


Figure 1 RT PCR quantification methods (Taken from study by Wetmur et al., 1995)

2.2.2.1. Absolute Quantification

In case of the absolute quantification assays, the concentration of the target molecule is expressed as an absolute value (e.g. copies, ng/µl, etc.). It uses a standard curve, calculated from the external standard sample with the known concentration and from the target molecule in the unknown sample. The system produces valid results only if the standard and the unknown samples are amplified and detected with the same efficiency. This absolute value is then used for the actual decision of whether the quality of DNA in the sample is sufficient to determine the DNA profile with a high degree of reliability (Rasmussen et al. 1998). The absolute quantification is very suitable for application on forensic genetics; virology and

microbiology, where you need to determine the copy number of a specific target, or for the determination of absolute gene copy number (Wang et al., 1998; Neiderstatter et al. 2007).

2.2.2.2. Relative Quantification

In the relative quantification assays, the target concentration is expressed as a ratio of the target and the reference gene in the same sample, rather than as an absolute value. The reference gene is an unregulated nucleic acid that occurs in a constant copy number in all samples. Relative quantification methods help to correct the sample with regard to the difference in quality and quantity, such as variations of the initial sample amount, cDNA synthesis efficiency, or sample loading / pupating errors (Witter et al., 1994). Relative quantification assays can be refined by applying two methods:

1. Normalization of the target/reference ratio in the sample with respect to the target/reference ratio in a calibrator,
2. Correction of any differences concerning the PCR efficiency of target and reference genes (Walsh et al.1992; Bustin et al., 2009; Richard et al.2003; Rasmussen et al.2001).

2.3. Quantification methods of DNA

Various methods have been developed for quantification of human DNA in forensic samples, e.g. Slot Blot method, UV spectrometry, Fluor metric method (Pico Green®; OlygoGreen®), Luminometry (AluQuant® Human Quantification System), Real-Time quantitative PCR – using (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA (SYBERGreen®) , and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits the detection only after hybridisation of the probe with its complementary DNA target (TaqMan™) (Walsh, 1992; Tolun et al.2003).

UV Spectrometry is a very useful method used in the forensic analysis for many kinds of trace evidence. It combines the microscope with the spectrophotometer so that the light absorption characteristics of a very small sample can be recorded. The concentration of a DNA sample can be checked by the use of UV spectrophotometer. DNA absorbs UV light very efficiently making it possible to detect and quantify at concentrations as low as 2, 5 ng/µl. The forensic use of the spectrophotometer is very inappropriate because of: (1) low sensitivity (low levels of captures); (2) zero accuracy (whatever increases the absorption at

260nm, is regarded as DNA); (3) measurement should be large numbers of isolate (Rapley, Whitehouse, 2007).

The commercial market is discovering new devices such as NanoDrop (NanoDrop 2000™, NanoDrop 2000c™, NanoDrop 8000™; Thermo Fisher Scientific Inc.), their function is to quantify nucleic acids and proteins and they are applicable for the quantification of buccal swabs (www.nanodrop.com).

Slot blot is a technique in molecular biology used to detect bio molecules. A Southern blot is an important method in the field of forensic science. This method is commonly used for the detection of a specific sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. This method is very laborious, it takes up to 2 days and involves rinses, incubations, pupating, washes, exposures, and developments. The semi-quantitation is conducted by manual comparison or by means of a scanner, and the obtained quantity may not reflect the final result due to the variations in PCR efficiency (Butler et. al., 2005).

In contrast, RT-PCR requires 2 hours for setup and time run. The quantity we obtain from this highly automated quantisation reflects the amplifiable result (Butler et al., 2005). RT PCR has lower detection limit and larger dynamic range. The above mentioned DNA quantification methods all represent the indirect measurement of the DNA concentration in a sample (Nielsen, 2008).

The quanti ABI blot method belongs among the commercially available slot blot methods. This particular method is based on hybridisation of a biotinylated oligonucleotide probe to extracted DNA samples immobilized on a nylon membrane. The Quanti Blot kit is useful for screening samples and for variety of applications because it provides the possibility of rapid quantification of both single- and double-stranded DNA. The quantisation of the DNA sample is extremely important for STR analysis in order to obtain optimal results (Applied Biosystem, product manual 2004).

The principle is that the isolate is applied to the membrane, and hybridized with a specific probe. The probe will show something, the stronger the signal, the more is the probe bound, i.e. the more target sequences probe can be found, the more DNA.

Fluorometric method is an analytical method using the transmission abilities of certain substances, after having transferred into the excited state of fluorescence radiation in the

ultraviolet or visible range. The tested substance is dissolved in a solvent or mixed solvents; the solution is transferred into a tube or a pipe fluorodensitometry (flow tube) and there is the light excitation, especially the excitation of the monochromatic light of a prescribed wavelength (Fähnrich, 2009).

Luminometry is a method of measuring the intensity of radiation generated by the following chemical reaction: ATP + luciferin + O2 → oxyluciferin + AMP + PPI + radiation. Luciferin and luciferase substances used in the experiment were isolated from the glowing fireflies. The emitted quantity of radiation is directly proportional to the amount of ATP in the sample and the amount of ATP thus provides the information about the number of cells contained in the sample. When a sample is taken for Luminometry, the blurring space is bounded by swab moistened by saline. The portion of the wool swab is transferred to a reagent ampoule, where the ball shakes out. Then the additional reagents are added. These reagents are necessary for the reaction and for measuring the intensity of the emitted radiation by a special device, the Luminometer. The results are expressed as relative light units (RLU) (Schram E., 1991). The quantification of the human DNA by Luminometry is practically done with *Alu sequences.*

Alu sequences are abundant in the human genome, numbering approximately 500,000 to 1,000,000 copies per genome. The sequences are about 300 base pairs long and they occur repeatedly several thousand times throughout the genome and are therefore classified as short interspersed elements (SINEs) amongst the class of repetitive DNA elements. In fact, this is the main advantage of *Alu sequences*, because the forensic samples are often contaminated with foreign DNA. An *Alu sequence* is a short stretch of DNA originally characterized by the action of the Alu restriction endonuclease. *Alu sequences* of different kinds occur in large numbers in genomes of primates. In fact, *Alu sequences* are the most abundant mobile elements in the human genome. They are derived from the small cytoplasm 7SL RNA, a component of the signal recognition particle. The Alu family is a family of repetitive elements in the Human genome. With regard to the fact that Alu sequences represent the most dispersed DNA sequences in the human genome, they are suitable for the forensic purposes, because there are many copies of Alu sequences, so they ensure satisfactory results in case they are used for the DNA quantification (Walker et al.2003; Niklas et al.2003; Frukmin et al.2002).

2.4. Principle of RT-PCR

Real-time PCR is based on the observation during the polymerase chain reaction (PCR) directly within the reaction (the so-called “real time“), using fluorescent probes or dyes that detect the amount of PCR product during the reaction by increasing its fluorescence activity (Haque et al.2003).

Its advantage over the conventional PCR is the precise and accurate determination of the initial number of copies of target template DNA sequences, and the ability to quantify (Bustin et al., 2004). Real-time PCR is performed by using devices called special cycler, which allows the implementation of both thermal cycling and fluorescence detection at each cycle of PCR. The detection of the resulting product can be used in different systems that are based on the determination of changes in fluorescence intensity during the amplification (Butler et al., 2005). The fluorescence is measured during each cycle of PCR and its intensity is directly or indirectly proportional to the amount of target copies in the reaction mixture. Concerning the quantitative detection of the product during PCR, there are three known general methods based on the use of: (1) Intercalation dye which binds to DNA; (2) Fluorescently labelled probe binding in the middle of the amplified product; (3) Fluorescently labelled primer.

Possible detection in Real Time PCR:

This probe and intercalation fluorophores are fluorescent substances releasing radiation in the presence of PCR. They are divided according to the specificity of the PCR amplicon to:

1. Nonspecific
   Intercalation and ”minor groove binding“ substances : (1) SYBR®Green I ;(2) LC Green® ;(3) Eva Green® ;(4) Pico Green® ;(5) Ethidium bromide
2. Specific
   Short oligonucleotide hybridizing with the PCR amplicon: (1) Hydrolysis probes (TaqMan™); (2) Hybridization Probes FRET ;(3) Molecular Beacons ;(4) Scorpions® (Didenko, 2001).

Some of the above probes are further described in this chapter.

2.5. RT-PCR quantification

The whole process of RT-PCR is characterized by a curve (Fig. 2.a, Fig. 2.b), which can be divided into three main phases: the exponential multiplication phase, the linear phase and the plateau phase, where there is virtually no further amplification of the sample.

Characteristics of the individual phases:

1. Exponential phase: in this phase, the reagents are abundant and the PCR product doubles every cycle.

2. Linear phase: during this phase, the reagents begin to run out. The PCR reaction slows down. One or more components of the PCR have decreased below a critical concentration, and the amplification efficiency begins to decrease. This phase is called linear because the amplification approximates an arithmetic progression.

3. Plateau phase: in this phase, the reagents are depleted and PCR reaction stops. The amplification plot achieves the plateau phase when the PCR stops, the Rn signal (normalized report signal) remains relatively constant, and the template concentration reaches a plateau at about 10^{-7}M (Bloch 1991).

Real-time PCR focuses on the exponential phase because it provides the most precise and accurate data for the quantisation. Within the exponential phase, the real-time PCR instrument calculates two values. The Threshold Line is the level of detection at which a reaction reaches a fluorescent intensity above the background. The PCR cycle at which the sample reaches this level is called the Cycle Threshold (Ct) (Figure 2.b). The Ct value is used in the downstream quantisation or the presence/absence of detection. By comparing the Ct values of samples of unknown concentration with a series of standards, the amount of template DNA in an unknown reaction can be accurately determined.

Figure 2.a shows the ideal amplification curve that remains at 100% efficiency versus a more realistic one showing a linear and plateau phase.

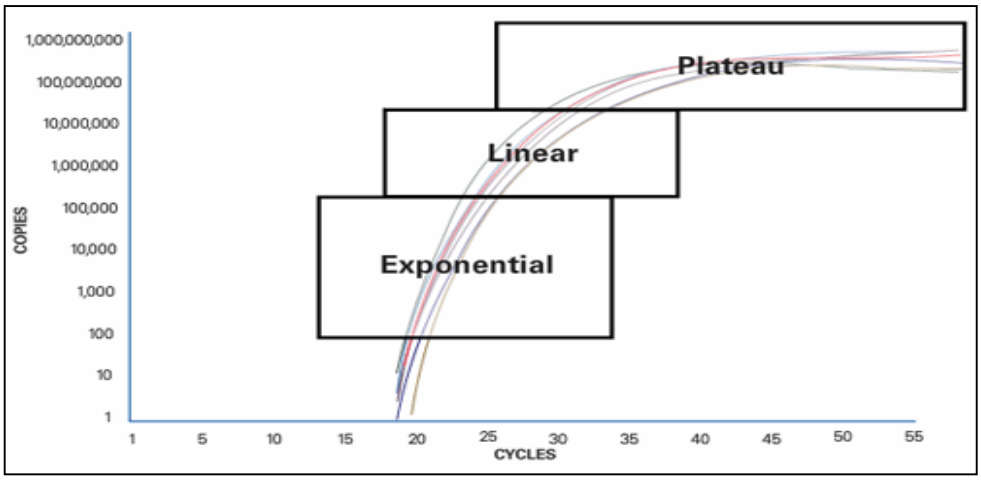


Figure 2.a Real-time PCR phase (taken from Applied Biosystem).

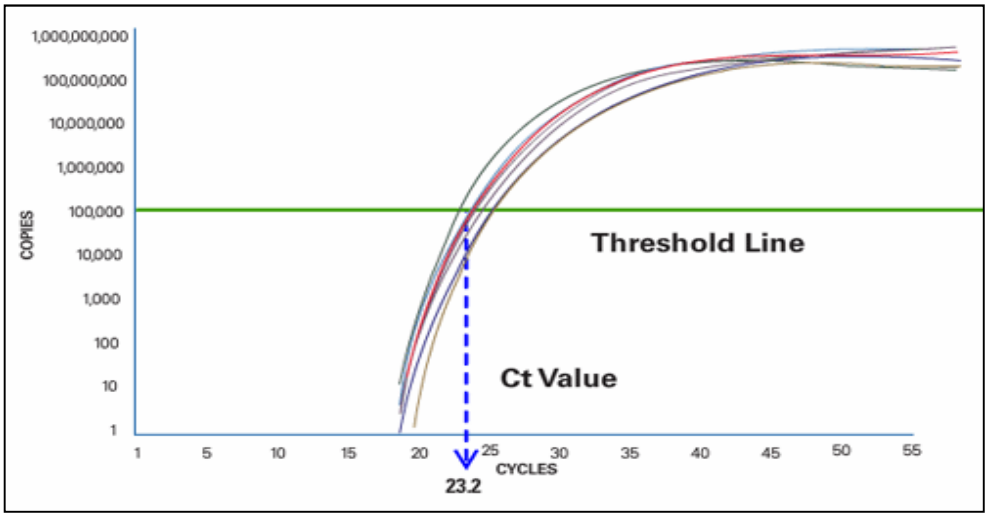


Figure 2.b The PCR cycle at which the sample reaches a fluorescent intensity above the background is the Cycle Threshold or Ct (taken from Applied Biosystem).

The study of Peters and Sanchez et al. may serve as another example: Figure 2.c shows the amplification curves for the Real-time PCR assay over a 7-log dilution series of synthetic oligonucleotide DNA template. All the curves are parallel to each other showing that the amplification for each template dilution has the same kinetics. The phases of amplification can be clearly seen within each individual curve (Peters, 2004; Sanchez, 2004).

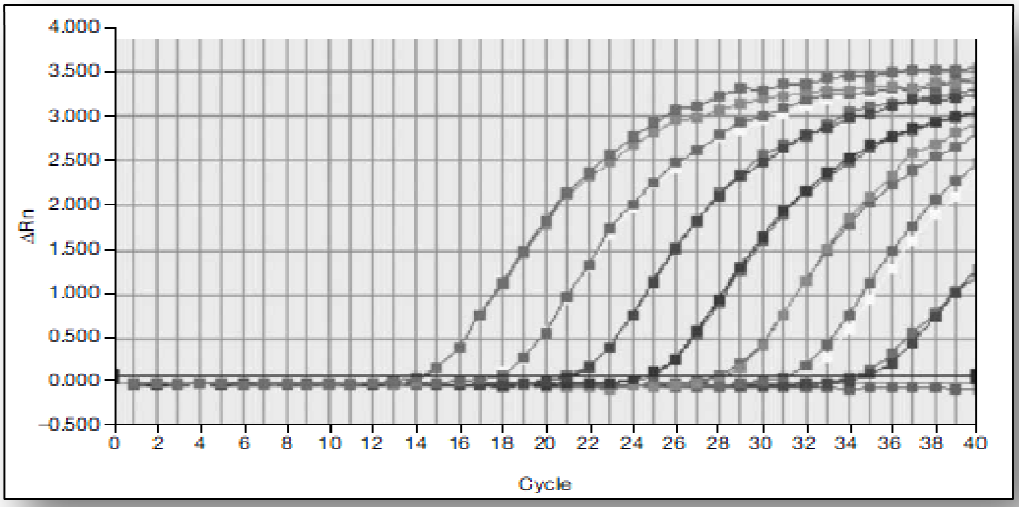


Figure 2c Sample of dilution series of an oligonucleotide standard over a 7-log range, from a real-time PCR experiment. Each amplification curve illustrates the four phases of a polymerase chain reaction experiment: baseline, signal being made but not detectable by the instrument; geometric, detectable signal with maximal PCR efficiency; linear, post-geometric signal with slowly declining PCR efficiency; and plateau phase, where there is either no product or the product occurs in a small extent.

During the exponential phase, the reaction can be described mathematically as (1):

$$N_c = N\ (1+ E) \qquad (1)$$

N_c – is the concentration of the amplified product at any cycle, N - initial concentration of the target template, E – efficiency of the system, and c – number of cycles.

Usually only 4-6 cycles from 40-cycle PCR show this exponential growth. After that, the reaction components become limiting (Witter et al., 1998).

In the plateau phase of PCR, the efficiency becomes variable and the amplification is best expressed as: (2)

$$N_c = N\ (1+ E_v) \qquad (2)$$

Because the E_v value is not known (it is constant to the E), it is not possible to calculate N during plateau phase as it is during the log phase (Witter et al., 1998).

