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Sensitive Forensic DNA Analysis

Application of Pyrosequencing and Real-time PCR Quantification

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Abstract
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The field of forensic genetics is growing fast and the development and optimisation of more sensitive, faster and more discriminating forensic DNA analysis methods is highly important. In this thesis, an evaluation of the use of novel DNA technologies and the development of specific applications for use in forensic casework investigations are presented.

In order to maximise the use of valuable limited DNA samples, a fast and user-friendly Real-time PCR quantification assay, of nuclear and mitochondrial DNA copies, was developed. The system is based on the 5' exonuclease detection assay and was evaluated and successfully used for quantification of a number of different evidence material types commonly found on crime scenes. Furthermore, a system is described that allows both nuclear DNA quantification and sex determination in limited samples, based on intercalation of the SYBR Green dye to double stranded DNA.

To enable highly sensitive DNA analysis, Pyrosequencing of short stretches of mitochondrial DNA was developed. The system covers both control region and coding region variation, thus providing increased discrimination power for mitochondrial DNA analysis. Finally, due to the lack of optimal assays for quantification of mitochondrial DNA mixture, an alternative use of the Pyrosequencing system was developed. This assay allows precise ratio quantification of mitochondrial DNA in samples showing contribution from more than one individual.

In conclusion, the development of optimised forensic DNA analysis methods in this thesis provides several novel quantification assays and increased knowledge of typical DNA amounts in various forensic samples. The new, fast and sensitive mitochondrial DNA Pyrosequencing assay was developed and has the potential for increased discrimination power.

Keywords: forensic, sensitive DNA analysis, DNA quantification, mtDNA, Real-time PCR, Pyrosequencing

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List of Papers

This thesis is based on the following publications, which are referred to in the text by their roman numerals.

I **Real-time DNA quantification of nuclear and mitochondrial DNA in forensic analysis.**
Andréasson H., Gyllensten U. and Allen M.
BioTechniques 33:402-4, 407-11 (2002)[1]

II **Nuclear and mitochondrial DNA quantification of various forensic materials.**
*Andréasson H., *Nilsson M., Lundberg H. and Allen M.
Manuscript

III **Rapid quantification and sex determination of forensic evidence materials.**
Andréasson H. and Allen M.
Journal of Forensic Sciences 48:1280-7 (2003)[2]

IV **Mitochondrial sequence analysis for forensic identification using Pyrosequencing technology.**
Andréasson H., Asp A., Alderborn A., Gyllensten U. and Allen M.
BioTechniques 32:124-6, 128, 130-3 (2002)[1]

V **Coding region mtDNA analysis for increased forensic discrimination using Pyrosequencing technology.**
Andréasson H., Nilsson M., Styrman H. and Allen M.
Manuscript

VI **Quantification of mtDNA mixtures in forensic evidence materials using Pyrosequencing.**
*Andréasson H., *Nilsson M., Frisk S. and Allen M.
Manuscript

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Contents

Abbreviations

DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
STR	Short tandem repeat
SNP	Single nucleotide polymorphism
nDNA	Nuclear DNA
mtDNA	Mitochondrial DNA
LCN	Low copy number
rCRS	Revised Cambridge reference sequence
D-loop	Displacement loop
HVI/II	Hypervariable region I/II
C_t	Threshold cycle
PPi	Pyrophosphate
SSB	Single-stranded DNA binding protein

Introduction

Forensic DNA typing

Forensic genetics involves the wide field of DNA typing for individual identification. It is probably most known to people as a tool for the identification of suspects in a criminal investigation where the offender is connected to the crime or, as in most cases, to the crime scene. It is maybe even more important to be able to exonerate the wrongfully convicted or exclude an innocent suspect early in an investigation. DNA typing can also be used in other areas of the forensic field, where identification of individuals from mass disasters, wars and mass graves are a few examples. For law enforcement, DNA typing can be useful even without actual suspects as the DNA analysis allows a distinction to be made between serial crimes and copycat crimes and also provides a genetic profile to search against in a reference database of convicted offenders. Furthermore, DNA typing can be used for ancient DNA analysis, examining family relationships (such as paternity testing) and phylogenetic studies.

Background

In 1953, James Watson and Francis Crick, working at Cambridge University, first described the double helix structure of DNA [1]. Since then, a wide range of DNA technologies have been invented. In the late 1970's, the first generation of DNA markers, the restriction fragment length polymorphisms (RFLPs), were introduced. This form of DNA typing used large restriction digested fragments of DNA that were separated based on size and further analysed by Southern blotting and hybridisation with radioactively labelled probes [2]. In the 1980's, a new group of markers called variable number tandem repeats (VNTRs) or minisatellites were introduced. The VNTR's have many alleles and high heterozygosity. In 1984, based on VNTR analysis, Alec Jeffreys developed "DNA fingerprinting", the first forensic DNA typing tool [3]. However, the use of Southern blotting and radioactively labelled probes was time consuming and costly. It required large DNA amounts and consequently only large blood or sperm stains could be analysed. When the polymerase chain reaction (PCR) was introduced in the mid 1980's [4], the potential in forensic DNA analysis was significantly

enhanced. It was now possible to analyse not only large blood, sperm and saliva stains but also more limited biological samples as DNA sources [5-7].

With the introduction of PCR it became possible to analyse another type of tandem repeats, the short tandem repeats (STRs), or microsatellites [8]. The STRs can be, for example, tri- or tetranucleotide units repeated approximately 5 to 50 times, resulting in many alleles and high heterozygosity [9, 10]. Today, several sets of STR markers can be amplified together in a multiplex PCR and it is possible to analyse DNA from many different types of evidence materials found at a crime scene. However, there are still a few cases where it is impossible to perform nuclear DNA (nDNA) typing. This is commonly seen for materials such as shed hairs and epithelial cells deposited in fingerprints. In these cases, analysis of single nucleotide polymorphisms (SNPs) in hypervariable regions of the mitochondrial genome has shown to be a helpful tool [11, 12]. The fist convictions based on analysis of mitochondrial DNA (mtDNA) as circumstantial evidence were in 1996 (Stockholm, Sweden and Tennessee, USA) [13, 14].

A new generation of forensic DNA analysis tools

Mitochondrial DNA analysis is primarily used because it is more sensitive than nuclear DNA analysis. Even though STR analysis today is much more sensitive than the former "DNA fingerprinting" analysis there are still several types of evidence materials that are unsuitable for routine nDNA analysis. Challenging materials, containing only limited amounts of degraded DNA, include older samples, shed hairs, teeth and bone specimens [13, 15-17]. In these cases, successful DNA analysis is often limited to the sequencing of the high copy number mitochondrial molecule.

Analysis of multiple autosomal markers is still, and will always be, much more discriminating than mtDNA analysis. Therefore, a sensitive quantification method, that simultaneously determines the nuclear and the mitochondrial DNA content within an evidence material, is very useful in forensic DNA analysis. The valuable DNA sample should not be wasted on an unsuccessful nuclear analysis, due to limited DNA amounts. Similarly, the material and efforts should not be spent on a less discriminating mtDNA analysis if nDNA typing is possible.

