DNA Analysis of PCR-Inhibitory Forensic Samples

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Till Maria

Populärvetenskaplig sammanfattning

I Sverige analyseras omkring 40 000 DNA-prover från brottsplatser varje år. DNA-profilerna jämförs mot DNA-profiler från misstänkta personer och kan på så vis användas för att knyta en person till ett brott. Alla typer av kroppsvävnader kan analyseras, såsom blod, saliv och hudceller. Vanliga bevismaterial är cigarettfimpar, flaskor, burkar och kläder. De material som cellerna sitter på kan störa DNA-analysen, PCR, och därigenom försämra DNA-profilens kvalitet.

I november 2010 modifierades DNA-analysen av brottsplatsprover vid Statens kriminaltekniska Laboratorium. Det enzym, DNA-polymeras, som dittills varit standard i Sverige och i resten av världen ersattes av en polymerasblandning som jag designat och prövat ut i detta doktorandprojekt. Den nya metoden gav kompletta DNA-profiler från ett ökat antal smutsiga prover med lite DNA. För saliv höjdes andelen från 38% till 87%, och för blod från 69% till 94%.

I inledande studier visade jag att de två polymeraserna som ingår i blandningen är mer robusta mot smuts jämfört med standardanalysen. De uppvisade också skillnader sinsemellan, exempelvis fungerade det ena bättre för snusprover medan det andra fungerade bättre för tuggummi och cigarettfimpar. Blandningen blev ett sätt att utnyttja dessa komplementära egenskaper till att skapa en mer generell metod. Förutom komplementaritet så uppvisade de två enzymerna synergi: de förstärkte varandras goda egenskaper.

Resultatet från en kriminalteknisk DNA-analys är ett komplext diagram, där toppar representerar DNA-profilen. Topparnas höjd och balans visar hur väl analysen fungerat. För att kunna jämföra prestandan mellan olika analysmetoder behöver man kvantifiera DNA-profilens kvalitet. I detta syfte utvecklade jag en matematisk modell som omvandlar analysdiagram till enskilda kvalitetsvärden. Modellen användes sedan för att bevisa synergin mellan de två DNA-polymeraserna i blandningen.

Saliv är vanligt förekommande på brottsplatser och kan säkras från exempelvis flaskor och burkar. Eftersom salivspår ofta är osynliga för blotta ögat ger många prover som förväntas innehålla saliv negativa DNA-analysresultat. För att minska andelen negativa prover utvecklade jag en enkel metod för att testa provtagningstops för närvaro av saliv. Metoden gav utslag ner till 0.5 µL torkad saliv, och kan användas som urvalsverktyg i exempelvis utredningar av inbrott. Testet kan utföras direkt på brottsplats utan laboratorieutrustning.

Abstract

DNA evidence, linking perpetrators to crime scenes, is central to many legal proceedings. However, crime scene samples often contain extraneous substances that may interfere with the PCR-based forensic analysis, resulting in partial or negative DNA profiles. Extensive DNA purification may remove inhibitors, but involves the risk of DNA loss. In this work, pre-PCR processing was applied to improve the success rate of forensic DNA analysis of "dirty" samples without interfering with the composition of the samples.

An experimental model system was developed to screen for inhibitor-tolerant DNA polymerase-buffer systems. The best-performing polymerases, Bio-X-Act Short, Ex*Taq* HS and PicoMaxx HF, were applied in STR DNA analysis of PCR-inhibitory crime scenes samples, i.e. samples that failed to produce complete DNA profiles in routine casework despite containing acceptable levels of DNA. A ranking index, called the forensic DNA profile index (FI), was developed to quantitatively describe DNA profile quality. The application of analysis of variance (ANOVA) to FI values confirmed that the three alternative polymerases significantly improved DNA profile quality for 20 of 32 problematic samples, compared with the standard polymerase Ampli*Taq* Gold.

Ex*Taq* HS and PicoMaxx HF showed complementary inhibitor-relieving properties. A blend of the two polymerases exhibited tolerance to a broader range of extraneous compounds, improving DNA profile quality in 34 of 42 PCR-inhibitory forensic samples. When used separately, Ex*Taq* HS and PicoMaxx HF improved the results of analysis for 26 and 23 samples, respectively. Apart from their complementarity, synergy between the polymerases was mathematically proven by calculating the geometric mean values of FI and applying ANOVA.