2.5.1. Standard curve

A calibration curve is a general method of determining the concentration of a substance in an unknown sample. This is done by comparison of the unknown sample with a set of standard samples with known concentration (Rutledge et at., 2003). A calibration curve is a possible approach to the problem of the instrument calibration; other approaches may mix the standard sample with the unknown sample, giving an internal standard. Used mathematical models operate with a value called Ct (threshold cycle), which is equal to the cycle where the amplification curve exceeds the stated threshold fluorescence, located in the exponential phase reaction. The standard curve is a graph of quantification standard reactions, while the Ct values are analyzed against the starting quantity values of the standards. The software calculates the regression line by calculating the best fit with the quantification standard data points. The regression line formula has the following form: (4)

$$Y\ (conc.) = -k\ x + b \qquad (4)$$

Where k is the slope, b is the y-intercept, and x is the starting DNA quantity. The values associated with the regression analysis can be interpreted as follows:

- **R^2 value** – measure of the closeness of fit between the standard curve regression line and the individual Ct data points of quantification standard reactions.

- Regression coefficients:

  – **Slope** – indicates the PCR amplification efficiency of the assay. A slope of −3.3 indicates 100% amplification efficiency.

  – **Y-intercept** – indicates the expected Ct value for a sample with Qty = 1 (for example, 1 ng/µL) (AppliedBiosystem Quantifier™ Kits Manual).

The assay efficiency is based on the slope of the line, and it is calculated by the formula (5) (Green et al. 2005):

$$Efficiency = [10^{(-1/slope)}] - 1 \qquad (5)$$

The efficiency is primarily an indication of how the PCR reaction has proceeded.

The integrity of the data fit to the theoretical line is described by the r^2.

This is a measure of the accuracy of the dilutions and the pupating. The y-intercept is an indication of the sensitivity of the assay and how accurately the template has been quantified (Bustin, 2004).

Once a calibration model is developed, a Ct value obtained from an unknown sample can be used to estimate the initial concentration of the target DNA of interest. A calibration curve must be thoroughly validated because the accuracy of the quantification estimation is entirely dependent on the accuracy of the known DNA standards. Factors such as the DNA standard source, the nucleotide base composition, the exact concentration determination, the dilution preparation and the stability during the storage can all introduce uncertainty into a qPCR calibration model (Sivaganesan et al., 2010). Several mathematical models have been proposed to estimate the DNA concentration from an unknown sample where an absolute calibration curve model is developed from a series of DNA standards with known concentrations and their associated Ct measurements (Pfaffl, 2001).

The most common approach employs a 'single' set of DNA standard Ct measurements for each instrument run. In this case, a series of known DNA concentration standards and several unknown DNA samples are analyzed in the same instrument run. Therefore, if the unknown samples are from this run, then the calibration curve data corresponding to this run are used to estimate the unknown samples. Another popular strategy utilizes a 'master' calibration curve derived from DNA standard Ct measurements generated from multiple instrument runs. These data are then used to generate a calibration curve for the estimation of unknown DNA target concentrations analyzed over the course of a study without the prerequisite that the unknown test samples and the corresponding calibration curve data originate from the same instrument run (Sivaganesan et al., 2010). Curves can be prepared externally using Excel, but modern instruments have incorporated the software evaluation model.

Figure 3 is an exaggerated depiction of the standard curve of a 'perfect' assay and what influences on the outcome would have the alteration of the parameters.

By expressing the template amount in the molecules, the formula below can be used to determine how many cycles are necessary to produce a specified number of molecules.

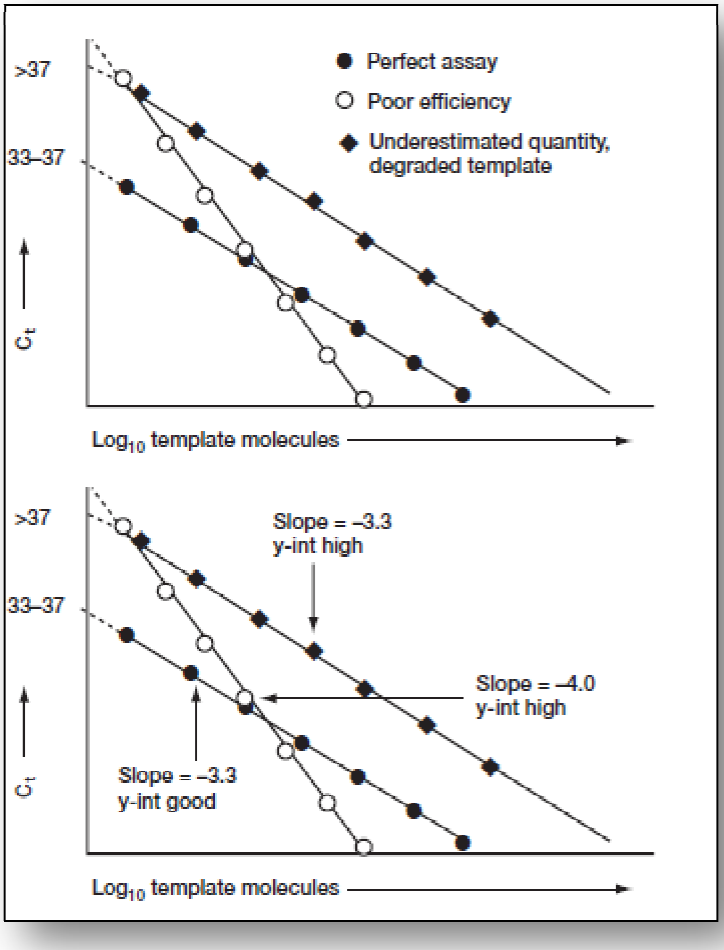


Figure 3 Standard curve diagrams. Exaggerated depictions of a 'perfect' standard curve (●-closed circles) and the effect that low efficiency (○-open circles) or inaccurate template quantification or degraded template has on the y-intercept (♦-closed diamonds) (Taken from a study by Pamela S. Adams, 2004).

2.5.2. Baseline and threshold

Each real-time PCR instrument has baseline-setting algorithms that determine the background noise of the detector and reagents using the fluorescence generated in a designated number of cycles at the beginning of the run.

This may be a fixed number of cycles for all samples or adaptive for each sample, depending on the type of instrument that is being used. Baseline anomalies may be slight or severe. The correct placement of the threshold is the next crucial step in the data analysis (Pfaffl et al., 2004).

For instance, SDS software (or Sequence Detection System; Apllied Biosystems), uses a threshold setting to define the level of detectable fluorescence. The threshold is presented by the manufacturer, but it can be set manually, depending on the height of the baseline.

A sample with a higher starting template copy number reaches the threshold earlier than a sample with a lower starting template copy number. To determine the Ct value, the SDS software uses the Rn (or normalized report signal, which means cycle by cycle changes) values collected from a predefined range of PCR cycles called the baseline:

- The software generates a baseline-subtracted amplification plot of ΔRn versus the cycle number.
- An algorithm defines the cycle where the ΔRn value crosses the threshold setting (the default threshold setting is 0.2) as the threshold cycle (Ct) (Quantifier Kits User's Manual, 2006).

2.6. RT PCR chemistries

As it was already mentioned at the beginning of this chapter there are few general fluorogenic methods to monitor the real-time progress of the PCR: by measuring Taq polymerase activity using double-stranded DNA binding dye chemistry (SYBER® Green or Ethidium bromide) (Higuchi et. al., 1993) or by measuring the 5' –nuclease activity of the Taq DNA polymerase to cleave a target –specific fluorogenic probe (Holland et al., 1991). This acceleration of the PCR is due to the increasing number of PCR products after each cycle.

Taq DNA polymerase is a thermo stable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated by Thomas D. Brock in 1965. It is frequently used in the polymerase chain reaction (PCR), methods for considerable amplification of short segments of DNA. Its single polypeptide chain, 94kDa enzyme that extends DNA strands 5 ` - 3`; it also has 5` - 3 ` exonuclease activity.

The commercially available, genetically engineered enzyme Ampli-Taq ™ has temperature optima of 75 to 80˚C. The enzyme is used for DNA sequencing by the Sanger methods, for cloning and for PCR procedures of DNA amplification. For latter amplifications, it is particularly useful because during the heat denaturation cycle it is not inactivated and it is not necessary to add new enzyme after each cycle. Phosphate buffers and EDTA are inhibitory to polymerization (Chien et al. 1976).

There are a number of technologies that can detect PCR products:

Quantification of the amplicons for commonly used fluorescent **dye SYBR ®** which fluoresces upon binding to the minor groove of dsDNA. The fluorescence of SYBR Green I is up to 1000 times higher after binding to DNA and the fluorescence signal increases with the increasing amount of PCR product. The signal is measured either at the end of elongation, or continuously. It is clear that the dyes that bind to DNA can be used in multiple reactions and their main limitation is the inability to distinguish non-specific products. Nonspecific signals generated by primer dimmers can be extinguished using primers labelled with specific fluorophores (http://www.ambion.com/techlib/basics/rtpcr/index.html).

TaqMan ™ probes are oligonucleotides longer than primers, with higher Tm value of about 10° C with a fluorescent label on the 5'-end of the arc at the 3'-end. The probe binds to the inner part of the amplified sequences, and if it creates a homoduplex, it is spread over
5 'exonucleases activity of Taq DNA polymerase. This causes the end of quenching and the fluorescence emission (Butler et al. 2004).

Plexor® HY System uses certain modified nucleotides that are recognized by DNA polymerase but do not form duplexes with normal, unmodified nucleotides. Plexor® HY System uses two modified bases in DNA - isoguanin (Iso-G) and 5'methylisocytosine (iso-C), which form a unique pair in dsDNA. The amplification of target sequences is done by using two primers: one primer is synthesized in iso-C as the 5'-terminal nucleotide and the fluorescent label 5'-end of the second primer is unmarked. Fluorescence quenching reactions are covalently coupled with iso-G. During the amplification, specific incorporation reduces the fluorescence quenching of fluorescent contrast, iso-C near fluorescent dyes located at the end of the primer (Promega Kit Manual).

In principle, the simplest method is using fluorescent dyes that interact with the DNA bases (e.g. SYBR Green). Increasing fluorescence corresponds to the increasing amount of DNA in the sample. At present, it is probably the most widespread method using 5-exonucleases activity of DNA polymerase. The key here is the use of oligonucleotide - probe (mostly the TaqMan probe), that specifically binds to the DNA sequence between the primers. The probe is at one end of the marked fluorescent substance and the quenching fluorochromes are at the other end. The synthesis of complementary DNA polymerase hydrolyzes the fibber TaqMan

probe, to release the fluorescent substance, and the increase of fluorescence is detected and recorded in real time. The amount of the sought cDNA sequences (our marker) in the sample can be accurately quantified on the basis of the standardization curves (Šmarda et al., 2005).

PicoGreen® dsDNA Quantisation Reagent is an ultra-sensitive fluorescent nucleic acid stain intended for quantitation of the double-stranded DNA (dsDNA) in molecular biology procedures. The fluorometer from Turner BioSystems together with PicoGreen® dsDNA reagent makes it possible to conduct the direct quantisation of dsDNA in as little as 100 µL of the total volume. In case the sample is contaminated by compounds such as salt, urea, ethanol or chloroform, the conjunction with PicoGreen and fluorimetric DNA eventually shows satisfactory results. The assay protocol minimizes the fluorescent contribution of RNA and single-stranded DNA (ssDNA). Using the PicoGreen dsDNA Quantisation Reagent and the Modulus Fluorometer, researchers can analyze dsDNA in the presence of equi-molar concentrations of ssDNA and RNA with the minimal effect on the quantitative results (Promega Kit manual, Tolun et al., 2003).

The **AluQuant™** Human DNA Quantisation System probes are obtained by denaturing the sample and incubating it with the AluQuant™ Enzyme and AluQ™ Probe solutions which are contained in the kit. The method employs a series of reactions that ultimately result in the production of adenosine triphosphate (ATP), which correlates with the amount of the present DNA. The amount of ATP is determined by using a Luminometer (Promega Corporation 2006; Frumkin et al.2002).

In the forensic analysis, the DNA quantification Luminometry is mainly used before PCR and STR analysis. This method does not involve electrophoresis, blotting, or DNA amplification. The calculations are simplified thanks to the AluQuant ® Calculator in Microsoft Excel (Pormega Corporation, 2006).

The use of the probe-based RT PCR to quantify human nuclear DNA in forensic analysis was described for the first time by Andreasson et al. (2003). The list below shows that the human DNA is possible to use commercially for the quantification of available probes.

By using RT PCR we can partly quantify the human nuclear DNA:

a) Single-copy autosomal DNA quantification by singleplex PCR: *HUMTH01, hTERT*; (Richard et al., 2003; Green et al. 2005).

b) Single –copy autosomal DNA quantification by duplex PCR: *TH01, CSF1PO*; (Swango et al. 2006).

c) Single –copy X and Y chromosome DNA quantification by singleplex PCR: *AMLE X, AMEL Y, SRY*; (Green et al. 2005; Swango et al. 2006; Alonso 2005; Andresson 2003).

d) Alu sequence quantification by singleplex PCR: *Alu sequence, Iter-Alu, Intra-Alu* (Nicklas et al. 2003; Walker et al. 2003).

e) MtDNA quantification by singleplex PCR: *HV1*; (Alonso et al. 2003).

f) Autosomal DNA and mtDNA quantification by duplex PCR: RB1, mtDNA coding region, HUMTH01, mtDNA region ND1; (Alonso et al. 2003; Timken et al. 2005).

g) Autosomal DNA, Y chromosome and mtDNA quantification by triplex PCR: Nuclear Alu sequence, Y-ch target, mtDNA target (Walker et al. 2005).

It is also possible to quantify the human mtDNA and non-human species (Alonso and Garcia, 2004).

2.7. RT-PCR platforms and kits

The world market is already full of wide range of manufacturers producing high quality equipment for real time PCR. This paper uses the methodology for using the devices of two reputable companies: Applied Biosystems (ABI Prism®System) and Roche (LightCycler™). There are many available commercial kits for quantification of the human DNA, such as Quantifier® Kit (Kremser et al.2009), Quantifier® DUO DNA Quantification kit (Applied Biosystem) or Plexor® HY System (Promega). The forensic analysis is able to detect markers of variability in different parts of the genome. These are also the trends which adjust the design of commercial kits designed for quantification. The first kits were aimed only at one target sequence, e.g. Quantifier® Kit. In many cases it is necessary to have the information about the quantity of male components in the biological material; especially in case of mixed samples, there were kits designed with the focus on the estimated number of copies of Y chromosomes such as Quantifier® Y Kit. The detection of the sample quantification is a very

specialized area and therefore special kits have been developed. These kits are represented by two components namely the Human Male DNA and Human DNA, one of Quantifier® DUO DNA Quantification Kit. In addition, all kits contain an IPC (internal PCR control) which I will mention further in the text. All the above mentioned components are part of the reaction and they are all contained in one kit and therefore it is not too financially demanding and the consumption of the samples is neither too high (www.appliedbiosystem.com; www.roche.com).

2.7.1. ABI Prism® System

The company Applied Biosystems (ABI) is a leading manufacturer of devices for real-time PCR, because it was the first company to produce these devices. Chemicals used in connection with these devices are TaqMan and SYBR Green, and this fact has been adapted and the software was installed (Bustin et al., 2004).

ABI Prism® 7900 HT System

This is the latest high-performance amplification and detection system for real-time PCR with 96-well micro plate. It is possible to process more than 5000 samples per day with this system. It uses a laser as the excitation light source, such as the detection equipment called the spectrograph and the cooled CCD camera (Bustin et al., 2004; Manual ABI Prism 7900).

2.7.2. Roche Applied Science

Roche is another well-known manufacturer of quality thermocyclers who produces for instance the following device: LighCycler® 480.

LightCycler™

LightCycler is one of the fastest air-heated thermocyclers with built-in micro-volume fluorometer for the detection and quantification of generated amplified PCR products. Amplified products accumulate in glass capillary tubes and they are detected by using the fluorescent dsDNA binding dyes or fluorescent probes. The sensitivity and accuracy of this device is lower, at the expense of speed, however it is comparable with the ABI 7900. The device can work with 32 samples placed in a circular carousel that rotates around blue LED light and the light which is headed to the tip of the capillary is filtered. Fluorescence radiation

emitted by the sample gets through the diachronic mirror and turns to three photo-detection diode with three band filters (530 nm, 640 nm and 710 nm). The increase of fluorescence was observed in the intervals of 10-100 ms and the data were collected once per cycle, continuously or progressively in accordance with specified temperature ranges (Bustin et. al, 2004; Dvořáková et al., 2007).