Although well established, the mtDNA sequencing analysis performed in the routine laboratories today can be further optimised for forensic analysis. When a cell dies, degradation of organic molecules, for example DNA, is initiated. Therefore, in some forensic samples, the DNA molecules are fragmented and the average lengths of intact DNA strands become shorter with time. The analysis of degraded DNA can therefore be improved by

designing shorter PCR fragments. In addition, the discrimination power in mtDNA analysis can be improved by including additional polymorphic sites or regions to those used in routine analysis.

Routine forensic DNA analysis

The nucleus of a human cell contains more than 99.5% of the entire genetic material. The nuclear genome is distributed between 24 different linear chromosomes (22 autosomes and two sex chromosomes). Each somatic cell is diploid, with both a maternal and a paternal contribution, resulting in a total of 46 chromosomes. The genome consists of approximately three thousand million nucleotides and variation between two unrelated individuals is estimated to occur once in every 1,000 bases. A large part of the genome is made up of non-coding DNA and in addition, it consists of a considerable amount of repetitive DNA, including both non-coding repetitive DNA and multiple copy genes or gene fragments.

STR analysis

STR markers consist of short, repetitive sequence elements, two to seven base pairs in length, which are well distributed throughout the human genome [8]. Several sets of autosomal STR markers have been selected for routine forensic casework analysis and are frequently used by the forensic community all over the world. For example, in the USA the Federal Bureau of Investigation (FBI) has selected 13 autosomal STR markers for typing. The use of a standard set of markers allows searching against convicted offender genetic profiles contained in their national database, the Combined DNA Index System (CODIS) [18]. The CODIS markers were chosen to overlap with the markers selected by the UK Forensic Science Services (FSS). The overlap of the markers selected by both the FBI and the FSS, facilitate international database searches with the help of organisations such as INTERPOL [19]. There are several commercial forensic typing kits available on the market, detecting these STR markers. A marker at the amelogenin locus is included in the commercial typing kits, allowing simultaneous sex determination of the donor of the samples.

Alleles at STR markers are differentiated by the occurrence of different numbers of repeat units contained within the amplified region. Primers for each STR marker are labelled with different fluorescent dyes that allow the PCR products to be detected after the fragments have been separated based on length. To allow multiplexing and simultaneous electrophoretic separation of multiple markers, the primers are designed to achieve non-overlapping allele size ranges (from approximately 100 to less than 500 base

pairs). An internal lane size standard is loaded together with each sample. For a more precise interpretation of the results, an allelic ladder containing the most common alleles for each of the STR markers is combined for each fluorophore dye and loaded into a separate gel lane or capillary. During electrophoretic separation, a laser beam causes the dyes to fluoresce and a CCD camera detects the signals at specific emission wavelengths. Genotypes are then assigned by comparing the allele sizes for the unknown samples with the sizes in the allelic ladder.

Discrimination power

When a DNA analysis has been performed on biological evidence material found on the scene of a crime, a comparison to a suspect is carried out. If the DNA profile of the evidence material does not match the DNA profile of the suspect it is an *exclusion*. In cases where the interpretation of the analysed markers fails, the results are *inconclusive*. This can be due to degraded or limited samples, contamination or technical problems during the analysis. However, if the DNA profile of the evidence material does match the suspect it is an *inclusion*, and the significance of the match has to be estimated.

Random match probability

Random match probability can be calculated from expected genotype frequencies based on individual allele frequencies in population databases, using Hardy-Weinberg and linkage equilibrium principles. Observed genotype frequencies are not possible to use since an incredibly large population database would be required to cover all possible genotype combinations. If the markers analysed are unlinked, for example autosomal STRs, *genotype identity by chance* can be calculated using the product rule by multiplying the frequencies for all genotypes observed in the DNA profile. Today the routinely used typing tests for forensic identification are based on 15 unlinked STR markers, together with the sex determination marker. The PowerPlex® 16 System from Promega reveals a random match probability in US Caucasians of about $1/1.83\times10^{17}$, while the AmpF*l*STR® Identifier® PCR Amplification Kit, from Applied Biosystems, reveals $1/2.00\times10^{17}$.

Population data and DNA databases

When interpreting the results of a forensic DNA analysis, the random match probability is calculated. Population data with allele frequencies for the routinely used markers are collected for different population groups (generally Caucasians, Africans, Americans, Hispanics and Asians). The population databases are statistically tested and rejected if the deviation from Hardy-Weinberg expectations is too high or linkage disequilibrium exists.

The population databases are used for discrimination power estimations and are compiled to contain only DNA profiles from anonymous donors of a specific population. On the other hand, specific DNA databases are compiled with DNA profiles of convicted offenders, allowing searches against DNA profiles obtained from crime scene evidence materials. For law enforcement, this permits the use of DNA analysis as an investigative tool in addition to providing evidence in criminal investigations with respect to a specific suspect. Obviously, the larger the database is, the higher is the probability that the searched DNA profile can lead to a match.

Optimisation towards more sensitive analysis

In order to be able to analyse valuable and limited DNA from evidence materials, forensic typing tests must have the capacity to be multiplexed. As the size of amplifiable DNA is reduced in degraded samples, the analysis is more likely to be successful using short fragments. To allow simultaneous gel separation of multiplexed STR loci, all the fragments need to differ in size. Some fragments also need to be longer as the STRs may contain long repeat units and a large allele spread or have other close repeat sequences preventing primers being located close to the repeat. Consequently, DNA typing systems used in the routine analysis are not optimal for the sensitive analysis of limited or degraded DNA samples.

Sensitive forensic DNA analysis

Due to its high copy number per cell, analysis of mtDNA allows greater sensitivity and has been used extensively in ancient DNA research and evolutionary studies. A drawback using of mtDNA analysis in forensic testing is the limited discrimination power, as well as the lack of the possibility of searching against profiles in national DNA databases of convicted offenders. Recently, a more sensitive autosomal STR analysis has been reported utilising an increased number of PCR cycles to facilitate analysis of more limited DNA samples. This analysis is referred to as LCN (low copy number).

Mitochondrial DNA

The role of mitochondria in the human cell is to generate energy by oxidative phosphorylation, the catabolic process that generates ATP by extracting energy from sugars, fats and other fuels with the help of oxygen. The mitochondria harbour their own genome, a double-stranded, circular DNA molecule of 16,569 base pairs [20]. The first complete mitochondrial genome sequence, referred to as the Cambridge reference sequence, was

reported in 1981 by Anderson et al. [20]. In 1999, Andrews et al. reported a modified version of the original sequence, the revised Cambridge reference sequence (rCRS), which is used as a universal mtDNA consensus sequence [21]. Each mitochondrion contains between two to ten copies of the mtDNA genome and each somatic cell can contain as many as 1,000 mitochondria [22]. In contrast to the diploid nuclear genome, the mitochondrial genome is a haploid molecule that is maternally inherited [23, 24]. Consequently, both males and females inherit their mtDNA from their mother. Therefore, maternal relatives can provide reference samples for forensic identification of, for example, the remains of missing persons.

Another difference between nuclear and mitochondrial DNA is that the mtDNA is not wrapped around histone proteins for protection. Also, due to the oxidative phosphorylation process, the mitochondria are exposed to high levels of mutagenic oxygen free radicals. Consequently, the mutation rate of mtDNA is higher than that of nDNA, in some regions approximately five to ten times [25, 26], and replication errors are often not repaired [27].