In November, 2010, the customised DNA polymerase blend was introduced in routine casework at the Swedish National Laboratory of Forensic Science, increasing the proportion of complete DNA profiles generated from impure samples from 38% to 87% for saliva, and from 69% to 94% for blood.

Many presumed saliva crime scene stains give negative DNA results if presumptive testing is not performed. In this work, amylase activity testing was evaluated as a tool for saliva screening. No direct correlation was found between amylase activity and the DNA content of saliva. However, the sensitivity of the developed swab screening procedure (positive results for 0.5 µL of dried saliva) makes it applicable in cases where the number of DNA analyses is limited due to cost.

List of publications

This thesis is based on the following papers:

I. **Evaluation of amylase testing as a tool for saliva screening of crime scene trace swabs**
Hedman, J., Dalin, E., Rasmusson, B., Ansell, R. 2011. Forensic Science International: Genetics 5:194-198

II. **Improved forensic DNA analysis through the use of alternative DNA polymerases and statistical modeling of DNA profiles**
Hedman, J., Nordgaard, A., Rasmusson, B., Ansell, R., Rådström, P. 2009. BioTechniques 47:951-958

III. **A ranking index for quality assessment of forensic DNA profiles**
Hedman, J., Ansell, R., Nordgaard, A. 2010. BMC Research Notes 3:290

IV. **Synergy between DNA polymerases increases polymerase chain reaction inhibitor tolerance in forensic DNA analysis**
Hedman, J., Nordgaard, A., Dufva, C., Rasmusson, B., Ansell, R., Rådström, P. 2010. Analytical Biochemistry 405:192-200

Other related publications by the author:

A fast analysis system for forensic DNA reference samples
Hedman, J., Albinsson, L., Ansell, C., Tapper, H., Hansson, O., Holgersson, S., Ansell, R. 2008. Forensic Science International: Genetics 2:184-189

Comparison of five DNA quantification methods
Nielsen, K., Smidt Mogensen, H., Hedman, J., Niederstätter, H., Parson, W., Morling, N. 2008. Forensic Science International: Genetics 2:226-230

Using the new Phadebas Forensic Press test to find crime scene saliva stains suitable for DNA analysis
Hedman, J., Gustavson, K., Ansell, R. 2008. Forensic Science International: Genetics Supplement Series 1:430-432

Pre-analytical sample preparation and analyte extraction
Rossmanith, P., Hedman, J., Rådström, P., Hoorfar, J., Wagner, M. 2011. In: Rapid Detection, Characterization and Enumeration of Food-borne Pathogens, ed. Hoorfar, J. ASM, Washington D.C., USA. pp. 121-136

Pre-PCR processing strategies
Hedman, J., Lövenklev, M., Wolffs, P., Löfström, C., Knutsson, R., Rådström, P. 2011. In: PCR Technology, Current innovations (3rd ed.), ed. Nolan, T. CRC Press, Boca Raton, USA. *In press.*

Strategies for overcoming real-time PCR inhibition in diagnostic PCR
Hedman, J., Rådström, P. 2011. In: Methods in Molecular Biology, PCR in Molecular Diagnostics of Microbial Pathogens, ed. Wilks, M. Humana Press, New Jersey, USA. *In press.*

Evaluation of three new forensic DNA profiling kits on PCR-inhibitory crime scene samples
Hedman, J., Albinsson, L., Norén, L., Ansell, R. 2011. Forensic Science International: Genetics Supplement Series. *In press.*

Applying a PCR inhibitor tolerant DNA polymerase blend in forensic DNA profiling
Hedman, J., Dufva, C., Norén, L., Ansell, C., Albinsson, L., Ansell, R. 2011. Forensic Science International: Genetics Supplement Series. *In press.*

Verification of alleles by using peak height thresholds and quality control of STR profiling kits
Albinsson L, Hedman J, Ansell R. 2011. Forensic Science International: Genetics Supplement Series. *In press.*

Abbreviations

AE_A	assay amplification efficiency
AE_R	reaction amplification efficiency
ANOVA	analysis of variance
BSA	bovine serum albumin
C_q	quantification cycle
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EPG	electropherogram
FI	forensic DNA profile index
FIgm	geometric mean of FI values
KOD	kinetic outlier detection
IAC	internal amplification control
LSR	least significant ratio
MLB	mean local balance
mRNA	messenger ribonucleic acid
PEG	polyethylene glycol
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
qPCR	quantitative PCR
RFU	relative fluorescence units
SH	Shannon entropy
SKL	Swedish National Laboratory of Forensic Science
STR	short tandem repeat
TPH	total sum of peak heights