LightCycler® 480

LightCycler® 480 is the newest system that enables the combination of the real-time online PCR with rapid cycling for 96 or 384 samples (depending on the type of the embedded heating block). The results can be quantified and analyzed simultaneously by monitoring the fluorescence during the amplification of nucleic acids. The optical system enables multiplex PCR reaction using sequence-specific detection with the use of different types of probes (e.g. HybProbe probe, SimpleProbe probes, Hydrolysis probes or other type of probe). Sequentially independent online detection may be conducted by using SYBR® Green I probe. The analysis of melting curves enables genotyping (SNP detection) with the use of specific probes or product using SYBR® Green probe (Bustin et.al, 2004). LightCycler 480 is the first device, which was developed by Roche (in cooperation with Idaho Technology Inc.) which makes possible to analyze high-resolution melting (High Resolution Melting (Hi-Res Melt, HRM) (Promega LightCycler 480 Manual).

2.7.3. Quantification kits

Table 1 Quantification Kits

Characteristics	Applied Biosystem	Promega
	Quantifiler® kit	Plexor® HY System
Gene Target	hTERT 5p15.33 SRY Yp11.3	Human autosomal DNA target human RNU2,99bp target on chromosome 17q8.15. Y-chromosomal , 133bp target.
Components	2.primers for amplification of human DNA or male DNA; 1 TaqMan® MGB probe with FAM ®dye	CAL Fluor® Orange 560dye CAL Fluor® Red 610 dye IC5 dye
IPC	IPC template DNA	IPC 150bp

CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1. Special chemicals

Dispolab (Copan Italia), 4'n'6 DNA Flocked Swab (Micro Rheologics SRL), Microcon® YM-100 (Millipore), Quantifiler® Human DNA standard, Microcon® YM-30 (Millipore), Tube 1.5 ml (Eppendorf AG), Tube 2 ml (Eppendorf AG), MicroAmp® Optical 96well reaction plate with barcode (Applied Biosystems50ml tube (Equimed®).

3.2. Used reagents

Water, Mol Bio grade, DNase – , Rnase – and proteinase free (5Prime), Nuclease-free Water (Eppendorf AG), Proteinase K (Sigma-Aldrich Co.), Dithiotreitol (Sigma-Aldrich Co.), Chelex® 100 Resin (Bio-Rad Laboratories), Quantifiler™ Human DNA Quantification Kit (Applied Biosystems), Plexor® HY System(Pormega Corporation), AmpFłSTR® NGM® PCR Amplification Kit (Applied Biosystems), AmpliTaq Gold® DNA Polymerase (Applied Biosystems), AmpFłSTR® Control DNA 007 (Applied Biosystems), PowerPlex® 16 System (Promega Corporation), PowerPlex® ESXI System (Promega Corporation), ESSplex Kit (QIAGEN), AmpFŁSSTR® NGM ™ Kit (Applied Biosystem), Hi-Di™ Formamid (Applied Biosystems), 0.1 % Na OH (Litolab), Internal lane standard 600 (Promega Corporation), CC5 internal lane standard 500 (Promega Corporation), GeneScan™ 500 LIZ™ size standard (Applied Biosystems).

3.3. Used technical equipment

ABI PRISM® 7900HT Fast Real-Time PCR System (Applied Biosystems), Concentrator 5301 (Eppendorf AG), Incubator 4020 (GFL ImbH), Thermomixer Comfort (Eppendorf AG), Centrifuge 5415R (Eppendorf AG), Mini Spin Plus (Eppendorf AG), Centrifuge 5415D (Eppendorf AG), VeritiTM 96 Well Thermal Cycler (Applied Biosystems), GeneAmp® PCR System 9700 (Applied Biosystems), Hybaid Limited Multiblock System 2S (Hybaid), ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems), ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems), 36 cm capillary with polymer POP-4™ (Applied Biosystems), IKA® Vortex Genius 3 (Vitrium®), LightCycler® 480 II (Roche), Thermomixer Comfort 1,5 ml (Eppendorf AG).

3.4. Biological material

We work mainly with the data concerning the biological material. This biological material is taken from the crime scene – cigarette butts, blood smears, bones, tissue, hair and traces of unknown origin. For the part which is purely an experimental study Quantifiler® Human DNA standard, Control DNA 007,Contorl DNA 9948 AND Control DNA 9947 A. Since it is work in forensic laboratories, certain principles must be adhered to, because the presence of external DNA contaminants can be absolutely devastating for the whole process of the DNA analysis. Even minimal amount of contaminated DNA, either human or microbial, may cause an incorrect interpretation of the results of the subsequent analysis and sequencing of the sample. For these reasons, certain measures must be met to minimize the probability of the contamination as much as possible. In order to prevent possible contamination, a number of steps have to be taken during the DNA analysis.

These steps are carried out during the analysis of DNA in order to protect it from possible contamination by a foreign DNA:

- One of the steps made to ensure the authenticity of the results, is the DNA analysis of all workers in the laboratory. This is done to determine the source of possible contamination.
- Of course, the use of protective equipment, such as gloves, masks, lab coat, and particularly the resistant pipette tips (Handt et al., 1996).
- The working environment should be regularly decontaminated and it is also recommended to use the UV decontamination.
- Pre - and post - amplification places must be physically separated so that the DNA, already amplified to several million copies, cannot be transferred into the non-amplified samples. Even trace amount of the already amplified DNA, transferred to the newly isolated DNA sample, would be absolutely devastating for the entire analysis.
- During the analysis only one item is to be used. The survey sample is always examined outside the known reference sample to prevent the transfer of DNA (the so-called cross - contamination) and the depreciation of the results.
- Instruments such as pipettes are decontaminated in the same way as the working environment, by isopropanol, 10% bleach bath or UV radiation (Handt et al., 1996) ideally they are decontaminated by combination of all mentioned means.

These decontamination steps are most important for the pre-extraction and amplification of DNA.

- During the extraction and amplification of the sample we use so called blank, or in other words the negative control - a mixture of all reagents without the presence of DNA from the sample. Negative control passes through all the steps as the analysed sample. If there is a sequence present in the results concerning the negative control, it is clear that there is a foreign DNA in the samples, and that the analysis should be repeated.

Positive controls containing known DNA are used in the amplification and sequencing step to monitor the correct course of action. It is not possible to continue to analyse the DNA, as the positive control fails during the amplification, or sequencing (Isenberg and Moore, 1999).

After finishing the work in the laboratory, the working surface and the laminar box must always be treated with the disinfectant spray Incidur and with the DNA Remover, which removes any remnants of DNA.

3.5. Isolation of DNA

Several primary techniques for the DNA isolation are used in today's forensic DNA laboratory: organic extraction, Chelex 100 extraction, and FTA or solid-phase extraction. The exact extraction or DNA isolation procedure varies depending on the type of biological evidence being examined. For example, whole blood must be treated differently from a bloodstain or a bone fragment. All the samples that pass the laboratory, including the samples from the crime scene (casework) must undergo the isolation method. Samples are used in the experimental part of this work. They were isolated mainly by Chelex 100 method and a smaller portion of the sample was isolated by the method of IQ and Qiagen.

3.5.1. Chelex® solution

Extraction procedures using Chelex® 100 are popular in the forensic science community because they save time, reduce costs, simplify extractions, reduce safety risks, and minimize the possibility of contamination. Chelex® 100, a chelating resin, is used to successfully extract DNA from many forensic samples, including bloodstains, tissue, hair, and bone (Walsh, P. S., D. A. Metzger, and R. Higuchi, 1991). Instructions for isolation using this method are as follows: take 2 - 10 µl of a blood sample and put it into 1 µl of dH_2O. Then centrifuge with maximum speed (13 000 rpm) for 3 minutes. Remove the supernatant and add 200 µl of 5% Chelex and 4 µl of Proteinase K. Vortex the sample tube (Eppendorf) and then incubate at 56 °C for 30 minutes. The next step is denaturation at 100 °C for 8 minutes, then vortex and centrifuge. The last step is the maximum speed (13 000 rpm) for 1 minute. In the event that buccal swabs and samples are immersed for 30 minutes in 1 µl dH_2O and then vortexed, cotton wool removal is preceded by centrifuge. The isolated DNA is found scattered in the supernatant.

3.5.2. Solid-phase extraction

We should also mention two other methods used to isolate small portions of the sample in the experimental section. These are methods of solid-phase extraction. With the desire to automate more steps in the DNA analysis, many laboratories have moved to various forms of solid-phase extraction, where DNA is selectively bound to a substrate such as silica particles and then released following stringent washes that purify the bound DNA molecules away from proteins and other cellular components. The most widely used solid-phase extraction methods are Qiagen columns, DNA IQ.

A) DNA IQ method :

Punched track is added to a 1.5 ml tube (Eppendorf). The next step of the isolation depends on the particular kind of track. In case it is a substance which is not heat-resistant, it is extracted without heating. If it is a skin, the lysing buffer does not work on some kinds of skin, and thus it is extracted in a small amount of aqueous buffer (100-200µ l) and after the successful removal of traces, adds 2 volumes of lysing buffer. In case of small feet, the material is placed directly into the basket with 100-150 µl of lysing buffer and incubated for 30 min at 95 °C. Most of the buffer remains in the basket. In the event that the sample is not totally cloudy, another amount of buffer should be added. While the punched track is added to the Eppendorf

tube, we add the lysing buffer: in case of chemical, we add 150_µl and 1.5µL of DTT, in case of a buccal swab; we add 250 µl and 2.5 µl DTT. This is followed by vortexing and incubation at 95°C for 30 minutes. We place the lysing solution together with the sample in 1.5 ml tube (Eppendorf) into the basket. This is followed by 2 minutes of centrifuge at 15 000 rpm, or by 5 minutes at 10 000 rpm. The basket is removed. Subsequently 7 µl of resin is added into the solution during constant vortexing. It is then vortexed and incubated for 5minutes. The tubes are placed into a magnetic stand and all the liquid is gradually removed. After that 100µl of lysing buffer is added into the tube and it is further vortexed. Then we place it on the stand and remove the buffer. We add 100µl of the washing buffer and vortex; we place the tube on the stand and remove the buffer.This step is repeated twice (there are altogether 3 washing cycles). The tubes are then left open in the stand for 5 minutes to air dry so that it can be replenished with another sample. Depending on the amount of the input material, we add 25 – 100 µl of the elution buffer (lower volume = higher final concentration of DNA).This is followed by vortexing and incubation at 65°C for 5 minutes. The tubes are vortexed once more and placed on the stand. DNA is then found in the supernatant. The final step involves putting the solution in a clean Eppendorf tube (Protocol form ICP).

B) Qiagen solution:

20 µl of protease or proteinase K is added into 1.5 ml tube (Eppendorf) together with 200 µl of sample. Subsequently, we add 200 µl of AL and votrex for 15 seconds. The sample is incubated at 56 °C for 10 minutes and then shortly turned down. The next step is the isolation. We add 20 µl of EtOH (96%) and vortex for 15 seconds and turn down shortly. Then we transfer the sample into the centrifuge box for 1 minute at 8000 rpm and turn down. The sample is further transferred to a new tube together with 500 µl of AW1. This step is repeated, and the sample is transferred to a new tube box. Then we add 500 µl of AW2. This is followed by the centrifuge for 3 minutes at 14 000 rpm. The column is then transferred into a new tube box and turned down at maximum speed for 1 minute. Then we transfer the column into a new 1.5 ml tube (Eppendorf) and add 200 µl of AE. The last step is the incubation for 5 minutes, and 1 minute of centrifuge at 8000 rpm (Protocol form ICP).

3.6. Quantification

After the isolation of DNA, we performed quantification with the following kits: Quantifiler™ Human DNA Quantification Kit (Applied Biosystems) and Plexor® HY System

(Promega) (Table 2). These kits are designed to quantify the real time PCR and are based on the principle of the TaqMan probe.

The preparation of the calibration curve requires several consecutive steps. The first step is to prepare standards in separate tubes. The buffer and the solution are pipetted into each tube in the following amount: 30 µl of buffer and 10µml of DNA are pipetted into the first tube. Given that the calibration curve consists of at least eight points, 20 µl of buffer is added into next seven tubes. The final volume in all eight tubes must be 30µl. 10 µl volume is taken from each of the previous tubes and delivered to the next tube. Thus we get eight tubes of different concentrations of DNA standards. We also use the RT-PCR method which involves a 96-well plate, on which various standards and quantification mixtures are pipetted. It is recommended to indicate the beginning and the end of the calibration line on the plate by different colours, to avoid possible errors in pipetting. The next step is the preparation of a mixture according to the manufacturer's instructions. The mixture consists of PCR mix and primer mix in the following proportions: 10.5 µl of primer mix and 12.5 µl of PCR reaction mix. The calibration range is always conducted in two parallel measurements. 23 µl of the reaction mixture and 2 µl of standard are added into the individual wells (together 16). In order to obtain the right calibration curve, the last two (four) wells contain positive control (Quantifiler Human DNA Standard) and negative control (water, dH_2O). Before we put the plate in the quantification device (ABI 7900 / LightCycler), it is necessary to cover the plate with the Optical Adhesive Cover to reduce the chance of well-to-well contamination and sample evaporation. It is necessary to cover the plate in case it is inserted in ABI PRISM ® instrument 7900HT Fast Real-Time PCR System (Applied Biosystems), but it is not necessary to use the cover in case of LightCycler (Roche).

Table 2 Sequences of amplified DNA kits Quantifiler™ Human Quantification Kit (Applied Biosystems), and Plexor ® HY System (Promega Corporation).

Kit	Gene Target	Location	Amplicon length
Quantifiler Human Kit	Human telomerase reverse transcriptase gene (hTERT)	5p15.33	62 bases
Plexor ® HY System	Human autosomal DNA target, human RNU2 Y-chromosomal	17q8.15 Yp11.3	99bases 133bases

3.6.1. Alternations to this procedure according Westring et al.

The reduce-scale reaction consists of 4.2 µL of Primer Mix, 5 µL of PCR Reaction Mix, and 0.8 µL of DNA extract to the final volume of 10 µL. This alternative method was tested as one of the future laboratory methods, where the initial amount of material is minimal (Westring et al.2007).

3.7 STR Genotyping

The quality and quantity of DNA recovered from forensic samples is often limited, and characterization would not be possible without the PCR method. The PCR process has dramatically improved the capability of DNA analysis to obtain valuable evidence in a wide range of forensic applications. This PCR product is sometimes referred to as an amplicon. Each cycle results in the doubling of amplicons. The result is an exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles of amplification performed. However, the process loses efficiency at higher cycle numbers. After 30 cycles, approximately a billion copies of the target DNA template are generated. Amplification is always performed in 0.2 ml PCR tubes in 25 µl of one of the following thermocyclers: VeritiTM 96 Well Thermal Cycler (Applied Biosystems), GeneAmp® PCR System 9700 (Applied Biosystems) with aluminium or gold pads, Hybaid. In addition to the amplification of the samples themselves, the positive and negative control was always amplified as well, while we used the DNA standard, resp. dH_2O, instead of the DNA isolate. The zone for PCR, where we isolated and prepared DNA samples for amplification, was always spatially separated from the zone of post-PCR, where we worked with the PCR product. Applied Biosystems and Promega manufacture most forensic STR DNA

amplification kits. Each manufacturer has modified their kits to meet the changing needs of the forensic science community. While other manufacturers have developed DNA STR kits, this module focuses on Applied Biosystems and Promega kits because they are currently the most widely used kits in the forensic science community. For the purpose of our study the following amplification kits were used: for the most part it was PowerPlex®16(Pormega), NGM ™ (Applied Biosystem), PowerPlex ESXI (Promega) and ESSPlex Kit (Qiagen).

3.7.1. PowerPlex 16

PowerPlex ® 16 System (Promega) is a multiple of the following loci: D3S13582, D5S818, D7S820, D8S11792, D13S317, D16S539, D18S512, D21S112, CSF1PO, FGA2, TH012, TPOX, vWA2, Penta D, Penta E, amelogenin for sex determination (Krenek et al., 2002). The large number of loci amplified and PowerPlex 16 has a high discriminatory power. According to the manufacturer´s instructions, the following items are used for the amplification of one sample with 16 PowerPlex: the PowerPlex, 2.5 µl of 16 10X Primer Pair Mix, 2.5 µl of buffer Gold Wed ST* R 10X Buffer, 0.5 µl of iTaq polymerase, 9.5 µl of dH_2O and 10 µl of DNA isolate. As the positive control we used 0.5 µl of DNA and DNA-9947A standard 9.5 µl dH_2O.

The course of the amplification cycles was the following:

- Initial denaturation:
  95 ° C for 11 min,
  96 ° C for 1 min,
- 10 cycles:
  Denaturation: 30 s at 94 ° C
  Get on primers: ramp 29sto 60 ° C, then 30 s at 60 ° C
  Extension chain: 23s ramp to 70 ° C, then 45 s at 70 ° C
- 24 cycles:
  Denaturation: 30 s at 90 ° C
  Get on primers: ramp 29s to 60 ° C, then 30 s at 60 ° C
  Extension chain: 23s ramp to 70 ° C, then 45 s at 70 ° C
- Final extension chain: 30 min at 60 ° C
- Storage: at 4 ° C.