Routine mitochondrial DNA analysis

The most frequently used sequencing technology for all applications in molecular and medical genetics, is the Sanger sequencing by chain termination technology invented in 1977 [28]. A sequencing primer is hybridised to the single-stranded DNA template and a new strand is synthesised by a DNA polymerase. The DNA synthesis is carried out in the presence of dNTPs and base specific ddNTPs, which lack the 3' hydroxyl group. The ddNTPs are incorporated into the DNA chain and cause chain termination, generating DNA fragments of different lengths. Four base specific elongation reactions are run in parallel using a mix of all four dNTPs and a small proportion of one of the four ddNTPs. As the concentration of the ddNTP is much lower than that of its dNTP analogue, chain termination will occur randomly at all the positions of the template, containing the base in question.

In the past, the Sanger sequencing fragments were radioisotope labelled. Today, fluorescent DNA sequencing is used [29] with fluorophore dyes attached to either primers or ddNTPs. Length separation by electrophoresis is performed, similar to analysis of STR fragments. A laser beam causes the dyes to fluoresce during electrophoretic separation and a CCD camera detects the wavelengths of the fluorophores. If four different fluorophores are used for the base specific reactions, all reactions can be loaded and run in a single lane. Further, when the ddNTPs are labelled with different fluorophores, and not the primers, the extension reactions can be performed in a single tube instead of in parallel.

Mitochondrial versus nuclear DNA

Typing mtDNA instead of nDNA in forensic analysis is particularly beneficial because of the high copy number of mitochondrial molecules per cell. In addition, the high mutation rate of mtDNA has resulted in a high density of polymorphism, enabling detection of much variation by sequencing of relatively short fragments. However, this variation is lower than what is obtained by multiple nuclear STR markers, which reveal higher random match probabilities.

Since the mitochondrial genome is maternally inherited and does not undergo recombination, all mtDNA SNPs are linked and the haplotype is the same for all maternal relatives. Uniparental inheritance is a useful factor for forensic identification, but also for studies of human evolution. However, the same features that make mtDNA useful for forensic purposes also make up to the main disadvantageous characteristics. For example, the maternal inheritance means that any maternal relative to the suspect could potentially be the donor of the evidence material.

Control region versus coding region

Unlike the nuclear genome, the human mitochondrial genome is very compact and approximately 93% of the DNA represents coding sequence. The genes are tightly packed and the coding sequences of a number of genes even overlap. The mitochondrial genome encodes 13 polypeptides involved in oxidative phosphorylation, 22 transfer RNAs and two ribosomal RNAs [20]. The only region lacking any known coding sequence is the control region, or displacement (D) loop, approximately 1,100 base pairs in length and containing the heavy strand origin of replication. The mutation rate of the control region is faster than that of the coding region [30]. Consequently, most of the mtDNA variation is concentrated in the two hypervariable regions of the control region, HVI and HVII (each approximately 610 base pairs). Despite this, as the coding region is much larger than the control region, it contains a majority of the total mitochondrial variation.

As the mitochondrial genome is monoclonal and does not undergo recombination, the mitochondrial SNPs are linked and considered as a haplotype. Individual haplotypes have been classified into a number of haplogroups, based on the evolutionary relationship of the sequences. In Europe, 99% of the mtDNA sequences can be classified into ten haplogroups; H, T, U, V, W, M, X, I, J and K. H is the most common Caucasian haplogroup and is widely distributed throughout Europe [31].

Heteroplasmy and DNA mixtures

The mitochondrial genome is normally homoplasmic, where every mitochondrial genome within an individual has an identical DNA sequence. However, heteroplasmy can occur where two or more types of mtDNA are present within the same individual. Variation in mtDNA is often caused by the high mutation rate and heteroplasmy can be thought of as the intermediary state between two homoplasmic DNA types. It is also possible to inherit heteroplasmy, either at germ line level or at the level of somatic cell mitosis and the replication of mtDNA. Consequently, the frequency of heteroplasmy can differ between different tissues [32].

It is important to distinguish heteroplasmy from mixed samples with DNA from more than one individual. Samples displaying several positions with two variants are more likely to be DNA mixtures than heteroplasmic. According to guidelines for forensic mtDNA analysis, a single base pair difference, between an evidence sample and a suspect, is considered inconclusive [33]. Exclusion is only reported if two or more differences are detected. This is to minimize the risk for erroneous interpretation of the DNA typing results, due to heteroplasmy, sequencing errors or sporadic mutations. At the same time, heteroplasmy can increase the power of the analysis in a forensic investigation. The heteroplasmy adds an additional level of variation when the evidence is compared to a reference sample sharing the heteroplasmy [33, 34]. The most well known forensic example is the analysis of the Romanov family remains. The Tsar Nicholas II and his brother the Grand Duke of Russia, Georgij Romanov, both shared a heteroplasmic position [35, 36]. With Sanger sequencing it is not possible to quantify the exact mixture ratio for either heteroplasmy or DNA mixtures due to multiple contributors, therefore new sensitive methods for this purpose would be of great value.

Mitochondrial DNA diseases

Due to the high mutation rate of mtDNA, the mitochondrial genome contains a large number of non-synonymous substitutions, which can affect mitochondrial function and ultimately cause various maternally inherited diseases. The tissues most sensitive to mutations in the coding mtDNA are the central nervous system including the eye, the heart and skeletal muscle, renal system, endocrine system and liver [37]. These tissues are often affected in mitochondrial inherited diseases associated with a variety of neuromuscular symptoms such as blindness, deafness, dementia, movement disorders, cardiovascular disease, muscle degeneration, diabetes and renal failure [38]. For example, Leigh's syndrome [39], Parkinson's disease [40], Leber's hereditary optic neuropathy (LHON) [37], Alzheimer's disease [41] and diabetes [42] have all been associated with variation in mtDNA.

LCN analysis of nuclear DNA

A major advantage using nuclear STR typing instead of mtDNA analysis, besides the higher discrimination power, is the possibility to search for the STR profile in national DNA databases. However, a traditional STR analysis performed in routine forensic laboratories today requires at least 0.5 to 1 ng of genomic DNA. Amplification of less DNA has been termed low copy number (LCN) analysis. Gill et al. define LCN analysis as amplification of less than 100 pg of DNA [43] and Budowle et al. define LCN as DNA analysis with results below the stochastic threshold for normal interpretation [44]. LCN analysis can be performed using standard multiplex STR typing kits, increasing the number of PCR cycles from 28 to 34 for increased sensitivity [43, 45].

As the definition by Budowle et al. is emphasising, LCN analysis may lead to stochastic amplification, resulting in an imbalance of alleles for heterozygous markers or allelic dropout. Due to stochastic variation, any apparent homozygote is considered to be a potential heterozygote. Potentially there is also a higher risk for stutters (enzyme slippage producing DNA bands shorter than the true allelic peak) and a higher risk of laboratory-introduced contamination (allelic dropin) when analysing LCN samples [43, 46]. Therefore, the interpretation of LCN typing results is critical. To start with, according to Budowle et al., LCN typing should not be used to make exclusions because of potential high background levels of DNA and potential detection of DNA contamination [44]. For the same reasons, it is difficult to analyse mixed samples with LCN typing. When performing LCN analysis, the sample extract is diluted into aliquots, to allow multiple replicate analysis of the same evidence sample. Following result interpretation, an allele is reported only if positive in several replicates, creating a consensus profile of the sample extract [43]. Moreover, the statistical part of the interpretation has to be modified for LCN analysis [43, 46, 47].