Content

1 Introduction

DNA analysis was first applied in the investigation of crimes in the United Kingdom in the mid-1980s (Gill et al., 1985). The invention of the polymerase chain reaction (PCR) (Mullis, 1987; Mullis and Faloona, 1987) enabled a continuous development of more rapid and sensitive methods with higher discrimination power (Cotton et al., 2000; Hochmeister et al., 1991a; Sparkes et al., 1996). In PCR-based analysis, only one or a few nucleic acid molecules are required, and partly degraded DNA can be successfully analysed.

In standard forensic DNA typing, short tandem repeat (STR) markers are amplified and detected, producing allele patterns, or DNA profiles, that are unique for every individual (apart from identical twins who have identical STR alleles) (Caskey and Edwards, 1994). The demand for forensic DNA analysis has grown steadily throughout the world during recent years, and many countries have set up extensive national DNA databases containing DNA profiles from known offenders and crime scene samples (Schneider and Martin, 2001; Werrett, 1997). The database in the United Kingdom alone holds profiles from 5.7 million individuals and 400 000 crime scene samples (http://npia.police.uk/en/13338.htm, accessed 2011-09-22). During the summer of 2011, the number of DNA profiles from suspects and convicted criminals in the Swedish National DNA database surpassed 100 000.

DNA reference samples secured directly from suspects or victims generally contain sufficient amounts of cells (blood or saliva) and are reasonably homogeneous. This has enabled forensic laboratories to set up completely or partly automated analysis chains with high throughput (Hedman et al., 2008a; Parson and Steinlechner, 2001). Biological stains found at crime scenes are analysed to link a perpetrator to a

crime, by comparing the DNA profiles to suspects in the case and running them against the national DNA database. Crime scene samples are heterogeneous by nature, as any kind of cellular material attached to any kind of surface or object may serve as evidence. It is therefore more difficult to streamline and automate the analysis of these samples, and the success rate is generally lower due to limited amounts of cells and the presence of substances interfering with the PCR amplification. This thesis describes efforts to improve the success rate of DNA analysis of crime scene samples by applying pre-analytical screening, customisation of the DNA polymerase-buffer system used in PCR, and quantitative DNA profile quality assessment.

The processing of crime scene DNA evidence is outlined in Figure 1. Sampling and sample treatment serve to generate PCR-compatible DNA extracts from the heterogeneous stains. Following DNA extraction, DNA concentrations are measured using quantitative PCR (qPCR) (Andreasson et al., 2002), and negative samples may be identified so that they are not included in further analysis, to reduce costs. Since DNA extraction and qPCR are rather laborious, it would be beneficial to be able to identify negative samples earlier in the process. Saliva is a common source of DNA in property crime (Bond and Hammond, 2008). Objects used for crime scene saliva sampling include bottles, cans, cigarette butts and foodstuffs (Abaz et al., 2002; Sweet and Hildebrand, 1999) (Papers I, II and IV). However, items are generally sampled based on the assumption that saliva is present, not by visually confirming its presence. Therefore, around 50% of presumed saliva crime scene stains may give negative DNA results if presumptive testing is not performed (Bond and Hammond, 2008) (Paper I).

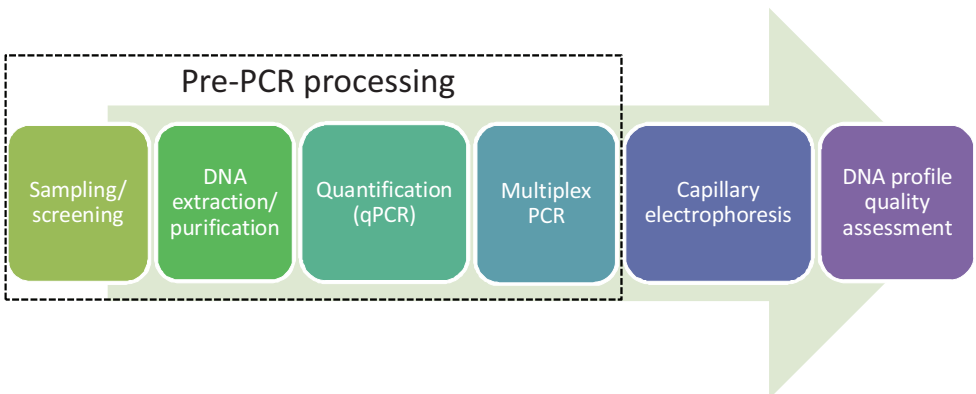

Figure 1. The processing of crime scene DNA evidence.