3.7.2. Power Plex ESX I

This kit amplified 16 autosomal STR loci - D3S1358, TH01, D21S11, D18S51, D10S1248, D1S1656, D2S441, D12S391, D19S433, SE33, and the amelogenin gene determining sex. In this kit, these loci are marked with different fluorescent colours and they also have a different

length amplicons. For the amplification by kits Power Plex ESXI from Promega Corporation, we used a reaction volume of 25 µl. To prepare a response, we used 5 µl of PowerPlex® ESI 5X Master Mix and 2.5 µl of PowerPlex ® 17 10X Primer ESI Pair Mix. The remaining 17.5 µl of sample was supplemented by DNA isolate or by water. As the positive control we used 0.5 µl of standard DNA 9947A (Promega Corporation), negative control reaction was used in 10 µl dH_2O. Concerning the thermocycler, we used the temperature profile recommended by the manufacturer.

3.7.3. NGM

The NGM™ Kit is a highly robust STR multiplex kit which uses single protocol to amplify the 10 SGM Plus™ loci (D2S1338, D3S1358, D8S1179, D16S539, D18S51, D19S433, D21S11, FGA, TH01, vWA, Amelogenin) together with the additional loci approved by the European Union Council for the expansion of the European Standard Set (D10S1248, D22S1045, D2S441, D1S1656 & D12S391). . For the amplification by kits Power Plex ESI 17 from Promega Corporation, we used a reaction volume of 25 µl. To prepare a response, we used 10 µl of AmpFlSTR® NGM ™ Master Mix and 2.5 µl of AmpFlSTR® NGM ™ Primer Set. As the positive control we used 10 µL of 007 controls DNA (AppliedBiosystem), negative control reaction was used in 10 µL of low TE buffer. Concerning the thermocycler, we used the temperature profile recommended by the manufacturer.

3.7.4. ESSplex

The ESSplex Kit serves as a multiplex application for special questions in forensic case work. The 15 polymorphic STR markers recommended by ENFSI and EDNAP as the new European Standard Set of loci D1S1656, D2S441, D2S1338, D3S1358, D8S1179, D10S1248, D12S391, D16S539, D18S51, D19S433, D21S11, D22S1045, FGA (FIBRA), TH01 (TC11), vWA, as well as the gender-specific Amelogenin, they are amplified simultaneously in a single PCR. We used a reaction volume of 25 µl. To prepare a response, we used 5 µl of Reaction mix B* and 2.5 µl of Primer mix and 0.4 µL Multi Taq2 DNA Polymerase. The remaining 16.1 µl of sample supplemented by DNA isolate or by water. Concerning ESSPLEX, we used the temperature profile for the thermocycler recommended by the manufacturer.

3.8. Amplification cycle number and formamide

To improve the quality of the resulting genetic profile, it is also important to observe the number of cycles and amount of formamide according to the instructions, or find their optimal values (Table 3).

Table 3 Number of cycles and formamide

Kit	Cycle number	Formamide
NGM	29	8.7
ESX(I)	30	10
ESS	30	12
PP16	34	12

3.9. Capillary electrophoresis

To determine the genetic profile, we used the device for capillary electrophoresis, ABI PRISM ® 3130xl Genetic Analyzer (Applied Biosystems). We prepared samples for genotyping in a 96-hole plate.

For amplification using commercial kits, we used 2 ml of the product generated during PCR and 12 µl of Hi-Di ™ formamide (Applied Biosystems). Prior to genotyping, we denaturized all samples by heating in the Thermocycler for 3 minutes at 95 ° C, and then by rapid chilling in the ice plate. Standard voltage was 3kV injection for 10 s, 15 s and 30 s. In addition to the PCR product and formamide, the internal standard was included in the sample before the electrophoresis. Internal standard is a length marker, which is marked with different fluorescent colour than the DNA sample fragments. This creates a clear marker peak representing fragments of a known number of bases at fixed intervals and according to them, then deducts the actual length of the investigated alleles. When amplified with PowerPlex Y kit, we used 1 µl of internal standard ILS 600. Concerning a series of kits Applied Biosystem, we used 0.3 µl of internal standard 500 LIZ ® (Applied Biosystems) and concerning the ESX and ESI kits, we used 1µl of CC5 ILS 500. The above described amount of internal standard is always applied to one sample (Butler et al. 2004)

For each of the multiplex kits and in each run of the sequencer, we also analyzed the corresponding allelic ladder. According to the allelic ladder, we subtracted the defined sample profiles, i.e. we assigned concrete alleles to the peaks in the electrophoretogram.

3.9.1. Commercially available software for analyzing data obtained by capillary electrophoresis

Data obtained by DNA analysis with the help of the capillary electrophoresis, can be displayed in the form of tables or electrophoretogram (in most cases there is a combination of both). The result of one single injection (fluorescent dye, i.e. the establishment of suitable fluorescent colours of different DNA fragments, allows analysis of many independent loci) is one electrophoretogram, showing the migration of fluorescently labelled fragments in time, during which they wander through polymer, within the operation of an electric field. The fluorescence intensity is given by the height of the peaks and is shown on the y-axis, the x-axis, and it shows the migration time of fragments, which influences their length. The higher the migration time, the longer the fragment is. To evaluate the collected data we use special software (Applied Biosystem, 2000). In assessing the electrophoretogram obtained from the analysis by capillary electrophoresis - sequencer we used GeneScan software version 3.7.1 and GeneMapper ID-X version 1.1 (both Applied Biosystems). GeneScan 3.7.1 is one of the older software which is still used for capillary electrophoresis and it is suitable for evaluating data obtained by the DNA analysis instruments such as the ABI PRISM 310, but the data can also be obtained from newer devices. However, concerning newer devices, the GeneScan 3.7.1 is often replaced by newer software GeneMapper ID-X 1.1 (Applied Biosystems, 2000, Butler et al. 2004).

The data were analyzed using four or five fluorescent colours, of which one is always reserved for the internal standard. Internal standard (GeneScan Internal Lane Size Standard) is applied to the same tube as the experimental samples. Thus it is subject to the same electrophoretic forces as the sample material and acts as a control. The software provides comparison of various fragments with an internal standard to determine their length. The company Applied Biosystems offers 5 different standards, which are labelled with fluorescent colours TAMRA or ROX. Each standard is suitable for different length of DNA fragments (Applied Biosystem, 2000).

The new software can include GeneMapper ID-X 1.1, which combines the advantages of GeneScan 3.7.1 with new and improved features. This means that several algorithms can recognize specific structures (e.g. asymmetric or cloven peaks), which were previously collected manually (Applied Biosystem, 2000). Other features include GeneMapper ID-X 1.1,

capturing the LOH (lost of heterozygosis) or AFLP (Amplified fragment polymorphism) (Applied Biosystems, 2004), which is a visualization of hundreds of amplified DNA restriction fragments together, which is used for example in seeking of some degree of similarity of isolates (Applied Biosystems 2000). For GeneMapper ID-X 1.1 software, the internal standards (Size Standards) are produced, which perform the same function as the internal standards with GeneScan 3.7.1 software. Individual standards correspond to a certain length of fragments (Tagliaro 2006).

3.9.1.1. Artefacts during DNA amplification

Nonspecific artefacts arise during the PCR amplification of STR alleles and appear in the resulting electrophoretogram .Common reason for such unusual peaks in the DNA sample is a degraded or contaminated sample, for example, unused primers. Their position or shape cannot be predicted nor identified. Because they can act as a sequence of different STR alleles or as part of renaturated PCR product. These artefacts can affect the outcome of the entire electrophoresis (Buckleton *et al*. 2005; Gill et al., 2000). It may be stutter peaks, heterozygous imbalance, A + / A-peaks, missing, or excess allele (Fig. 5).

- *Stutter peaks* - peaks corresponding to the allele by one repeat shorter than the true allele; stutter peaks arise from the prematurely finished operation of polymerase during the amplification, which then leads to a shorter DNA fragment (Butler et al., 2005).
- *Heterozygous imbalance*, i.e. the imbalance of peak height of individual alleles in one locus;
- *A+/A- peaks* -Taq polymerase adds to 3-end adenosine amplicon nucleotide sequences without regard to the template. This adenosine has a tendency to non-specific amplicon from release, so it is possible that some peaks appear in two forms, differing by 1 nucleotide. It reflects the emergence of the peak with two peaks.
- *Drop-in*, or sporadic contamination, therefore accidental occurrence of an isolated peak in a given profile, which is not a donor sample (but not the normal contamination, which manifests itself as a complete profile of foreign and which occurs even in normal PCR);
- *Drop-out*, which arises when none of the alleles can be provided, heterozygous locus can then falsely appear to be homozygous;

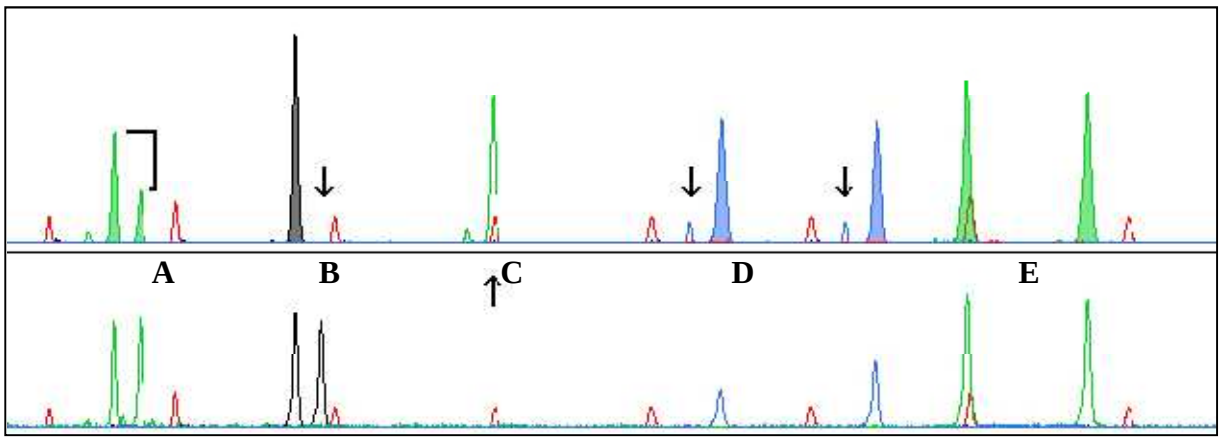


Figure 4 Artefacts that may occur during amplification. ***A****.-Heterozygous imbalance;* ***B****. - Drop-out;* ***C****.-Drop-in;* ***D****.-Stutter peak;* ***E****. - no artefact*

CHAPTER 4: RESULTS

4.1. Optimization of the calibration curve

To be able to use the values of the absolute DNA concentration in forensic samples, it was necessary to verify the accuracy of these measurements in case of the first experiments. The method of Real time PCR used in the laboratory of the Institute of Criminalistics Prague (ICP) belongs to the most commonly used methods and the quantification process is done with the help of the the external calibration curve derived from a serial dilution of DNA standards. A linear relationship between the cycle threshold (Ct) and the logarithm of the original concentration of DNA is assumed, and the regression coefficient helps to obtain the curve consisting of best matching points (Grgicak et al., 2010). The construction of the calibration curve on the basis of dilution series of the standard (Quantifiler Human DNA Standard) according to the instructions is presented in Figure 4.1.

Standard	Concentration (ng/µL)	Example Amounts	Minimum Amounts
Std. 1	50.000	50 µL [200 ng/µL stock] + 150 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [200 ng/µL stock] + 30 µL $T_{10}E_{0.1}$ buffer
Std. 2	16.700	50 µL [Std. 1] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 1] + 20 µL $T_{10}E_{0.1}$ buffer
Std. 3	5.560	50 µL [Std. 2] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 2] + 20 µL $T_{10}E_{0.1}$ buffer
Std. 4	1.850	50 µL [Std. 3] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 3] + 20 µL $T_{10}E_{0.1}$ buffer
Std. 5	0.620	50 µL [Std. 4] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 4] + 20 µL $T_{10}E_{0.1}$ buffer
Std. 6	0.210	50 µL [Std. 5] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 5] + 20 µL $T_{10}E_{0.1}$ buffer
Std. 7	0.068	50 µL [Std. 6] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 6] + 20 µL $T_{10}E_{0.1}$ buffer
Std. 8	0.023	50 µL [Std. 7] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 7] + 20 µL $T_{10}E_{0.1}$ buffer

Figure 4.1 Standards dilution series (Taken from Quantifiler® Kits User Manual)

Preparation of the calibration curve requires several consecutive steps, which are described in Chapter 3 (3.6. Quantification). Based on the above table (Fig.4.1), the dilution series standards (DNA standard) were made reduced methodology (3.6.1. quantification) and then used in the experiment.

The first experiments consisted of verifying the accuracy of the calibration curve points.
Before the use of any commercial quantification kit, it is necessary to assess whether the values stated by the manufacturer are consistent with the methodology used in the laboratory.

A core value that was observed in the early experiments was the number of amplification cycles Ct needed to cross the value of threshold signals (it is due to the rising value of fluorescence during RT PCR).

Because this experiment was focused only on the accuracy of the calibration curve, the concentration units were not used within the evaluation.

In order to eliminate the effects of possibly different standard DNA stock solution, which is part of every quantification kit used to the preparation of dilution series (standard curve), an experiment was carried out for **several months**, always with **different batch** of amplification kits. All measurement were doubled to make real values of the calibration points more accurate. Altogether 16 individual calibration experiments were evaluated and conducted within 6 months.

The experiment consisted of 16 single measurements (Table 4.1.) that were performed with the instrument ABI® Prism 7900 HT System.

Table 4.1.Series of 16 instruments runs (ABI® Prism 7900 HT System) of DNA calibration standards

ng/ul	Average	1 calibration	2 calibration	3 calibration	4 calibration	5 calibration	6 calibration	7 calibration	8 calibration	9 calibration	10 calibration	11 calibration	12 calibration	13 calibration	14 calibration	15 calibration	16 calibration
50	23.258	24.845	24.780	24.886	24.898	24.671	24.707	24.774	24.711	25.057	24.468	25.050	24.336	24.630	25.250	24.677	24.837
		24.875	24.796	25.009	24.882	24.804	24.689	24.770	24.782	24.974	24.720	24.964	24.340	24.645	25.372	25.036	24.752
16.7	24.872	26.453	26.474	26.690	26.563	26.716	26.653	26.678	26.581	26.655	26.618	26.547	26.085	26.146	27.025	26.431	25.890
		26.498	26.519	26.719	26.490	26.639	26.589	26.572	26.434	26.725	26.703	26.599	26.064	26.270	27.056	26.893	25.867
5.56	26.402	27.894	27.915	28.216	28.044	28.363	28.485	28.554	27.983	28.635	29.009	28.123	27.626	27.847	28.478	27.961	27.228
		27.999	27.938	28.299	27.906	28.345	28.355	28.416	27.910	28.679	29.127	27.973	27.573	27.737	28.461	28.243	27.446
1.85	27.966	29.595	29.645	29.716	29.782	29.883	30.113	30.026	29.524	30.054	31.308	29.475	29.516	29.338	29.955	29.608	29.525
		29.626	29.359	29.649	29.452	29.982	29.925	29.884	29.376	29.997	32.845	29.409	29.189	29.337	29.948	29.630	29.468
0.62	29.674	31.147	31.233	31.797	31.344	33.057	32.716	32.640	30.742	32.246	34.046	31.154	30.756	30.858	31.401	31.058	30.572
		31.023	31.009	31.999	30.832	31.949	32.370	32.526	31.076	32.518	33.971	31.133	30.778	30.971	31.349	31.099	30.363
0.21	31.235	32.487	32.939	33.595	33.118	34.773	34.285	34.944	32.133	33.020	N/A	32.877	31.715	32.616	33.054	32.255	34.128
		33.066	32.745	34.292	32.848	34.506	34.454	34.810	32.297	33.478	36.691	32.458	31.805	32.563	33.270	32.313	34.312
0.068	32.956	34.286	34.794	35.261	34.807	37.583	36.175	36.484	33.944	36.302	36.456	33.971	33.276	34.066	34.518	33.383	34.932
		33.695	34.241	34.683	34.742	37.978	36.942	37.780	34.571	36.131	36.982	34.476	33.416	33.669	34.140	33.890	34.993
0.023	33.687	35.268	37.441	36.774	N/A	38.219	N/A	36.946	35.557	36.482	37.689	35.512	35.361	35.033	35.244	35.501	35.888
		36.454	36.576	38.828	36.754	37.194	N/A	37.766	35.640	35.650	35.409	35.158	34.409	35.086	36.141	35.537	35.141
R²	0.9981	0.9981	0.9936	0.9987	0.9996	0.9898	0.9976	0.9862	0.9979	0.9862	0.9791	0.9985	0.994	0.9972	0.9951	0.9956	0.9781

R^2 – regression coefficient, N/A- without measuring

The calibration accuracy which is expressed by the regression coefficient R^2 can be found in the last row of the Table 1. The average Ct values for the individual points of the calibration curve can be found in the second column of Table 4.1.