Contamination

The risk of contamination has always been an important issue for forensic DNA analysis. Due to the high copy number per cell, mtDNA analysis is more prone to contamination than routine STR analysis. To facilitate the use of mtDNA typing, the forensic community has established guidelines for the performance and interpretation of the analysis. LCN typing is also sensitive to contamination and the same practices should be undertaken as for mtDNA analysis [46]. However, there is no risk of allelic dropout when analysing mtDNA and sequencing does not generate the stutter problem, as seen in STR analysis.

Guidelines

For forensic mtDNA analysis, all equipment, solutions and laboratory areas should, if possible, be exposed to UV light to be sure they are DNA-free. DNA from evidence materials should be extracted in duplicates on separate occasions whenever possible (if possible also by different persons) and analysed prior to the reference samples, preferably in separate rooms. The DNA samples should also be PCR amplified and sequenced in duplicate reactions to confirm the obtained sequence. Pre- and post PCR areas should be separated physically. Both reagent blanks and negative controls should be used for each extraction and amplification reaction. Finally, all laboratory personnel working with forensic analysis should be DNA typed and competency tested [33, 34, 48].

Technologies facilitating sensitive DNA analysis

DNA quantification

Traditionally, forensic DNA samples have been quantified by hybridisation with radioactively labelled or biotinylated human-DNA specific oligonucleotide probes [49, 50]. Recently, several quantification assays have been described for forensic purposes. The AluQuant™ system (Promega), for example, is based on a luciferase reaction [51]. However, these assays are not highly sensitive and are limited to detection of nDNA and thus not able to quantify mtDNA. With the introduction of the Real-time 5' exonuclease detection assay (TaqMan®) [52-55], new possibilities for DNA quantification have emerged.

TaqMan® probe assay

In the TaqMan® assay, a specific oligonucleotide probe is annealed to a target sequence located between two primer-binding sites (Figure 1). The probe is labelled with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end. Modification of the probe with a 3'-blocking phosphate prevents extension of the annealed probe during amplification. Cleavage of the probe by the 5'-3' exonuclease activity of Taq polymerase during strand elongation releases the reporter from the probe, the proximity to the 3' quencher is lost and the increase in reporter emission intensity is detected by a CCD camera. The sample emission is corrected for fluorescence fluctuations (Equations 1 and 2) and the difference is calculated using the equation $\Delta R_n = (R_n^+) - (R_n^-)$.

$$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Quencher}} \quad \textit{PCR with template} \qquad (1)$$

$$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Quencher}} \quad \textit{PCR without template} \quad (2)$$

The cycle at which the emission intensity of the sample rises above baseline and a specific threshold, is referred to as threshold cycle (C_t) and is inversely proportional to the target concentration [56]. The higher the target concentration is, the lower is the number of amplification cycles required to detect the rise in reporter emission above baseline (Figure 1).

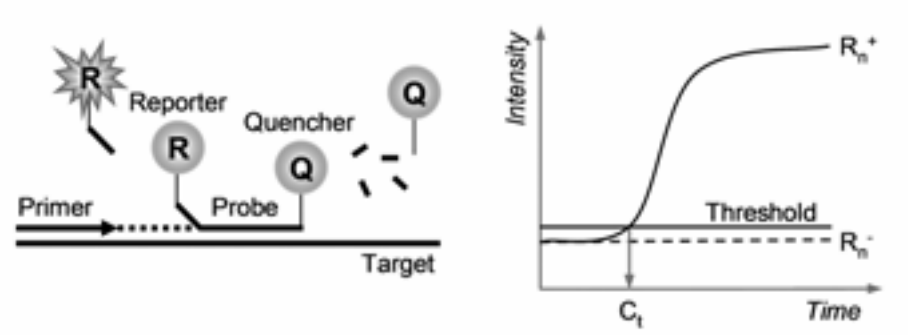

Figure 1. Schematic illustration of the Real-time 5' exonuclease detection assay (TaqMan®). Strand elongation results in cleavage of a dually labelled probe and increased reporter emission intensity. Light proportional to amplification is detected by the instrument and illustrated as a curve in a diagram.

Standard curves and absolute quantification

With the TaqMan® probe assay it is possible to perform absolute quantification if the absolute quantity of a standard is known. There are several important aspects to have in mind for the use of absolute standard curves. The target must be a single copy gene, pipetting must be precise since the standards are serially diluted, and finally, the standards should be stored as aliquots at -80°C [57].

Multiplexing

With DNA quantification based on the TaqMan® probe assay, it is possible to multiplex the analysis of several targets using fluorescent dyes with large differences in emission wavelength. The optimal multiplex assay detects targets of approximately equal quantity. Consequently, multiplex nuclear and mitochondrial DNA quantification is not optimal. In a multiplex PCR contains targets of different quantity, the primer concentration for the majority target (e.g. mtDNA) has to be limited to achieve equal amplification efficiencies and maintain the threshold cycles for both targets. The amplification of the majority target will therefore be stopped before it

limits the amplification of the minority target (e.g. nDNA). The best compromise is found when the chosen primer concentrations yield lower ΔR_n (the amplification plateau) than for single reactions, without affecting the C_t value.

SYBR® Green assay

In this assay, a double-stranded DNA intercalating dye, SYBR® Green, is used. The PCR product is detected by an increase in fluorescence caused by interaction with SYBR® Green. As for the TaqMan® probe assay, amplification of higher target concentrations requires fewer cycles to yield a rise in emission above the baseline which can be detected by the instrument. Quantification is possible by extrapolation of DNA quantities in unknown samples according to a standard curve. With the SYBR® Green assay, it is possible to perform a dissociation curve analysis. After Real-time detection, the amplicon is slowly heated up and the SYBR® Green emission is detected while the temperature increases. When the melting point for the amplicon is reached, the emission will drop in intensity, indicating that the double strands of the DNA amplicon have been separated. Since amplicons differing in only one or a few base pairs have different melting temperatures this can, for example, be used for genotyping or detection of insertions and deletions in the same assay as DNA quantification.

Pyrosequencing technology

Pyrosequencing is a novel sequencing-by-synthesis method that is non-electrophoretic and does not require dyes or specific labels [58-61]. It is performed in a single-tube format, in which a cascade of enzymatic reactions enables nucleotide incorporation and pyrophosphate (PPi) release to yield detectable light (Figure 2). The light is produced after enzymatic conversion of PPi, which is released after nucleotide incorporation. The nucleotides are added one at a time in a known order, cyclic or directed. If a nucleotide is not incorporated, it will be degraded by the enzyme apyrase. The produced light is detected in real-time, using a CCD camera and the amount of released PPi, and therefore the light intensity, is proportional to the number of incorporated nucleotides. The light emission is shown as a peak in a pyrogram, where the peaks represent each nucleotide in the template sequence (Figure 2). When the sequence contains two (or more) identical nucleotides consecutively, they will result in a double peak (or in a proportionally higher peak).

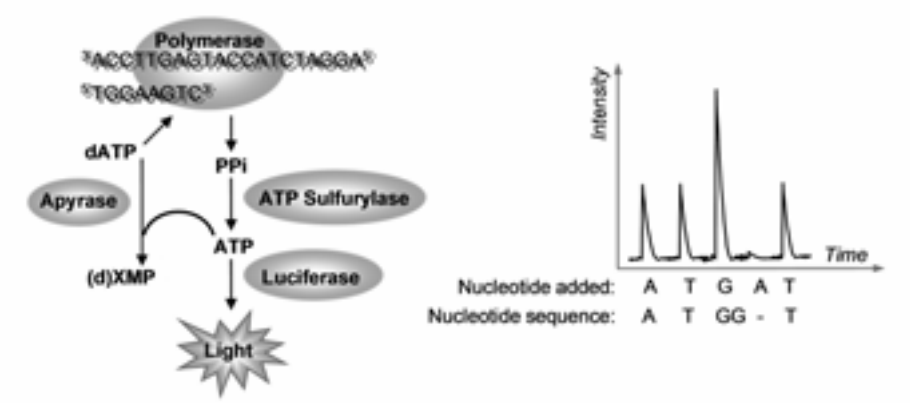

Figure 2. Schematic illustration of Pyrosequencing, a sequencing-by-synthesis technology. An enzyme cascade produces light proportional to nucleotide incorporation. The light is detected by the instrument and illustrated as peaks in a pyrogram.

Quantification

The proportional relationship between incorporated nucleotides and released light enables Pyrosequencing to be used for quantification [62]. Consequently, allelic contribution or SNP-variant proportions of pooled samples or DNA mixtures can be calculated from relative peak heights (Equation 3). Due to the approximately 20% higher peak of incorporated A nucleotides, correction according to homozygous or unmixed samples must be performed for certain reactions.

$$\%\ \text{SNP variant}\ 1 = \frac{\text{Peak height 1}}{\left(\text{Peak height 1} + \text{Peak height 2}\right)} \times 100 \qquad (3)$$

Read lengths

The read lengths possible to obtain using Pyrosequencing are much more limited than Sanger sequencing. However, in contrast to Sanger sequencing, Pyrosequencing can obtain readable sequence data from the first base after the primer. The read lengths can be increased by several different strategies. Single-stranded DNA Binding Protein® (SSB) can be added to the primed template prior to Pyrosequencing to increase the read lengths and to reduce the background signals [63]. When the sequence is known, except for the possible variable positions, the dispensation order can be entered according to that sequence. This will decrease the number of nucleotide additions not leading to incorporation and increase the additions leading to actual extension of the template, and consequently extend the sequence readout. If directed dispensation is used, non-extended nucleotides must be added after

homopolymeric regions to limit the occurrence of shifts in the sequence readout.

Until recently, the maximum read length reported was 100 nucleotides [64]. However, further developments, such as the use of a new dATP analog (c^7dATP) and an alternative DNA polymerase (Sequenase), have facilitated read lengths of 140 bases [65, 66]. Pyrosequencing at higher temperatures (37°C), using glycine betain as stabiliser of the sensitive luciferase enzyme, has also been reported [67]. In a recent version of the Pyrosequencing reagents (Pyro Gold SQA Reagents) SSB is included, prolonging the read lengths.

Ethical aspects

Several different ethical issues have to be considered when working with forensic DNA analysis. The history of forensic genetics is short and questions regarding forensic databases have been raised. There is a fear for misuse of individual DNA information by insurance companies and employers through access to the information in the databases. Can we be sure that these databases contain solely information for identification and not information about, for example, disease predisposition? When it comes to the issue of storing DNA profiles in databases, it can be discussed whether the database should cover all individuals convicted for any recordable offence (e.g. shoplifting), as is done in the UK, or if it should be limited to offenders convicted for severe or violent crimes, as in the USA and most other countries. A larger DNA database would facilitate identification of recurrent offenders and prevent the tendency to progress to more severe crimes. On the other hand, for reasons of integrity, it is very important to weight what is desirable from the forensic point of view against what could be acceptable with regards to the interest and protection of the rights of the individual.

Due to the risk of typing disease-associated variants, it is very important to develop forensic identification assays with caution. Today, both the nuclear STR markers and the mitochondrial SNP typing kits only detect variation in non-coding regions. As mentioned previously, variation in mtDNA can cause a variety of different diseases. It has therefore been suggested that only synonymous variants should be used in forensic mtDNA analysis. However, Parsons et al. argue that in particular cases, sites that theoretically could be disease related but are known to segregate widely in the population, can be considered for forensic identification [68].

Present investigation

Aim

The overall aim of the papers presented in this thesis is to evaluate the use of novel DNA technologies and to develop specific applications for use in forensic investigations. The need for more sensitive, faster and more discriminating systems is essential and has been the driving force for all six papers. In the first three papers, Real-time PCR was developed and used for quantification of the amount of nuclear and mitochondrial DNA in different forensic evidence materials. The following three papers describe mtDNA control region and coding region sequencing, based on Pyrosequencing technology. Two of the papers, III and VI, illustrate alternative applications of the two technologies, sex determination and mixture quantification, also very important issues in casework investigations.

Paper I

Background

The amount of DNA extracted from evidence materials in a forensic investigation is critical for subsequent genetic analysis. Until recently, no sensitive Real-time PCR quantification assay was available for use in routine forensic analysis. For cases where mtDNA had to be analysed due to limited amounts of nDNA, a quantification method was not developed at all. This paper describes the development of a nuclear and mitochondrial quantification system based on the Real-time PCR TaqMan® assay.

Results and discussion

The quantification system was developed to detect both nDNA and mtDNA in any given evidence material. For absolute quantification, serial dilutions of control DNA were used as standard curves for extrapolation of DNA amounts in unknown samples. This application was proven to be very sensitive with accurate quantification down to single copies of DNA, even though the limit was set to 10 copies. This high sensitivity meets the requirements of detection levels for all the different methods used in routine

analysis today; autosomal STR and SNP analysis, LCN analysis and mtDNA analysis. Also, the reproducibility was proven to be high, with average standard deviations of the standard series ranging from 0.14 to 0.21 for nDNA and between 0.60 and 0.67 for mtDNA. The C_t difference between the samples in the ten-fold dilution series (approximately 3.3 cycles for nDNA and 3.6 for mtDNA) illustrates an optimal slope of the standard curves and hence close to 100% amplification efficiency (Table 1).

Table 1. Nuclear and mitochondrial DNA quantification of standard serial dilutions with known concentrations.