In order to estimate potential differences from the standard course of RT PCR, these values were compared with developmental validation performed by the manufacturer. This

difference is illustrated in Table 4.2. The difference is evident in case of Ct values and there is a large difference in case of the standard deviation as well.

Table 4.2 Comparison of the average Ct values and standard deviation (STD) for internal ABI validation and the developmental study of ABI-1

ng/µl	Ct ABI	STD ABI	Ct ABI-1	STD ABI-1
50	24.81	0.24	23.09	0.10
16.7	25.52	0.29	24.64	0.17
5.56	28.14	0.43	26.19	0.16
1.85	29.81	0.67	27.67	0.17
0.62	31.61	0.95	29.09	0.17
0.21	33.34	1.10	30.31	0.19
0.068	35.08	1.35	31.9	0.28
0.023	36.16	1.08	33.45	0.48

STD – standard deviation, Ct- cycle threshold, Ct ABI – cycle threshold of internal validation, ,Ct ABI-1- cycle threshold of developmental validation, STD ABI- Standard deviation internal validation, STD ABI-1- Standard deviation developmental validation.

It is not obvious what is the source of the differences between Ct values in internal experiments and developmental validation but the way of pippeting seems to be fundamental problem. Another reason of different Ct values between the internal and developmental study of the Quantifiler™ kit may be different equipment, which is necessary to consider as well.

Also with regard to the fact that the equipment was changed during the experiment, it was necessary to conduct the recalibration to a different type of RT PCR instrument. Specifically it was Roche LighCycler®480. The same experiment as indicated in Table 4.1 was conducted but only 4 independent calibration curves were performed.

Even if the LightCycler®480 was used much less for the measurement than the ABI Prism® 7900 HT System, we observed very little difference in Ct values between the ABI Prism® 7900 HT System and LightCycler®480. The same calibration ranges serve as a platform. This allows the usage of both devices for obtaining absolute values of qualification without any difference.

The results of 16 experimental measurements (ABI Prism® 7900 HT System) and 4 experiments (LightCycler®480) are shown in Table 4.3.

Table 4.3 Quantification outcome measurement

Concentration of DNA standards (ng/µl)	Ct values ABI	Ct values LC
50	24.8122	24.27
16.7	25.5263	25.88
5.56	28.149	27.67
1.85	29.8168	29.09
0.62	31.6166	31.08
0.21	33.3499	32.71
0.068	35.0802	34.63
0.023	36.1606	36.08

Ct - cycle, which has exceeded a certain threshold concentration of DNA, ABI- Applied Biosystem (ABI Prism® 7900 HT System),LC- LightCycler®480.

Ct values may serve for raw estimation of DNA concentration in the sample. However, for the comparison of different methods or the usage of quantification value directly in PCR setup is important to know the absolute value of DNA concentration in the sample, in order to conduct the normalization of the sample volume, which is added into PCR, with regard to the optimal amount of DNA in PCR recommended by the manufacturer of the amplification kit. This amount may differ for different amplification kits and it ranges from 0, 5 to 1, 5 ng of input DNA in PCR.

Values of the absolute concentration are calculated for the individual Ct values on the base of the particular calibration curve: $Y\ (conc.) = -kx + b, [ng/\mu l]$[1]

The absolute values of DNA concentration in the sample will be influenced not only by the absolute Ct values for single calibration points but also by the course of the calibration curve itself due to fluctuations in the amplification process. It is necessary to consider the particular value of regression coefficient R^2, which is mainly determined by the accurate pipetting. The homogenizing of the control DNA stock solution before starting the calibration curve preparation may play very import role.

[1] *In the category of the sample where the absolute value of DNA concentration is near 0.03 ng / µl, we have in order to better converting the units reported in pg / µl. Explanations of shortcuts in the formula are listed on page 21.*

Also the number of calibration points has to be considered. Generally the more points the calibration curve contains, the more demanding its extrapolation is.

As resulted from table 4.1 some calibration curves show very low values of R^2 especially in calibration curves numerated 7, 10 and 16. There was a reason to evaluate the influence of recalculation of absolute quantification value according to different calibration curves. Two real casework samples with expected different concentration which was determined on the basis of the original calibration curves were chosen. The Ct values of the sample R 2090-01(blood) Ct = 32.8 and the sample H 1697-02(swab) Ct = 39.23 were then recounted to the absolute concentration according to:

1) Original calibration curves that were run together with samples on one plate.
2) Pooled calibration curve that was generated from average Ct values (see table 4.1).
3) Calibration curves with markedly different Ct values (calibration curves numerated 7, 16, see table 4.1).

Table 4.4 Effect of R^2 (calibration curve) on calculation of absolute concentration of casework samples

	Concentration of DNA pg/µl	
R^2	H 1697-02 Swab	R 2090-01 Blood
0.9521(H 1697-02 Swab)	22	N/A
0.9901(R 2090-01 Blood)	N/A	113
0.9862 (7.calibration)	10	469
0.9781(16.calibration)	4.2	235
0.9981(pooled calibration)	3.8	265

R^2 – regression coefficient, N/A- without measuring, H1697-02, R 2090-01-sapmles ID

As the results imply (see Table 4.4), the differences in absolute DNA concentrations obtained according to different calibration curves, are significant. If expected **cut off** value is determined somewhere between up 10 to 20 pg/µl than the selection of samples than could be omitted from further workflow would be very problematic and strongly dependent on preparation of particular calibration curve.

On base of results presented above calculation of calibration curve according to pooled calibration curve was implemented for all measurements of absolute quantification be means Quantifiler™. The course of pooled calibration curve is shown in figure 4.2.

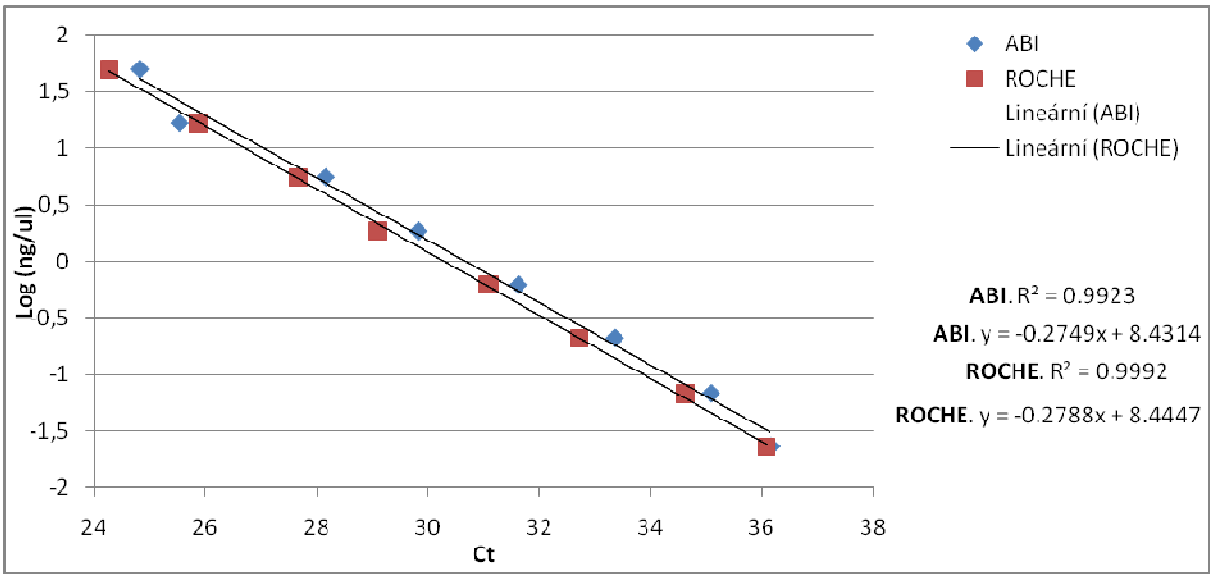


Figure 4.2 Course of pooled calibration curve for both instruments (ABI Prism® 7900 HT System, LightCycler®480) Ct values. Single Ct values for construction of pooled calibration curve are taken from Table 1.

Table 4.5 shows the expected absolute concentration of DNA in the sample as Ct values based on pooled calibration curve. This table enables rough estimation of volume of isolated DNA during PCR setup.

Table 4.5 Empirical absolute concentration values for quantification process using Quantifiler™ Kit on the ABI PRISM ® instrument 7900HT Fast Real-Time PCR System.

Dependence of the concentration of DNA in the sample on the Ct	
C_t	ng/µl
26	34.3637
27	17.9597
28	9.3864
29	4.9057
30	2.5639
31	1.34
32	0.7003
33	0.366
34	0.1913
35	0.1
Ct	pg/µl
36	52.3
37	27.3
38	14.3
39	7.5
40	3.9

Ct - cycle, which has exceeded a certain threshold concentration of DNA.

Within Quantifiler^{TM} kit the lowest point of calibration curve is set on 23 pg/µl. It means that all measurements of DNA quantity in casework samples under this value should be approximately considered as low template samples (LT samples) because approximately 200 pg input DNA may be theoretically used in PCR.

As was mentioned above one of the goals of quantification process in forensic casework samples is looking for **cut off** value which would enable to omit some samples from further workflow due to the reduced probability of performing reliable DNA profiles. It seems to be reasonable to expect such as value somewhere in the range of low quantification points of the calibration curve. This is a reason why detailed analysis of low calibration points is necessary.

An addition the large proportion of samples proceeded in the ICP (Institute of Criminalistics Prague) DNA laboratory shows the concentration values of DNA on the level of these points or often even far below. On the other hand when concerning the analysis of the Ct values of concentration points 23 and 66 pg/ µl, it was shown that the measurement of these values is relatively inaccurate due to high standard deviation (see Table 4.2).

This is a critical moment for the possibility of finding a real **cut-off** level of DNA due to the success of subsequent PCR.

The measured absolute concentration of casework samples between the two lowest concentration levels (23 and 66 pg/ µl) should not be considered in any case as a prediction of LT (low template DNA) status.

In order to examine how concentration of DNA in samples directly influence the PCR results in terms of final electrophoretograms attained after capillary electrophoresis, real data from casework samples were used. Total number of samples tested in this part of study was **40**. These samples were quantified on ABI Prism® 7900 HT System. Data were divided originally on base of Ct values to 4 groups, namely: Ct 28-32, Ct 33-35, Ct 36-37 and Ct 37 –U (Undetected data). The single categories correspond to certain absolute concentration of DNA determined according to pooled calibration curve and they are listed in the table 4.6. Biological material of analysed samples varies substantially and corresponds to common crime scene material. Quantification measurements were doubled for every sample of isolated DNA.

Table 4.6 The single concentration categories correspond to certain absolute concentration of DNA (pg/µl)

	No. of samples (pg/µl)									
Ct category	1	2	3	4	5	6	7	8	9	10
28-32	1515.2	1247.7	368.5	925.8	1411.5	975.1	737.7	1477	994.3	7008
	477.6	956.3	513	483.9	1430	1013.8	792.3	1649.3	1054	7054
33-35	315.3	238.5	135.6	98.7	57.6	50	280.5	129.6	182.8	61.1
	191.3	196.3	95.6	138.3	47.1	195.1	228	187.6	114	60.6
36-37	37.3	19.9	56.1	56.9	26.8	35	30.3	28.8	46.2	78.1
	45.1	66.1	17	40.6	43.6	47.7	68.64	35	29.7	50.2
37-U	U	U	30	26.7	U	11.3	10.7	21.9	U	12
	61	24.6	17.8	U	18.6	U	25.6	31.1	U	U

U-Undetected value

Theoretically according to table 4.5, the concentration of DNA in PCR concerning samples from the category 37-U should not exceed the level of 300 pg in PCR (if standard PCR setup is applied) and probably most of samples should be marked as LT samples because concentration of DNA does not exceed 100 - 200 pg /µl.

However, the dispersion of concentration values within the measured samples in the category 37-U is relatively distinctive. In addition some samples have undetermined value of concentration at least in one parallel measurement. The level of reliability of the amplification process should be much higher in the quantification category 36-37, because the average value of the absolute concentration of DNA is near to 50 pg/µl. By comparing the electrophoretogram of samples from both these categories, it is possible to align the average height of peaks and the number of dropouts in loci as a prediction of quality of DNA profile.

Table 4.7 Comparison of number of dropouts and height of peaks for both quantification categories

	Ct category 37-U	Ct category 36-37
The average height of the peak(RFU)	number of samples	number of samples
> 1500	5	2
1000	1	2
800	1	0
600	2	4
600 drop out	4	0
400 drop out	1	1
0 drop out	3	1

RFU-relative fluorescence unit

As shown in Table 7 there is no remarkable difference in the number of DNA profiles with occurrence of dropouts in these two quantification categories. Also the comparison of height of peaks in DNA profiles from both quantification categories appears to be very problematic. More accurate assessing of DNA profile quality was performed after evaluating of dilution experiment (see page 59) where heights of peak were compared with single quantification level of other control DNA (007). According to this the dilution experiment, the average value of the peaks height in concentration group 36-37 should be around 1000 RFU, which does not precisely match.

The number of samples that could be used for reliable genotyping of biological material was assessed in category 37-U. At least 7 samples out of 17 (40%) that were tested provided the reliable DNA profiles that could be used to direct comparison with reference samples.

These results do not enable to make any reasonable conclusion about predictability of quantification results attained with Quantifiler^{TM} kit for set cut off quantification value.

4.2. Testing relationship between input DNA in PCR and quality of DNA profiles.

In order to verify how much is this concentration limit reflected in the quality of DNA profiles, we conducted a dilution experiment. The aim of the experiment was to compare the effect of different concentration levels on the number of alleles detected in the subsequent PCR. We used a new control DNA marked 007, primary concentration of the stock solution is 0.01 ng/µl and it is therefore assumed that the dilution series will be less prone to pipetting error than in the case of starting diluting of the stock solution with a concentration of 50 ng / µl. The dilution experiment is shown in the Table 4.8. Third column presents DNA concentration in the each dilution level concerning calibration points measured by Quantifiler^{TM} .The fifth column contains the amount of DNA (in the pg), which was added to the amplification reaction. Thus, if the condition of the optimum amount of DNA inserted into the PCR would be met, only the highest concentrations 0.05 and 0.1 ng / µl respectively would be appropriate. In case of these concentrations there should not be any inhibition effect of the increased amount DNA. The concentration level numerated from 3 to 6 represents the category of samples that can be called LT DNA, although this limit is not entirely clear. The allelic dropout should be reflected in the amplification results of some markers. At concentration level numerated 5 or 6 representing the lowest concentration of input DNA in the resulting PCR, complete dropout loci can be expected to occur. In fourth column is expressed by single cells of template DNA.

Table 4.8 The method of calibration ranges preparation

Identification of the sample	Expected concentration (pg/ul)	Measured concentration-quantifiler (pg /ul)	Number of cells in response	Amount of DNA in response (pg)
0	100.00	72.7 / 92.1	167	1000.00
1	50.00	43.7 / 37.2	83	500.00
2	25.00	17.1 / 18.8	41	250.00
3	12.50	7.76 / 15.3	20	152.00
4	6.25	2.03 / Undetected	10	62.50
5	3.13	9.01 / Undetected	5	31.30
6	1.56	U / U	2	15.60

U-Undetected value

The concentrations lower than 10 pg/µl, measured by kits Quantifiler^{TM}, and are virtually undetectable (labelled as "U"). This is shown in the Table 8 in the seventh and eighth rows.

There are large variations in the two measured values at maximum concentration of 100 and 50pg / µl as well.

During the usage of the Quantifiler™ kit, it is possible to detect lower absolute concentration of control DNA 007 than it would be expected from the value gained by diluting the stock solution 007 DNA especially at concentration level numerated form 0 to 2.

Within the rough estimation, it is possible to claim, that Ct values lower than 36 would represent concentration level below 50 pg/µl which the limit is recommended by manufactures of amplification kits. Ct values below 37 represent concentration level less than 10 pg/µl. These samples are of LT samples nature and it is possible to expect their problematic PCR results what was partly proved be experiment demonstrated whit real casework samples (see table 4.6).

4.2.1 Internal validation of amplification kits.

Decision making process regarding selection of real casework samples with insufficient DNA for subsequent amplification, as shown by the results obtained by using the Quantifiler™ kit is burdened with considerable error. Results that would not theoretically provide sufficient amount of DNA gave a positive PCR result and are therefore false negative.