	C_t Value / nDNA copies				C_t Value / mtDNA copies			
Run	10^4	10^3	10^2	ΔC_t	10^5	10^4	10^3	ΔC_t
1	25.82	28.91	32.54	3.36	23.19	26.89	30.22	3.51
2	25.56	28.88	32.10	3.27	23.35	26.93	30.46	3.56
3	25.58	29.03	32.37	3.40	23.33	26.95	30.57	3.62
4	25.51	28.76	32.07	3.28	23.49	27.16	30.08	3.30
5	25.56	28.83	31.96	3.20	22.28	26.04	29.70	3.71
6	25.34	28.83	31.94	3.30	22.45	26.33	30.08	3.82
7	25.44	28.67	32.15	3.35	22.10	25.73	29.46	3.68
8	25.21	28.58	31.88	3.34	22.15	25.74	29.54	3.70
9	25.16	28.60	32.02	3.43	23.69	27.34	31.03	3.67
10	25.48	28.78	31.91	3.21	23.81	27.29	31.27	3.73
Average	25.47	28.78	32.09	3.31	22.98	26.64	30.24	3.63
SD	0.19	0.14	0.21	0.08	0.67	0.63	0.60	0.15

Furthermore, the assay was tested on evidence materials that had previously been Sanger sequenced in casework investigations for the mitochondrial HVI and II region. The samples were divided in six different categories; hairs, bloodstains, fingerprints, skin debris, saliva stains and others. In total, 236 samples were analysed for their mtDNA content and 154 were nDNA determined, with approximately half of the samples showing less than 10,000 mtDNA copies or less than 1,000 nDNA copies. In an evaluation of samples with known HVII sequence or DRB1 data, the limit for successful results using these markers was determined to be approximately 80 mtDNA or 200-300 nDNA copies, respectively.

The Real-time PCR quantification assay was also designed for multiplexed analysis for the simultaneous estimation of nuclear and mitochondrial DNA content. The assay is performed in single tube reactions, minimizing the risk of laboratory-induced contamination, and saving valuable forensic evidence material. Moreover, casework materials often contain inhibitors that substantially reduce the PCR efficiency. The TaqMan® quantification assay was shown to produce a somewhat altered shape of the amplification curves for samples containing inhibitors. With addition of BSA (0.16 mg/ml)

however, these samples showed normal amplification curves with amplification efficiencies similar to other samples.

In conclusion, the sensitive quantification system described in this paper was the first Real-time PCR assay developed for quantification of forensic samples and has since been followed by many similar assays [69-74]. The Real-time PCR format makes the assay very easy to perform and provides the opportunity of single or multiplex detection of nuclear and mitochondrial DNA copies for use in casework investigations. The quantification is very fast and facilitates the choice of the optimal method for subsequent genetic analysis (nuclear or mitochondrial DNA), to achieve as high discrimination power as possible for the specific material. The choice of the optimal DNA amount to use for successful analysis, but without the risk of allelic dropouts and preferential amplification, is also very important.

Paper II

Background

Information on the expected DNA content of different types of commonly found evidence materials would be of great importance in routine forensic analysis. Until now, no extensive quantification study has been presented. Paper II describes a material evaluation based on the quantification system presented in Paper I. Both nuclear and mitochondrial DNA amounts have been determined in a variety of different types of evidence materials.

Results and discussion

Determination of DNA content was performed on samples of epithelial cells collected from various accessories, such as rings, watches and necklaces as well as on fingerprints visualised by two different detection methods. A detailed study of human hair, one of the most commonly found evidence materials at a crime scene, was also carried out. The hairs were cut and extracted in 3 cm pieces, beginning one cm from the root.

The analysis illustrated large differences in DNA content between plucked and shed head hairs. A 74-fold difference in the average mtDNA content was seen within the first cm of the hairs, possibly explained by the hairs different growth phases. In addition, large differences in copy number were noted within hairs, evaluated at different lengths. Shed hairs showed a 2-fold reduction in mtDNA copies per cm between the first and the second piece, while the difference in plucked hairs was 80-fold. There is a large overall inter-individual variation in the first cm of hairs, illustrated by a 1 and 2

order(s) of magnitude variation, for shed and plucked hairs respectively. Intra-individual variation was also evaluated and resulted in somewhat less variation compared to the inter-individual variation. In quantification of nDNA, no DNA was detected in shed hairs. However, 95% of all plucked hairs were determined to contain enough DNA for successful STR typing, according to the kit manufacturers (Applied Biosystems, Promega) requirement of approximately 170 copies or more. Of the shed hairs, the majority contained more than 1,000 mtDNA copies, sufficient for mtDNA sequence analysis.

Differences could also be detected between the two different fingerprint visualisation methods, with the prints visualised with black powder containing somewhat more DNA than the magnetic powder treated prints. Almost all prints contained sufficient DNA amounts for successful mtDNA analysis. Also, variation in DNA content between the different categories of accessories was apparent. For example, the highest DNA amounts among accessories were seen in samples collected from earrings. In 70% of all samples from accessories, sufficient amounts for successful STR analysis were detected. Large variation was also seen between samples of the same accessory category.

This paper describes a unique quantification study of DNA extracted from different materials commonly found at crime scenes. The analysis was performed utilising the fast and sensitive Real-time PCR technology. Valuable information, regarding the typical DNA content of various materials and inter- and intra-individual variation, were provided in this study. This information can be of importance for future development of new sensitive assays for use in forensic DNA investigations.

Paper III

Background

Sex determination of the donor of a sample can be important as an investigative tool in casework analysis. Today, sex determination is not always possible to perform using standard typing technologies. Although the routinely used STR-typing kits include a sex-determining marker, analysis of degraded or limited DNA might not be possible. In Paper III we present an assay for quantification of nDNA in combination with sex determination, based on another Real-time PCR assay that utilises the intercalation of the SYBR® Green dye to double-stranded DNA. Using this dye, detection of multiple targets is not possible but a melting curve analysis can be performed in addition to the quantification. The dissociation curve analysis

is used here to determine sex by analysis of a 3 base pair deletion on the X-chromosome [75, 76]. The assay is designed to amplify a short target of 73 base pairs that enables sex determination of evidence materials with limited DNA amounts, where routine analysis fails.

Results and discussion

For the quantification part of the system, standard curves were constructed of control DNA diluted from 10,000 copies down to single copies. A linear relationship was found down to 10 DNA copies and the reproducibility fell within expected ranges. To evaluate the system on limited samples, 40 actual casework materials were tested. Furthermore, an overall correlation for both control samples and evidence materials was seen compared to quantification with the TaqMan® probe assay described in Paper I.

Sex determination was successfully tested on 23 randomly picked control samples with known gender. The evidence materials were also successfully analysed using the SYBR® Green assay. Forensic samples, however, often contain mixtures with a DNA contribution from more than one individual. Therefore, male control DNA was mixed with female control DNA in different ratios and evaluated by the melting curve analysis. The mixtures showed proportional differences in melting curve patterns but, as exact ratios were not possible to define using this assay identification, rather than quantification, of mixtures is obtained. This system will enable DNA quantification and sex determination of more limited samples where routine analysis cannot be performed. Therefore, information that is valuable for casework investigations will be obtained that has previously not been possible on these samples.

Paper IV

Background

Mitochondrial DNA analysis of evidence materials is performed in a forensic investigation when the extracted DNA amounts are too limited for an nDNA analysis [13]. Analysis of mtDNA has mainly been performed by Sanger sequencing of the two hypervariable regions (HVI and HVII) in the mitochondrial control region, or D-loop. Sanger sequencing of mtDNA is very sensitive due to the high copy number per cell, but also time consuming to perform. The main drawback with mtDNA analysis compared to nDNA analysis is the limited discrimination power. Paper IV describes an alternative mtDNA assay for forensic analysis based on Pyrosequencing of 13 PCR fragments covering highly informative regions of the entire

mitochondrial genome. The mtDNA sequencing is performed using 19 separate Pyrosequencing reactions, 4 HVI, 4 HVII and 11 coding region reactions (Figure 3).

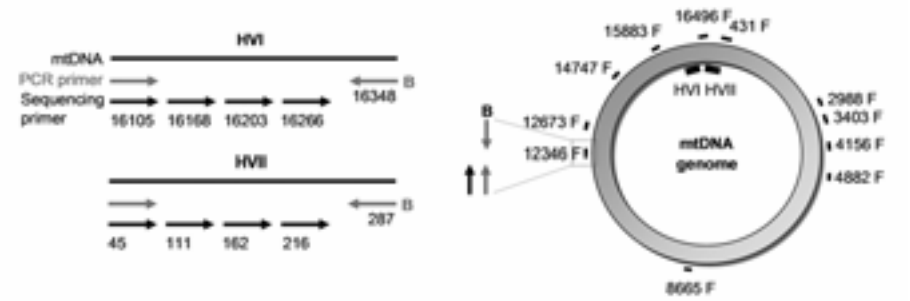

Figure 3. Schematic illustration of the fragments for the mtDNA control region and coding region assays. Both PCR and Pyrosequencing primer locations are indicated.

Results and discussion

D-loop analysis was performed on 190 and 120 previously Sanger sequenced HVII and HVI control samples, respectively. The Pyrosequencing results were therefore confirmed with previous sequencing data. The average HVII fragment read length was more than 30 nucleotides before manual editing and over 50 nucleotides after editing. For HVI, the average read length was, after the addition of SSB, up to 27 nucleotides before editing and 50 nucleotides after editing. The large difference before and after editing is due to incorrect base calling by the software when scoring homopolymeric peaks. Overall, the longest read length was 71 nucleotides and the shortest was 45. To compare the coverage of this Pyrosequencing system with Sanger sequencing, the fragments were aligned to the rCRS. For HVII, 95% was covered and for HVI, 82% coverage was obtained.

The coding region analysis was tested on 36 control samples and, as with the D-loop fragments, revealed identical results compared to Sanger sequencing. For the coding region fragments, the average read length was 25 before manual editing and over 50 after editing. The longest read length was 67 nucleotides and the shortest was 31. When aligning the coding region fragments to the rCRS, a total of 20 different SNPs were detected among the 36 individuals, ranging between zero and four SNPs per fragment. Finally, 50 forensic evidence materials were successfully analysed. These samples were from different sources and included material recovered from robber masks, wigs, a fake moustache, shoes, mobile phones, watches, knives, guns, fingerprints, old letters and paraffin-embedded tissues (Figure 4).

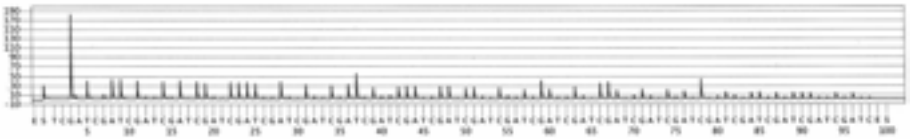

Figure 4. Pyrosequencing of DNA extracted from a paraffin-embedded tissue with primer II45F and cyclic dispensation of nucleotides.

The Pyrosequencing application developed in this paper was the first assay for use in forensic investigations that has been described for the analysis of coding region sites as a complement to D-loop analysis. The use of shorter PCR fragments will enhance the sensitivity even further in analysis of evidence materials with limited or degraded DNA. In conclusion, this fast and sensitive mtDNA sequencing system has the potential for higher discrimination power.

Paper V

Background

Extended routine analysis of mitochondrial control regions would increase discrimination through determination of additional polymorphic sites. However, most agree that sequencing the entire genome would not be possible, due to the time consuming nature of Sanger sequencing and the limited amounts of DNA in forensic evidence samples. Paper V involves optimisation of the Pyrosequencing system described in Paper IV, including additional coding regions suitable for forensic mtDNA analysis (Figure 5). A larger database was also compiled and the detected SNPs were evaluated for their discriminatory capability.

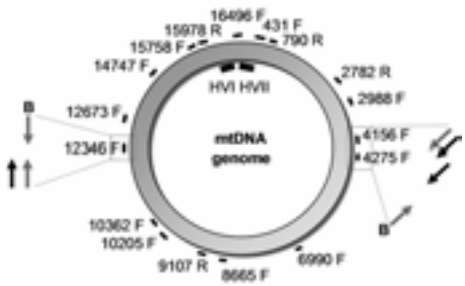

Figure 5. Schematic illustration of the optimised and complete mtDNA coding region assay. Pyrosequencing primer locations are indicated.

Results and discussion

Pyrosequencing analysis was performed on 15 PCR fragments from the DNA of 60 individuals with zero or one difference from the rCRS in HVI/II (0/1 individuals), as well as on 75 individuals with at least 2 differences compared to the rCRS (+2 individuals). The 15 fragments were successfully analysed in 17 Pyrosequencing reactions and aligned to the rCRS. The read lengths per fragment varied between 20 and 122 nucleotides, with an average of 83 nucleotides. Since directed nucleotide dispensation was used, an average of 1.2 nucleotides could be read for every nucleotide dispensation. In total, 20 variable positions were detected among the 0/1 individuals and 43 variable positions were detected among the +2 individuals. Altogether, 52 SNPs, with frequencies ranging between 1% and 47%, were detected, of which 18 were singletons. Out of the 60 0/1 individuals, only 12 could not be resolved by at least 2 differences when adding the coding region information.

To illustrate how the coding region SNPs can be used to resolve the DNA types of different individuals sharing the same HVI/II variations, an analysis of samples in a cold case investigation was carried out. The case involves letters sent in 1952 displaying only a single D-loop difference between evidentiary and reference samples. In the Pyrosequencing coding fragment analysis, an additional three positions were detected between the samples from the evidence letters and official reference letters, concluding that the suspect did not seal the letters (Table 2). An example of a pyrogram can be seen in figure 6.

Table 2. Pyrosequencing results in an mtDNA coding region analysis of samples from four evidence letters and four official reference letters from 1952.

Sample	HVI 16239	NC[a] 16519	C[b] 3010	C[b] 7028
rCRS	G	C	C	T
Evidence letters	A	C	T	C
Reference letters	G	T	C	T

[a] NC denotes noncoding. [b] C denotes coding.

Figure 6. Pyrosequencing of DNA extracted from an old letter with primer C9107R and directed dispensation of nucleotides.

The Pyrosequencing assay described in this paper can be used as either a pre-screening test for selection of informative samples prior to sequencing or a complete analysis tool replacing Sanger sequencing. The coding region

information facilitates higher discrimination power for mtDNA analysis, illustrated by an increase in the number of individuals with exclusive mtDNA types. Compared to other coding region assays available today [77, 78], this system detects all variation in selected regions, including very rare SNPs, with the potential to substantially increase the power of discrimination in specific cases.