In order to predict better behaviour of the whole amplification process depending on the input amount of DNA to PCR, the individual concentration levels of the control DNA 007 were subjected to the amplification process. Because it was necessary to test a few amplification kits in terms of internal validation studies in ICP DNA laboratory, the individual concentration levels were tested by following kits: PowerPlex®16 System (Promega), AmpFlSTR® NGM™ (Applied Biosystem), ESSplex (Qiagene) and ESX (Promega). From each concentration level of control DNA 007, three PCR were performed for each amplification kit. The number of alleles in different loci was compares with the control DNA 007 profile for each amplification kit. Amplification parameters for individual kits were taken according to the manufacturer. Parameters are shown in Table 4.9. In case of the kit PowerPlex® 16 System (Promega), the internal protocol used in IPC DNA laboratory was used. All crucial features of PCR setup and postPCR steps are summarised in table 4.9.

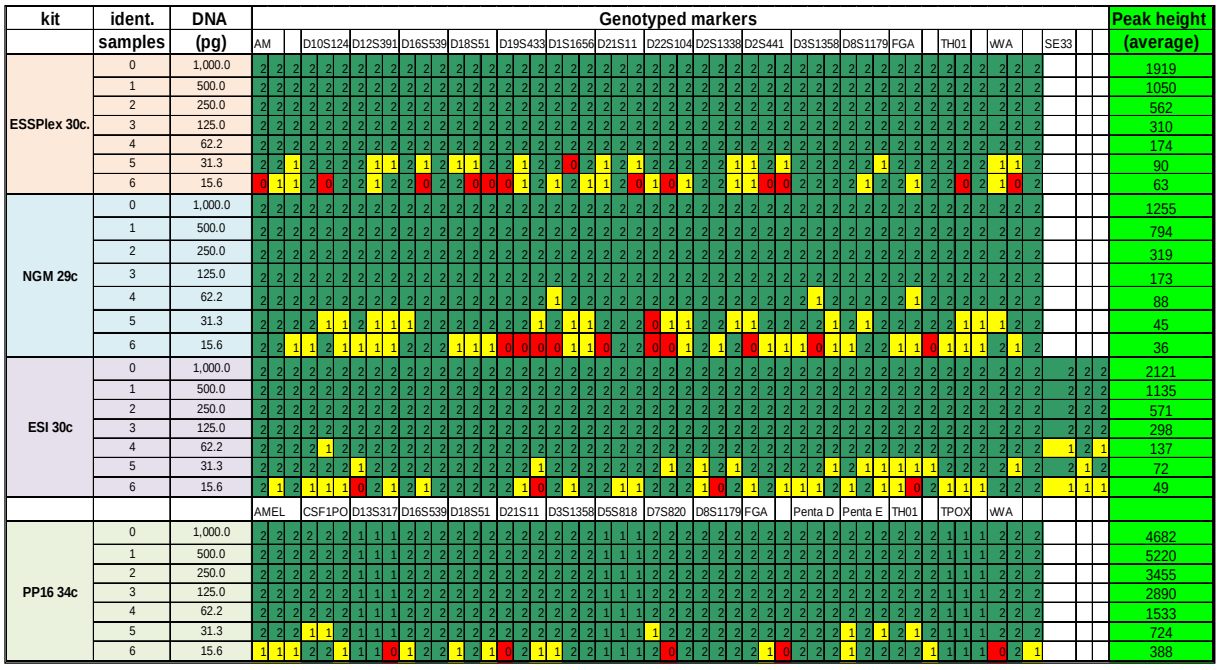
Table 4.9 PCR setup and post PCR steps in genotyping process be means of above mention kits.

Kit	Numbers of cycles	Formamide	IS	Mixture + PCR product
PP16	34	12	1	12+2
NGM	29	8.7	0.3	9+1
ESSplex	30	10	0.5	12+1
ESX	30	10	1	11+1

PP16- PowerPlex ® 16 System (Promega), NGM- AmpFlSTR® NGM™ (Applied Biosystem) , ESSplex (Qiagene), ESX (Promega), IS- internal standard

The results of the sensitivity of all amplification kits at different levels of quantification of the template DNA is presented in Figure 4.3.

Figure 4.3 Result of internal validation kits



30c.,29c.,30c.,34c. -The number in the first column means the number of cycles used in amplification.

The figures in the dark-green coloured part of the table mean the number of detected alleles in particular loci in the positive control DNA of tested kits. The yellow coloured squares mean allelic dropouts and the red coloured squares mean loci dropouts.

High levels of quantified DNA control 007 numerated from 0 to 3 always provide complete genotype for all four amplification kits. This result infers even higher sensitivity of tested amplification kits in terms of input DNA in PCR because 500 pg is generally recommended as

the limit amount of input DNA. The first allelic dropouts were observed at quantification level numerated 4 (62, 2 pg of input DNA) within NGM, and ESX amplification kits. PowerPlex 16 and ESSplex amplification kits have higher sensitivity due the amounts of input DNA that is exhibited in occurrence of the first allelic dropouts at concentration level 5 (31,5 pg of input DNA). As was shown in table 8, it is difficult to measure the concentration level numerated 4 (65.2 pg) by means of Quantifiler kit.

However, this level of concentration provides reliable amplification results. This is the basic discrepancy for using the Quantifiler kit for determination of the cut off value.

In case of PowerPlex 16 kit the number of amplification cycles is probably the main reason why the first allelic loci occurred at quantification level 5 (31,3 pg of input DNA) therefore low quantity of the template DNA in the sample may be improved by increased number of cycles in PCR.

On the other hand this increased sensitivity of PCR is probably connected with the presence of other peaks (drop-ins, contamination or non-specific PCR for such low concentrations of DNA). Figure 4.4 shows the results of the three lowest concentration levels and differences in the number of peaks in case of the individual kits. The kits ESI and NGM give us theoretically better results than other two kits (PP16, EESplex) because there are less additional peaks (labelled whit blue squares) .

Figure 4.4 The number of nonspecific peaks (except off leader), heights of reported peaks is more than 30 RFU

Amount of DNA	62.5pg			31.25 pg			15.62 pg			Amount of DNA	62.5pg			31.25 pg			15.62 pg		
Conc. level	4			5			6			Conc. level	4			5			6		
ESSPlex										ESX									
AM	2	2	2	2	2	1	0	1	1	AMEL	2	2	2	2	2	1	2	0	1
TH01	3	1	2	2	2	2	2	0	1	D3S1358	2	2	2	2	2	1	0	0	2
D3S1358	2	2	3	2	2	2	3	1	2	D19S433	2	1	2	1	2	1	2	0	0
vWA	2	1	2	1	1	2	1	0	2	D2S1338	2	2	2	2	2	1	1	0	2
D21S11	2	2	2	1	2	1	1	2	0	D22S1045	2	2	2	2	1	2	1	2	2
D16S539	3	2	2	2	1	2	2	0	2	D16S539	2	1	2	2	2	2	2	1	2
D1S1656	2	2	2	3	0	2	1	2	1	D18S51	2	2	2	2	1	2	2	1	2
D19S433	2	3	2	2	1	2	0	1	2	D1S1656	2	2	2	2	1	2	2	1	2
D8S1179	2	2	2	2	2	1	2	1	2	D10S1248	2	1	2	2	2	1	1	1	1
D2S1338	3	2	3	2	2	1	2	2	1	D2S441	2	2	2	2	2	2	1	2	1
D10S1248	2	2	2	0	3	3	3	0	1	TH01	2	2	2	2	2	2	1	0	1
D22S1045	2	2	2	2	0	2	2	0	1	vWA	2	2	2	0	1	2	2	2	2
D12S391	3	2	2	2	0	1	2	1	2	D21S11	2	2	2	2	2	1	0	1	1
FGA	2	2	2	2	3	2	3	0	2	D12S391	2	2	2	1	0	2	0	2	1
D2S441	2	1	2	1	1	1	0	0	0	D8S1179	1	1	0	0	0	1	1	0	1
D18S51	2	2	1	0	0	1	1	0	0	FGA	2	2	2	0	1	1	1	0	2
PP16										SE33	1	2	1	2	1	2	1	1	0
D3S1358	5	4	2	2	2	2	1	2	2	NGM									
TH01	2	2	2	2	1	2	2	3	1	D10S1248	2	2	2	1	1	0	1	0	1
D21S11	3	2	2	2	3	2	0	2	1	vWA	2	2	2	1	2	2	2	0	2
D18S51	3	2	2	2	2	2	1	2	1	D16S539	2	2	2	1	2	2	2	1	2
Penta E	2	2	2	2	2	1	1	2	2	D2S1338	2	2	2	2	2	1	2	1	2
D5S818	2	1	1	1	1	1	1	1	1	AMEL	2	2	2	1	2	1	0	2	0
D13S317	1	1	1	1	2	1	1	1	0	D8S1179	2	1	2	2	1	2	1	0	1
D7S820	4	2	3	1	3	2	2	0	3	D21S11	2	2	1	2	2	1	0	1	2
D16S539	2	2	2	2	2	2	1	2	3	D18S51	1	2	1	0	1	1	1	1	1
CSF1PO	2	2	2	2	1	2	2	2	1	D22S1045	2	2	2	0	1	1	0	0	0
Penta D	3	2	2	2	3	2	2	2	3	D19S433	2	1	2	1	2	1	0	0	0
AMEL	2	2	2	2	2	2	1	1	1	TH01	1	2	2	1	1	1	1	0	1
vWA	2	2	2	2	3	2	0	2	1	FGA	2	1	2	2	1	2	1	1	0
D8S1179	3	3	2	2	2	2	2	2	2	D2S441	2	2	2	1	1	2	0	1	0
TPOX	1	1	1	1	1	1	1	1	1	D3S1358	2	1	2	2	2	0	1	0	1
FGA	5	4	3	3	2	3	4	1	0	D1S1656	1	2	2	1	0	1	0	1	1
										D12S391	2	2	2	1	0	1	0	1	0

Another group of non-specific artefacts represents stutter peaks. They result from slipping process in the polymerase extension (elongation) of DNA. Source of allelic drop-in or sporadic contamination is another donor of biological material. In case of ESSplex kit (Qiagene) there are non-specific artefacts that occurred very frequently. Table 4.10 presents the percentage of the stutter peak and allelic drop-in at various kits reserved in internal validation study of all kits.

Table 4.10 Percentages of stutters and drop-ins in the internal validation study of all kits

Non-specific artefacts	DNA (pg/µL)	Kits			
		ESX(I)	ESSplex	NGM	PP16
S	1,000.0		20%	2.5%	5.1%
T	500.0		20%		7.6%
U	250.0		12%		
T	125.0		10%	2.5%	
T	62.2		23%		10.2%
E	31.3		7.6%		10.2%
R	15.6		5.1%		
D	1,000.0	5.1%	2.5%		
R	500.0				
O	250.0				
P	125.0				
	62.2		5.1%		
I	31.3				2.5%
N	15.6				

PP16- PowerPlex ® 16 System (Promega), NGM- AmpFlSTR® NGM™(AplieedBiosystem) , ESSplex (Qiagene), ESX(Promega), IS- internal standard

Another outputs deduced from experiments dealing with relationships of quantity of input DNA in PCR and quality of DNA profile is the determination of stochastic limit. It is approximate value of peak heights at which the probability of dropouts is close to zero. Specific results show that the peak heights in different quantitative levels of the control DNA 007 for single kits differ remarkably especially between PowerPlex 16 kit and others kits as it can be seen in the last column of Figure 4.3. Again, this may be explained by increased number of PCR cycles within PowerPlex® 16 kit. Even if dilution experiment with control DNA 007 represents artificially prepared data, it may infer behaviour of real casework samples in terms of reliability of attained data.

4.2.2. Evaluation of data from real casework samples quantification within Plexor ® HY System quantification kit.

In order to make the quantification process more efficient, Plexor® HY System quantification kit was used. According to manufactures information this kit should be more sensitive to detection of the lower concentration of DNA in challenged samples, due to higher number of target probes for quantification. The main aims of this experiment was finding if another commercial quantification kit is more suitable for prediction the quality of DNA profiles on

base of quantification of real casework samples with low expected amount of template DNA. Much more samples could be analysed even due to more user friendly software utilised in process of the evaluation of quantification data.

All measurement was done with LightCycler® 480 RT instrument. The construction of the calibration curve on the basis of dilution series of the standard (Plexor® HY Male Genomic DNA Standard) according to the instructions is presented in Figure 4.5.

Figure 4.5 Print screen of serial dilution of the Plexor Genomic standard (taken from Promega Corporation).

Concentration	Volume of DNA	Volume of TE^{-4} Buffer
50ng/µl	Use undiluted DNA	0µl
10ng/µl	10µl of undiluted DNA	40µl
2ng/µl	10µl of 10ng/µl dilution	40µl
0.4ng/µl	10µl of 2ng/µl dilution	40µl
0.08ng/µl	10µl of 0.4ng/µl dilution	40µl
0.016ng/µl	10µl of 0.08ng/µl dilution	40µl
0.0032ng/µl	10µl of 0.016ng/µl dilution	40µl

All data were collected during 6 months in ICP DNA laboratory. The absolute concentration of DNA in the sample was evaluated and then the results of each experiment were divided into three categories according to the attained values:

1) **Category I - < 1 pg/µl**
2) **Category II - 1-10 pg/µl**
3) **Category III - >10 pg/µl**

For every quantification category description of typical characteristic DNA profile was done. All samples were amplified with PowerPlex® 16 kit according to the standard protocol used in the ICP DNA laboratory (instructions in Chapter 3, sub-section 3.7. STR Genotyping). **All data (the calibration and casework samples) were generated in single amplification runs. As is obvious from figure 4.5, the lowest calibration point represents concentration level 3** pg/µl what is approximately 8 times less than in case of the Quantifiler™ kit

Category I - < 1 pg/µl

Altogether **167** samples were analysed in this category. Especial emphasis was put on negative results after PCR. As a negative result were considered:

1. Such as course of electrophoresis, where no detectable alleles (loci) are captured. Such effect is very rare, virtually no such sample was found.

2. Such as course of electrophoresis, where there are loci dropouts, at least two, while one of them is not occurred in the locus Penta E, Penta D or FGA.

3. The loci dropout is not observed but height of the peak does not exceed the value of 300 RFU.

In order to confirm results of the first amplification run, **66** samples were subsequently subjected to the re-amplification to verify the presence of dropouts in the 1st amplification reaction. To verify the match or the difference in the amplification runs, a system for evaluating the quality of the individual loci was established. Only the absolutely identical genotypes in the individual loci were considered as the same course in both PCR reactions. The table 4.11 shows the number of loci in this category, category I.

There were found **10** samples, representing **6%** of the total number of samples in this quantification category. These profiles fulfil all requirements for full-value DNA profile that is allowed to compare with other DNA profile. The sources of biological material represent:

1. Six swabs of different surface without any detailed knowledge about donor.

2. Swabs of surfaces where the single donor of biological material is expected (cigarette butt, relevant part of T-shit, gunstock and handle of plastic bag).

All these samples could be considered as **false negative**.

Category II - 1-10 pg/µl

Altogether **200** samples were analysed in this category. According to the results of the first amplification, samples with detected massive loci dropout were found even in this category, and therefore it would be possible to classify them virtually into category I according to quantification results. In case of **123** samples the height of alleles do not reach the

interpretation (stochastic) limit set by dilution experiment (see Figure 4.5), and therefore the second amplification was used to verify the real occurrence of dropouts. Numbers of identical loci were also evaluated, and the results shown in Table 4.11.

About **43%** of the total number of processed samples in this category, which were amplified by the second amplification, showed more than **8** repeatable loci. According to internal rules in ICP DNA laboratory at least **8** loci are compulsory for putting DNA profile into database. This percentage is too high in order to exclude entire quantification category from further workflow only on the basis of quantification results. These samples would be considered as **false negative results**. This result also partially answers to the globally discussed question whether it is possible to obtain reliable results in samples where less than 100 (200) pg of input DNA is amplified.

Category III - >10 pg/µl

Altogether **180** samples were analyzed in this category. This category includes samples, which should provide more reliable results with respect to quantification results. **85 %** of them provide the electrophoretograms in which the peak heights reliably exceeded the stochastic limit. None of the evaluated samples in this category showed dropouts, but the second amplification had to be carried out in case of a few found DNA profiles. The reason for the second amplification of these DNA profiles, where the DNA concentration in PCR exceeds 200 pg, is mostly the testing of reliability of minor component of mixed DNA profiles. The peak heights of these minor profiles rarely exceed the stochastic limit.

Also the mixed samples without clearly differentiated major and minor portion of the DNA profiles and the peak heights bordering with the stochastic limit are necessary to amplify in the second amplification. Amplification of **26 samples** in this quantification category was performed**.** Even within this quantification category there were samples with peak heights below the stochastic limit.

As the table 4.11 shows it is possible to use 19 of these samples (73 %) for the next direct genotyping biological materials. Seven of these samples have false positive results in the third quantification category.

Table 4.11 Number of matching loci for tree quantification category

The number of matching loci	<1pg	1-10pg	>10pg
0	0	0	0
1	8	9	0
2	7	7	0
3	4	9	1
4	6	3	0
5	1	6	0
SUM	26	9	2
Num. samples	37		
6	2	4	4
7	2	8	2
8	2	4	0
9	0	4	0
10	3	7	3
SUM	34	27	20
Num. samples	81		
11	0	2	0
12	0	7	2
13	0	5	5
14	2	3	2
15	0	3	7
SUM	1	9	16
Num. samples	26		

Reliability and utilization of amplification replicates is summarised in Tab 4.11. Where number of identical loci in each quantification category is recorded. Within each category amplification replicates were further divided according to the number of matching loci. The category numerated 1 – 5 represents DNA profiles that are not suitable for any comparison with reference samples. The category numerated 6-10 represents unambiguous DNA profiles. These profiles could be further evaluated by the third amplification. The category numerated 11 – 15 represents DNA profiles that could be use for comparison purposes even on base of consensus strategy (only repeatable alleles are reported).

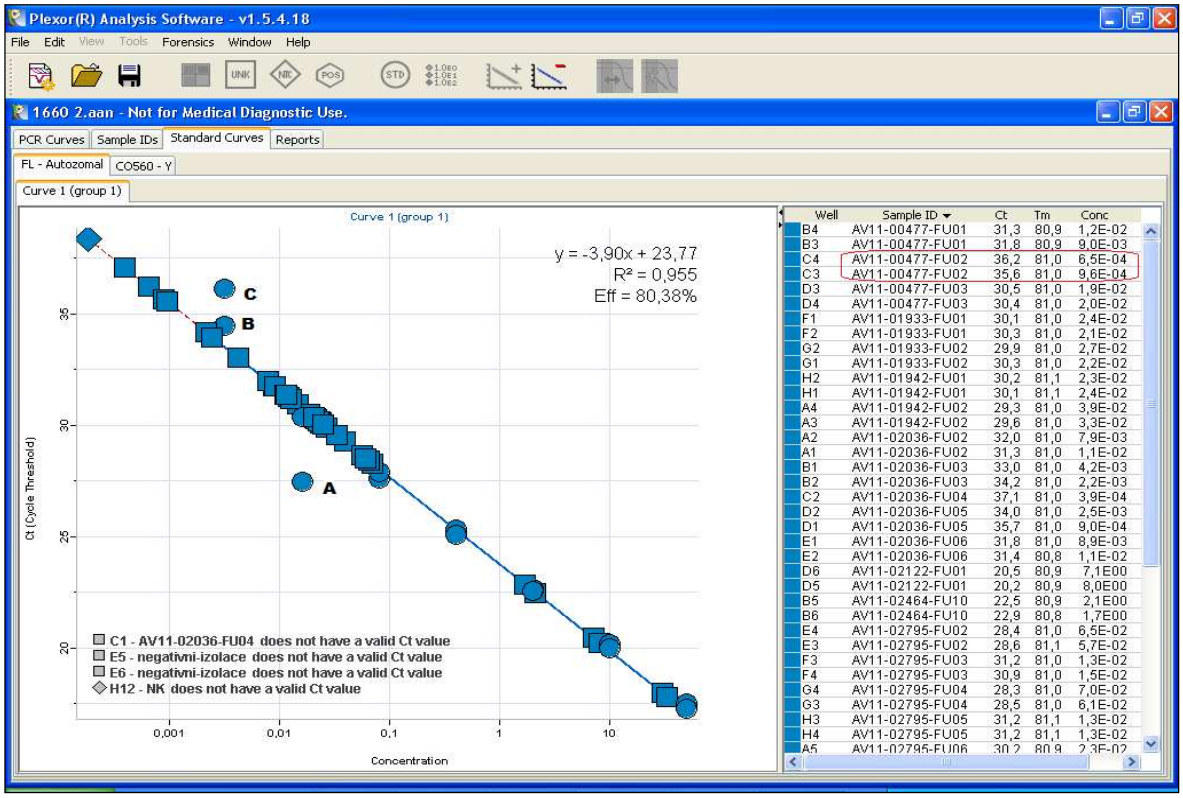
4.2.2.1. Influence of calibration points changes of quantification categories on determination within Plexor ® HY System quantification kit.

Concerning routine utilization of absolute quantification, to make the decision about the next method of the analysis, there are problematic concentration values which occur around the **cut-off value**. Since these concentrations are generated by using the calibration curve, all variations during its construction (mathematical evaluation) directly affect the absolute value of DNA quantity in the sample.

Due to graphical imaging of Plexor ® HY System quantification kit evaluating software it is very easy to observe both the small deviations in Ct values of individual points of the calibration curve, and the slope of the calibration curve which is determined by the efficiency of quantification PCR reaction.

In order to determine how these changes are reflected in absolute concentration of DNA in the sample, a one curve where the value of $\mathbf{R^2}$ is less than 99% (Figure 4.6) were changed by removing several concentration points. The progressive changes of calibration curve and the resulting changes in the absolute concentration of DNA near 1pg were observed.

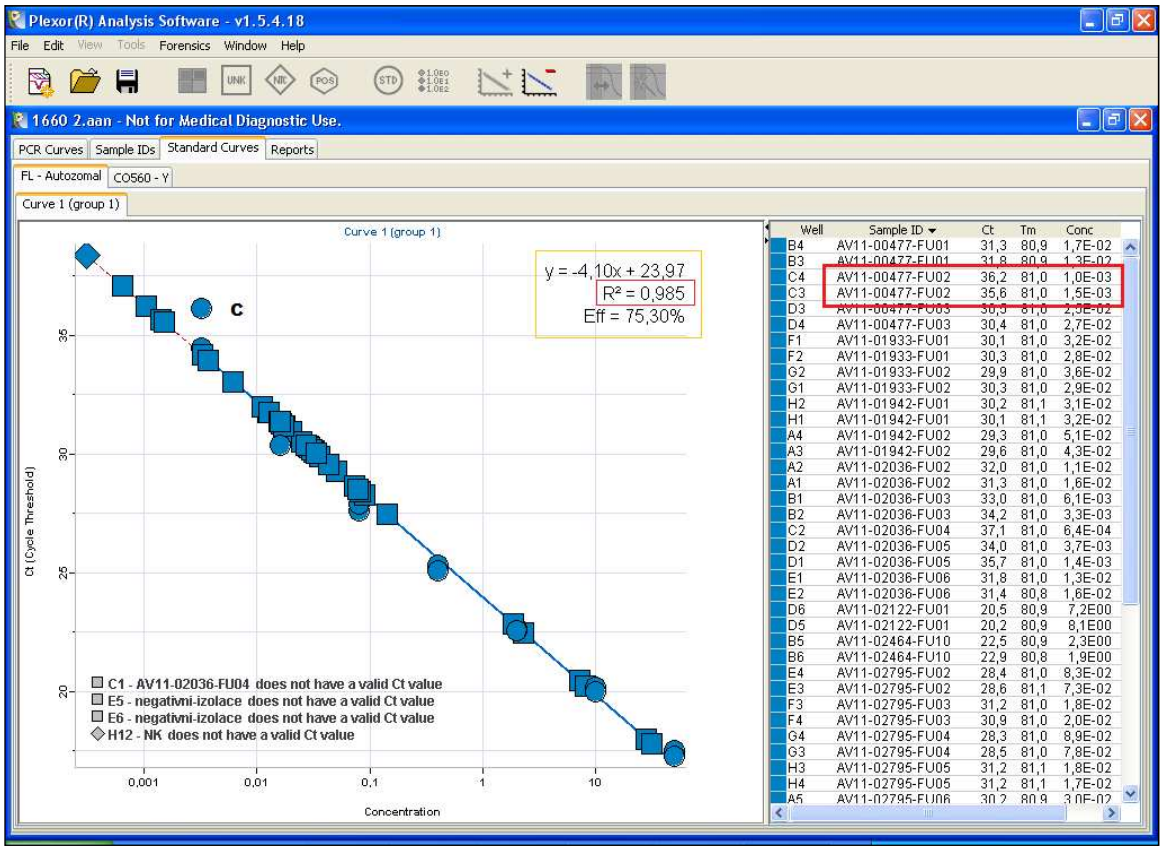
Figure 4.6 Print screen calibration curve from Plexor® HY system without any calibration points omission



The values of Absolute concentration which are measured in ng / µl are listed in red picture frames.

If the points of the calibration curve are removed (point **A, B**), than this change is reflected by increasing of R^2 value and also by change of the absolute DNA concentration in the sample *AV11-00477-FU02* (the value is increased above the suggested cut off value 1 pg) (Figure 4.7).

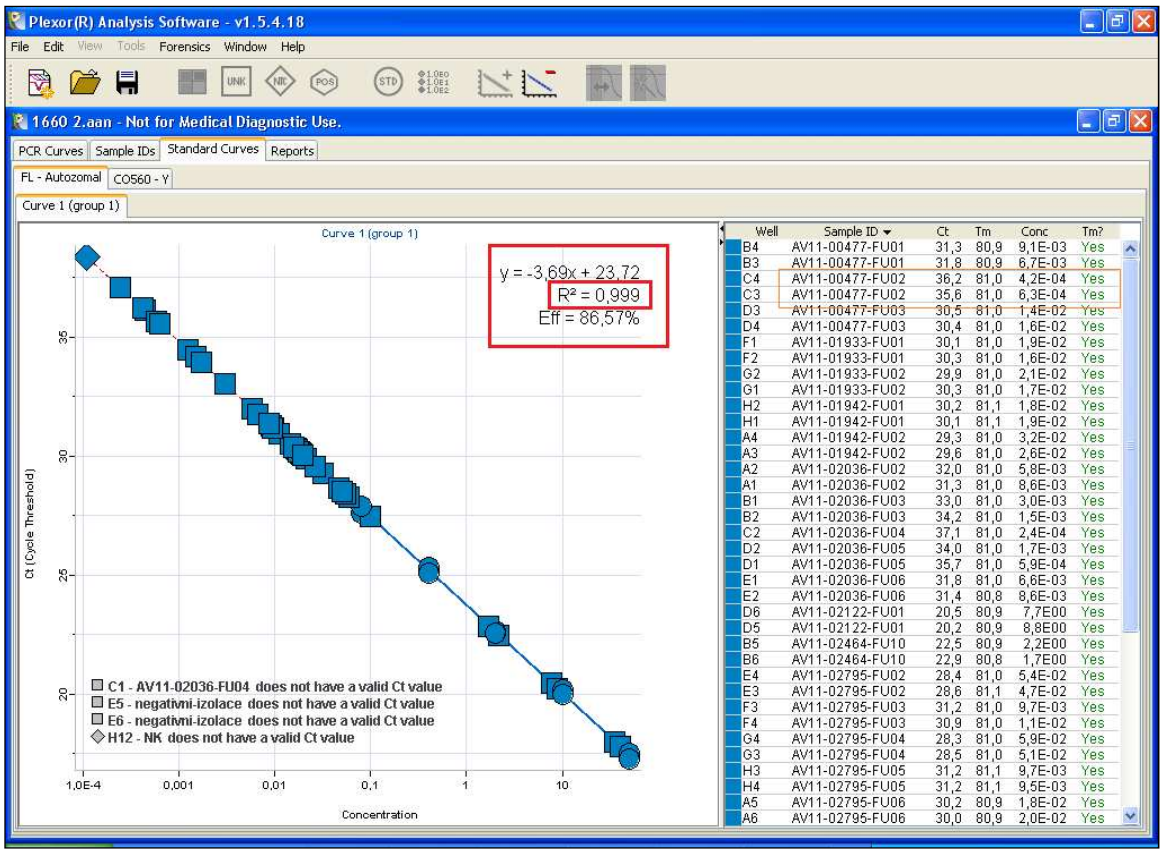
Figure 4.7 Print screen calibration curve from Plexor® HY system with two omitted points (A,B).



The values of Absolute concentration which are measured in ng / µl are listed in red picture frames.

By removing another point from the calibration curve (**C**), the R^2 value is changed to the value above 99% and the absolute value of DNA concentration in sample *AV11-00477-FU02* is shifted (the value is again reduced under the suggested cut off value 1 pg) (Figure 4.8).

Figure 4.8 Print screen calibration curve from Plexor® HY system with one omitted point (C).



The values of Absolute concentration which are measured in ng / µl are listed in red picture frames.

CHAPTER 5 DISCUSSION

Genotyping of biological samples is nowadays a discussed issue not only in terms of extraordinary strong evidence in the field of forensic practice, but also in terms of the financial demands. This process is closely connected with the rules of the legal system where the expenses invested in processing of the forensic samples are often neglected.

The reliable quantification method seems to be the essential instrument for these purposes. It helps to optimalize of the amount of DNA which enters the amplification process and thus reduce the necessity to carry out PCR in the replicates. Even though the laboratories have the chance to use any approach to search the optimal method, the emphasis is put mainly on the specificity of the quantitative determination of human DNA in forensic samples.There is a reason why RT PCR was choose. At least semi-automatization of the whole process including suitable software evaluation is also very important. All these requirements are met by the quantification kits.

Every introduction of a new method and commercial kits in the forensic DNA laboratory has to undergo the process of internal validation. The goal is to verify the agreement of the introduced procedures with the development validation study of the manufacturer.Part of this internal valiation could be issues conected to quantification method itself. The matter of using half volume of the quantification chemistry set up was involved in previous internal ICP DNA laboratory validation studies. As it is implied in study (Westring et al. 2007) this method is more and more used worldwide.

The Quantifiler method was used in the ICP laboratory as an approximate method and the accuracy of this method was not critical with regard to the wide range of input DNA recommended by the manufacturer. Because the number of samples in the ICP laboratory continuously increases, the goal was to make use of another possibility of the quantification process, which is determination of the quantification cut off value. It colud lead to the possibility to stop the analysis of any sample that has a quantification value at or below the minimum value by forensic DNA analyst(Cupples C.M. et al 2009).

This value should enable the selection of such samples of isolated DNA which most likely did not provide reliable DNA profiles during the whole workflow. Because this expected value is located on the lower limit of the calibration curve, it is necessary that the method provides reliable results in this area. Commercial kits are generally tested by the manufacturer for a

specific kind of instrument. In case of change of the instrument it is necessary to test the sensitivity of the new device and compare it with the sensitivity of the previous device. The accuracy of the measurement sensitivity has a fundamental impact on the accuracy of determination of cut off quantification values.

Given that the laboratory routinely use two quantification instruments Light Cycler ®480 and ABI® Prism 7900 for Quantifiler® quantification kit ,comparison of calibration curves of two independent instruments based on the slope and the R^2 (standard deviation) value was done and it was showen there are no differences. For both instruments and Quantifiler® kit pooled calibration curve was performed but for long accurancy would be important to take measurements of the calibration curves from time to time. Therefore it shows that both instruments work under those same conditions and their resulting outcomes are not much different. The similra result was achived in other study(Sivaganesan et al. 2010).

The measurement accuracy in the area of very low concentration is essential. However, in case of the Quantifiler ®kit there was a very high level of inaccuracy within low concentration values which was demonstrated through high dispersion of absolute quantification values in casework samples. Therefor, even in very small number of casework samples that were tested weak predictive ability of the absolute quantification value for determination of unreliable DNA profiles was shown.

For the purpose of more reliable estimation of the usability of absolute quantification, the dilution experiment was done where the quantitity of DNA in PCR could be regulatively assessed even in connection with the peak height, the so called stochastic limit could be created. The experiments proved its direct dependency on the number of cycles in PCR. Concerninig the amplification kit (pp16,ngm,esxi, essplex) was not prove any significant influence.

Finally we managed to approach set a limit amount input of DNA to detect and reliably interpret of STR profiles (Figure 4.3).

It is generally assumed that the sensitivity of RT PCR amplification kits is depended of the number of fluorescent probes that are used in kit chemistry. Because of possibility in ICP DNA laboratory to start using Plexor kit that should fulfilled these assumption the comparison

of prediction of the cut off value for selection some casework samples for the further workflow was evaluated.