Paper VI

Background

Forensic evidence samples commonly contain a mixture of DNA from several contributors [79]. This can be due to a mixture of biological fluids during the criminal event (e.g. rape case samples contain a mixture of seminal and vaginal fluids), or contamination introduced later. In routine STR analysis, the specific ratios of DNA from multiple contributors can be quantified [80]. However, in analysis of SNP markers and mtDNA sequences, quantification of mixture ratios is impossible due to the nature of the used technologies. Consequently, no optimised quantification system for mixture analysis based on these markers is available today [81, 82]. In this paper, a system for mtDNA mixture quantification using Pyrosequencing technology is described. Pyrosequencing has previously been used to sequence short stretches of mtDNA [83] and has recently been used in a number of quantitative studies [84-89]. The sequencing is based on light production that is proportional to the amounts of nucleotides incorporated and will therefore allow quantification.

Results and discussion

Based on the system described in Paper IV and V, five fragments were selected for analysis of seven different variable sites to allow for mtDNA mixture quantification. The sites were selected based on the frequency of the minor variant, ranging from 14 to 56% among Caucasians, and haplogroup property. Three positions in the mtDNA control region are covered and four positions are located in the coding region. Mixture series were constructed of control DNA in known proportions, one for the control region and one for the coding region SNPs. The variable sites were tested and the result shows a linear relationship between measured and expected mixture ratios for all variable sites. All positions except one have linear regressions with regression coefficients ranging from 0.89x to 0.99x and intercepts between 0.0 and 7.4. SNP 73 shows a regression coefficient of 0.86x and an intercept of 15.3, for which a correction were made to extrapolate mixture ratios in unknown samples. The R^2 values for all variable sites were close to one, 0.98 or 0.99.

The quality of the control DNA standard mixtures, as well as quantification of individual SNPs, was evaluated and standard deviation values were calculated. Overall, the average standard deviation per SNP varies between 1.2 and 1.7. According to the manufacturer (Biotage AB), standard deviations in optimal assays, designed according to their recommendations, are expected to be approximately 1-2%. Although, due to the combined forensic application, our assay is developed on the same fragments as used for mtDNA sequencing, standard deviation values within the expected range were obtained. Pyrosequencing quantification was finally tested on a number of evidence materials known to be mixtures by previous D-loop Sanger sequencing (Figure 7).

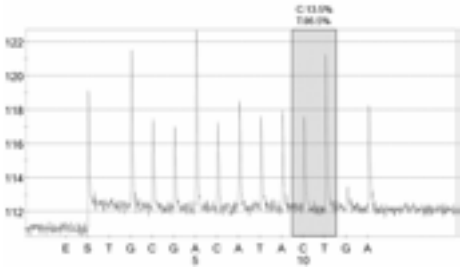

Figure 7. Pyrosequencing quantification of a forensic sample with DNA extracted from a piece of tape found at the crime scene. The control region SNP position 73 reveals, after correction to the standard curve, 84 % T (95% CI, 76-92) and 16 % C (95% CI, 8-24) contribution to this mixture. Interestingly, the suspect showed a T and the victim a C in the same position.

In conclusion, this Pyrosequencing based system is a novel method for fast and simple quantification of mtDNA contribution in evidence materials with mixed profiles from multiple contributors or of heteroplasmic origin. Although Sanger sequencing allows detection of mixtures, no ratio quantification can be performed. Pyrosequencing is therefore superior to Sanger sequencing in the sense that quantification is allowed. The assay design is very flexible and here we showed how previously designed PCR fragments, for other Pyrosequencing applications, could also be utilised as templates for quantification with only minimal optimisation.

Concluding remarks

Development and optimisation of sensitive forensic DNA analysis methods is highly important. DNA analysis is commonly used as a valuable tool by law enforcement in casework investigations, as a complement to traditional criminalistic tools. A more sensitive typing system will allow a wider range of evidence materials to be analysed, for example limited and degraded samples as well as cold case samples or even ancient DNA samples. DNA analysis provides very useful evidence in court trials. As prosecutors reapply for extended custody periods every second week, a faster, more sensitive DNA analysis would be a significant advantage. An mtDNA analysis system revealing a higher power of discrimination than previously possible would also enhance the significance of the evidence in casework investigations and the following court trials.

In this thesis, a sensitive system for quantification of nuclear and mitochondrial DNA amounts extracted from different types of evidence materials was developed and evaluated. This system allows fast and easy determination of optimal DNA amounts for use in subsequent identification analysis to allow a successful analysis without dropouts or preferential amplification. In a forensic investigation, it is important that the genetic analysis gives high discrimination power. It is also important not to waste limited or degraded DNA on an nDNA typing method that will be ultimately unsuccessful if an mtDNA analysis would have been successful. Similarly, an mtDNA analysis should not be performed if a more discriminating nDNA analysis will be successful. The development of a novel Pyrosequencing system for the analysis of short stretches of DNA in the mitochondrial control and coding regions is also included in this thesis. The short PCR fragments facilitate sensitive analysis and the coding region fragments increase the discrimination power for mtDNA analysis, compared to D-loop analysis routinely performed today. With this system, all variation within the selected regions is detected, including rare SNPs, increasing the power of discrimination even further. In addition to sequence information for individual identification, a number of other features of a DNA assay can be of importance and useful for casework in law enforcement. In this thesis, a system enabling sex determination of limited samples, which cannot be analysed with a routine method, is described. This additional information can be of great value during the first critical days of an investigation. Finally,

a Pyrosequencing-based system for fast and simple quantification of mtDNA mixtures, with contribution from more than one individual, was developed and described. No mixture ratio quantification method was previously available for SNP markers and mtDNA and hence these samples were not interpreted. The assay design was proven to be very flexible and other positions could easily be added and quantified if required.

The continued development and optimisation of novel sensitive forensic DNA analysis methods in the future is highly important. The still growing sequence databases reveal new information that will provide new markers that can be used in forensic analysis. Of special interest is SNP variation that can be analysed in very short fragments with high sensitivity. The growing awareness among perpetrators of the possibilities of DNA analysis puts even greater requirements of sensitivity on future identification assays, as attempts to destroy DNA evidence are common. As the DNA methods become more sensitive, and as new markers are developed and new types of materials can be analysed, the guidelines and recommendations developed by the forensic community need to evolve with them.

In conclusion, the field of medical genetics is growing fast and so is the development of new DNA analysis tools for use in forensic investigations. The assays developed and described in this thesis provide a fast and sensitive mtDNA analysis, as well as an increased discrimination power. These assays are of great importance for casework analysis, as discriminatory DNA analysis of challenging samples previously not possible to analyse, can be performed quickly. A number of social benefits for the public can also be foreseen by a broader use of DNA analysis. For example, the wrongfully convicted can be exonerated, innocent suspects can be excluded and severe violence crime offenders can be prosecuted faster than today, resulting in a higher degree of legal security.

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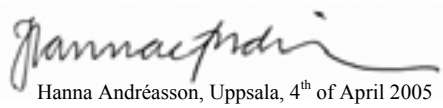
Lena, Lars, Karl, Gustav
Mamma, Pappa

PETER♥



Hanna Andréasson, Uppsala, 4th of April 2005

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