Long time experiment was carried out that collected data from several independent quantification runs of casework samples and 547 samples could be approximate divided into tree quantification categories which are described in results. On the base of data from dilution experiment the quantification data were connected to character of particular electrophoretogram especially in terms of heights of peaks or occurrence of dropouts .The lowest quantification category (< 1pg) contained the height percentage (94%) of unreliable DNA profiles and could be set as cut off value. However the accuracy of calibration curve points in one calibration run may play partial role in strict arrangement of particular sample into quantification category. In addition attention must be devoted to character of casework samples especially in terms of source of biological material and expected number of donors because falsely positive samples were found even in this category. 50 % of samples in category (1-10pg) are possible to consider as ambiguous result. And reliability DNA profiles should be evaluated in the second amplification.

CHAPTER 6 CONCLUSION

The experiments showed that the measurement of very low concentrations with Quantifier® kit on the ABI® Prism 7900 HT instrument is loaded with relatively large an accuracy resulting from pipetting errors and a lower sensitivity of this kit in comparison with the Plexor®HY System kit.

Using DNA controls 007 allow a better estimate of the interaction between the input quantity of DNA and detectable alleles. This was applied to assess the sensitivity of individual amplification kits which laboratory was tested under internal validation. It was documented a crucial effect of number of cycles of PCR for total detection capability of the amplification kits.

Based on the results we can say that it is not possible to define the absolute minimum of the cut-off value for Quatifiler® kit. That would allow samples to make a reliable selection highly anticipated low-template DNA. For Plexor® HY System kit was set this quantification level up to 1 pg / µl, however, and below this value is possible to detect samples that give false positive results.

This data should be useful for forensic society seeking to define laboratory policies regarding further analysis of case samples that are “undetected” for human DNA by Quantifier ™.

BIBLIOGRAPHY

ADAMS P.S., Data analysis and reporting, (Bustin, S. A.): A-Z of quantitative PCR, *International University Line,* 2004

ANDREASSON H., ALLEN M. (2003) Rapid quantification and sex termination of forensic evidence materials. *J. Forensic Sci*. 48: 1280-1287.

ALONSO A., MARTIN P. (2005) A real-time PCR protocol to determinate the number of amelogenin (X-Y) gene copies from forensic DNA samples. *Methods Mol.Biol*. 297: 31-44.

ALONSO A.,MARTIN P., ALBARRAN C., GARCIA P., PRIMORAC D., GARCIA O., FERNANDEZ S., GARCIA-HIRSCHLFELD J., SNACH M., FERNANADEZ-PIQUERAS J. (2003) Specific quantification of human genome from low copy number DNA samples in forensic and ancient DNA studies.,*Croat. Med.J.* 44: 273-280.

BATZER M.A.,DEININGER P., ALU REPEATS AND HUMAN GENOMIC DIVERSITY 2002 *Nature Publishing Group* VOLUME 3 370-380

BUTLER, J. M. (2005) Forensic DNA typing - biology, technology and genetics of STR markers, 2nd edition. *Elsevier Academic Press,* 241-299.

BUTLER J. M., BUEL E., CRIVELLENTES F., MCCORDS B. R., 2004, Forensic DNA Typing by Capillary Electrophoresis Using the ABI Prism 310 and 3100 Genetic Analyzers for STR Analysis, *Electrophoresis,* 25(10-11), 1397-1412.

BUSTIN S.A, BENES V.,GARSON J.A., HELLEMANS J., HUGGETT J., KUBISTA M.,MULLER R.,NOLAN T.,PFAFFL M.W.,SHIPLEY G.L.,VANDESOMPLE J.,WITTWER C.T. *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments Clinical Chemistry 55:4 *Papers in Press. Published February* 26, 2009

BUTLER, J.M. (2005) Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers, *2nd edition*, Chapter 16 “Laboratory Validation”, pp. 389-412.

BUTLER J.M, Fundamentals of Forensic DNA Typing, Preface © 2009, *Elsevier Inc,* ISBN : 978-0-12-374999-4

BULANDER N, ROLF B, Comparison of the Plexor® HY System, Quanitifiler™ and Quantifier DUO kits using the Roche LightCycler® 480 System and the ABI 7900 real time PCR instrument, FSI,2,2009, 104-105

BUSTIN S.A. A-Z of quantitative PCR, *International University Line*, 2004

BLOCH W (1991) Biochemistry, 30, 2735-2747.

BUCKLETON J., TRIGGS CH., WALSH M., 2005, Forensic DNA Evidence Interpretation, *CRC press*, p. 531

CHIEN A, EDGAR DB, TRELA JM (1976).Deoxyribonucleic acid polymerase from extreme thermopile Thermis aquaticus.. *J. Bact.* 127 (3): 1550–7.

COLLOPY CH. (2008) Maximizing DNA Recovery ,*Forensic* Vol. 5 | No. 1

CUPPLES CH.M, M.S.;CHAMPAGNE J.R.,M.S.; LEWIS K.E, M.S.; CRUZ T.D., Ph.D. STR Profiles from DNA Samples with ‘‘Undetected’’ or Low Quantifiler^{TM} Results *J Forensic Sci*, January 2009, Vol. 54, No. 1

DIDENKO VV (2001) DNA probes using fluorescence resonance energy transfer (FRET) : ADESING AND APPLICATIONS. *Biotechniques* 31 : 1106-1116,1118,1120-1121.

FRUMKIN D.,WASSERSTROM A.,DAVIDSON A.,GRAFIT A. Authentication of forensic DNA samples FSIGEN-486;

FANRICH K, CRAUSE C, HUBER C, 1999, Synthesis and characterization of new chloride sensitive indicator dye based of dynamic fluorescence quenching J *Photochem Photobiol A: Chem* 128: 111-120.

DVOŘÁKOVÁ M., Základní principy a využití kvantitativní PCR s ohledem na onkologickou diagnostiku, *Brno,* 2007

GREEN R, ROINESTAD I, BOLAN C., HENNESSY L., Developmental Validation of the Quantifiler RT PCR Kits for the Quantification of Human Nuclear DNA Samples. *J.Forensics Sci*, 2005, Vol.50, No.4.

GILL P., WHITAKER J., FLAXMAN C., BROWN N., BUCKELTON J., (2000) An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA, *Forensic Sci.Int*. 112: 17-40.

GRIGCAK CH.M,Ph.D.; URBAN Z.M., B.S.; COTTON R.W., Ph.D. Investigation of Reproducibility and Error Associated with qPCR Methods using Quantifiler®Duo DNA Quantification Kit**J Forensic Sci*, 2010

HANDT O.,KRINGS M.,WARD R.H.,PAABO S., (1996) The retrieval of ancient human DNA sequence. *Am J Hum Genet 59, 368-376.*

HILL C.R.,DUEWER D.L.,KLINE M.C.,SPRECHER C.J.,MCLAREN R.S.,RABBACH D.R.,KRENKE B.E.,ENSENBERGER M.G.,FULMER P.M.,STORTS D.R.,BUTLER J.M., 2010, Concordance and Population Studies along with Stutter and Peak Height Ratio Analysis for PowerPlex® ESX 17 and PowerPlex® ESI 17, *Forensic science international: Genetics*, xxx(xxx), xxx-xxx (p. 7) – Article in Press.

ISENBER A.R., MOORE J.M. (1999). Mitochondrial DNA analysis at the FBI Laboratory. *Forensic Sci Commun 1.*

HAQUE K.A.,PFEIFFER R.M.,BREEMAN M.B.,STRUEWING J.P.,CHANOCK S.J.,BERGEN A.W., Performance of high-throughput DNA quantification methods *BMC Biotechnology* 2003, 3:20

HANSSON O., FINNEBRAATEN M., HEITMANN I.K., RAMSE M. and BOUZGA M., Trace DNA collection – performance of minitape and three different swab, *FSI ,2*, 2009, 189-190

HIGUCHI R.,FOCKER C.,DOLLINGER G.,WATSON R. (1993) Kinetic PCR analysis : real time monitoring of DNA amplification reactions. *Biotechnology* 11, 1026-1030.

HOLLAND,P.M., ABRAMSON,R.D.,WATSON,R., GELFLAND,D.H. (1991) Detecting of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of Thermus aquaticus DNA polymerase .*Proc.Natl.Acad.Sci*.88,7276-7280.

KREMSER A, BAYER B, JUNG S, ANSLINGER K, (2009) Quantifiler Human DNA Quantification Kit (Applied Biosystems) as a screening kit fot DNA profiling. *Forensic Science International: Genetics Supplemetn Series 2* : 106-107.

KRENKE BE, TEREBA A, ANDERSON SJ, BUEL E, CULHANE S, FINIS CJ, TOMSEY CS, ZACHETTI JM, MASIBAY A, RABBACH DR, AMIOTT EA, SPRECHER CJ. Validation of a 16-locus fluorescent multiplex system. *J Forensic Sci*, 2002, vol. 47, s. 773–785.

MULLIS KB (1990) Target amplification for DNA analysis by polymerase chain reaction. *Ann Biol Clin (Paris)* 48: 579-582.

NIKLAS J.A., BUEL E. (2003) Development of Alu-based, real-time PCR method for quantification of human DNA in forensic samples. *J.Forensic Sci.* 48: 936-44

PETERS IR, HELPS CR, HALL EJ, DAY MJ (2004) Real – time PCR : consideration for efficient and sensitive assay desing . *J Immunol Methods* 286: 203-217.
PFAFFL M (2004) Quantification strategies in real-time PCR. In : A-Z of Quantitative PCR (ed BUSTIN SA) pp 441-492.*International University Line*,La Jolla, CA.

PFAFLL M.W., A new mathematical model for relative quantification in real time PCR, *Nucleic Acids Research* , 2001, Vol.29, No.9

RICHARD M.L., FRAPPIER R.H., NEWMAN J.C., (2003) Development validation of a real time quantitative PCR assay for automated quantification of human DNA. *J.Forensic Sci*. 48 : 1041-1046.

RAPLEY R., WHITEHOUSE D., Molecular Forenics, 2007, *John Wiley & Sons Ltd.*

RAYMAOND J.J.,VAN OORSCHOT R.A.H., GUNN P.R., WLASH S.J. and ROUX C., Trace DNA success rates relating to volume crime offences, *FSI, 2*, 2009, 136-137

RASMUNSSEN, R (2001) Quantification on the LightCycler .In : MEUER S, WITTWER C, NAKAGAWARA K (eds) Rapid cycle real-time PCR, methods and applications. *Springer Press*, Heidelberg.

RASMUNSSEN,R.P., MORRISON,T.B.,HERRMANN,M.G. and WITTWER,C.T.(1998) Quantitative PCR by con-tinuos fluorescence monitoring of a double strand DNA specific binding dye. *Biochemica* 02/1998,8-11.

RUTLEDGE R.G, COTE C., (2003) Mathematics of quantitative kinetic PCR and the application of standard curve. *Nucleic Acids Research* 31 (16), e93.

SANCHEZ JA, PIERCE KE, RICE JE,WANGH LJ (2004) Linear-after-the-exponential (LATE)-PCR: an advanced method of asymmetric PCR and its uses in quantitative real-time analysis. *Proc Natl Acad Sci* USA 101 : 1933-1938.

SIVAGANESAN M.,HAUGLAND R.A.,CHERN E.C.,SHANKS O.C., Improved strategies and optimization of calibration models for real-time PCR absolute quantification water. *r e s e a r c h xxx (2 0 1 0) 1 e1 0*

SWEET D., D.M.D, Ph.D.; LORENTE M., M.D., Ph.D., LORENTE J.A., M.D., Ph.D.; An Improved Method to Recover Saliva from Human Skin: The Double Swab Technique *J Forensic Sci* 1997;42(2):320-322.

SWAGO K.L, TIMKEN M.D, CHONG M.D and BOUNCRISTIANI M.R., A quantitative PCR assay for the assessment of DNA degradation in forensic samples, *FSI* , 158 (2006), 14-26

SAIKI RK, SCHARF S, FALOONA F, MULLIS KB, HORN GT, ERLICH HA, ARNHEIM N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 1985, vol. 230, s. 1350–1354

SCHRAM E., 1991. Evolution of Bioluminescent ATP Assays. In: Bioluminescence and Chemiluminescence: Current status. Eds. Staney P.E., Kricka Lh., p. 407-412.

TAGLIARO F.,SMITH E.P., 1996, Forensic Capillary Electrophoresis, *Trends in analytical chemistry*, 15(10), 513-525.

TAGLIARO F., BORTOLOTTI F., 2006, Recent Advances in the Application of CE to Forensic Science (2001-2004), *Electrophoresis*, 27(1), 231-243.

TOLUN G.,MYERS R.S., A real-time DNase assay (ReDA) based on PicoGreen® fluorescence *Nucleic Acids Research*, 2003, Vol. 31, No. 18 e111

TIMKEN M.D., SWAGO K.L, ORREGO C., BUONCRISTIANI M.R. (2005) A duplex real-time qPCR assay for the quantification of human nuclear and mitochondrial DNA in forensic samples : implications for quantifying DNA in degraded samples. *J.Forensic Sci*.50 : 1044-1060.

UTZ S., MALIK N., Trace DNA : Comparison of DNA recovery using the classical swab technique versus the hydrosoluble adhesive tape., *Swiss.*

VANGILDER HD, VRANA KE, FREEMEAN WM (2008). "Twenty-five years of quantitative PCR for gene expression analysis". *Biotechniques* 44 (5): 619–626.

WALSH P.S., METZGER D.A., HIGUCHI R. (1991). Chelex® 100 as a medium for simple extraction of DNA for PCR - based typing from forensic material. *BioTechniques 10, 506-513.*

WALSH P.S., ERLICH H.A., HIGUCHI R. (1992) PCR methods and application, 1, 241-250.

WALKER J.A., HEDGES D.J., PERODEAU B.P., LANDRY K.E., STOILOVA N., LABORDE M.E., SHEWALE J., SINHA S.K., BATZER M.A. (2005) Multiplex polymerase chain reaction for simultaneous quantification of human nuclear, mitochondrial , and male Y-chromosome DNA: application in human identification. *Anal.Biochen*. 337: 89-97.

WALKER J.A., KILROY G.E., XING J., SHEWALE J., SINHA S.K., BATZER M.A. (2003) Human DNA quantitation using Alu element-based polymerase chain reaction. *Anal. Biochem*. 315 : 852-855.

WESTRING CH.G., KRISTINSSON R., GILBERT D.M., DANIELSON PH.B., Validation of reduced-scale reaction for the Quantifiler tm Human DNA KIT , *JFSCI*, 2007, Vol.5, No.6

WANG,Z.,SPANDORO,J. (1998) Determination of target copy number of quantitative standards used in PCR-based diagnostic assays. In: Gene Quantification (FERE,E,ed.), *Birkhauser*,Boston,pp.31-43

WETMUR,J.G.(1995) In: Molecular Biology and Biotechnology (MEYERS,R.A.,ed.),*Publishers Inc.*,*New York*, pp.31-43.

WITTER,C.T.,REED,G.B.,RIRIE,K.M. (1994) Rapid cycle DNA amplification. In: The polymerase Chain Reaction (MULLIS,K.,FERRE,F. And GIBBS,R.,ed.) *Springer-Verlag*,*Derrfield* Beach,FL,pp.174-181.

ŠMARDA J., DOŠKAŘ J., PANTŮČEK R., RŮŽIČKOVÁ V., KOPTÍKOVÁ J. (2005), Metody molekularní biologie, *Brno.*

Used Internet sources:

www.ambion.com/techlib/basics/rtpcr/index.html

www.appliedbiosystem.com

www.nanodrop.com

www.promega.com

www.roche.com

Material of commercial companies:

Applied Biosystems 2000, Chemistry Reference for the ABI PRISM®310 Genetic Analyzer, GeneScan®Reference Guide, http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040961.pdf

Applied Biosystems 2003, ABI PRISM®3100/3100-Avant Genetic Analyzer, User Guide,

http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041428.pdf

Applied Biosystems 2004, GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis, Tutorial, http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_041475.pdf

Applied Biosystems 2009, AmpFℓSTR® NGM™ PCR Amplification Kit**,** User's Guide, http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_074250.pdf

Applied Biosystem 2006, Quantifiler™ kits, Users Manual

Applided Biosystem 2008, 7900HT Fast Real-Time PCR System, Users Manual

Promega Corporation, 2008, PowerPlex® 16 System - Instructions for Use of Products DC6530 and DC6531, Technical Manual, http://www.promega.com/tbs/tmd012/tmd012.pdf

Promega Corporation, 2009, PowerPlex® ESX System - Instructions for Use of Products DC6720 and DC6721, Technical Manual, http://www.promega.com/tbs/tmd024/tmd024.pdf

Promega Corporation, 2009, PowerPlex® ESI 17 System - Instructions for Use Of Products DC6780 and DC6781, Technical Manual, http://www.promega.com/tbs/tmd028/tmd028.pdf

Promega Corporation 2008, Validation Guide for the Plexor® HY System.

Roche 2009, LightCycler® 480 System, Users Manual