Amylase activity testing has been applied for forensic saliva screening for over thirty years (Willott, 1974; Willott and Griffiths, 1980). The amylase presumptive tests available generally require laboratory equipment and are time-consuming, and thus not suitable for quick screening or crime scene applications. In this work, the applicability of amylase testing for saliva screening was analysed, and a screening tool for dried saliva recovered on cotton swabs was developed (Paper I).

Since the introduction of commercial STR DNA typing kits such as AmpFlSTR SGM Plus (Applied Biosystems) and PowerPlex 16 (Promega), Ampli*Taq* Gold is the DNA polymerase of choice throughout the world of forensic DNA analysis. It is also an integral part of the new AmpFlSTR NGM kit. However, *Taq* DNA polymerases in general and Ampli*Taq* Gold in particular have been found to have lower tolerance to various PCR-inhibitory samples and substances than other polymerases (Abu Al-Soud and Rådström, 1998; Belec et al., 1998; Eilert and Foran, 2009). The common approach to dealing with PCR inhibitors is removal using various DNA purification methods (Akane et al., 1993; Bourke et al., 1999; Lantz et al., 1994; Shutler et al., 1999), or dilution (Mahony et al., 1998; Tsai and Olson, 1992a). However, extensive DNA purification increases the workload and includes the risk of DNA loss and contamination, and dilution is only possible if the amount of DNA is sufficiently high.

Pre-PCR processing (Rådström et al., 2004) (Figure 1) is an integrated concept where the issue of analytical detection limit and impact of PCR inhibitors is addressed in all steps leading up to PCR amplification (for a more recent review, see Hedman et al., 2011a and Hedman and Rådström, 2011). Ideally, the first part of assay optimisation using pre-PCR processing should be to identify the DNA polymerase-buffer system most suitable for the chemical content of the samples to be analysed. This may reduce the need for extensive DNA purification, thereby streamlining the analytical process and improving the DNA yield. When this project was initiated, 12% of saliva/secretion crime scene samples analysed at the Swedish National Laboratory of Forensic Science (SKL), containing low or moderate DNA concentrations (0.025-0.25 ng/µL), gave negative DNA profiles due to the presence of PCR inhibitors affecting the polymerising activity of Ampli*Taq* Gold (Paper II).

Through the application of pre-PCR processing, an experimental model system for quantitative screening of DNA polymerases was developed in order to identify polymerases with the potential of improving crime scene sample amplification. The most promising DNA polymerases were subsequently evaluated in multiplex STR DNA typing using PCR-inhibitory crime scene samples (Paper II).

1. Introduction

Electropherograms (EPGs) resulting from forensic DNA analysis are complex diagrams in which allelic peaks represent the amplification of each allele (see Figure 2 in Paper II). The alleles present in the EPG represent the DNA profile for that particular crime scene sample or individual. The quality of an EPG or DNA profile is determined by the heights of the allelic peaks, and by the balance between the peaks. Quality assessment of EPGs is vital to determine their evidential value, and in evaluating the performance of different DNA purification methods or DNA polymerase-buffer systems. In general, the quality of EPGs is compared manually by experienced scientists, based on empirical knowledge, and/or by comparing allelic peak heights (Abaz et al., 2002; Castella et al., 2006; Li and Harris, 2003; Moss et al., 2003). Manual comparisons may, however, be biased and difficult to quantify, and using only peak heights means that important peak balance information is not taken into consideration.

In order to describe the quality of DNA profiles in a quantitative and unbiased way, a ranking index was developed, combining intensity with intra-locus and inter-loci balance (Papers II and III). This Forensic DNA profile index (FI) expresses the quality of a complex EPG as a single value, enabling quantitative comparisons of DNA profile quality. The application of analysis of variance (ANOVA) to FI values from STR DNA typing of PCR-inhibitory crime scene samples verified the significance of the improvements achieved by using the alternative DNA polymerases Bio-X-Act Short (Bioline), Ex*Taq* Hot Start (TaKaRa Bio Inc.) and PicoMaxx High Fidelity (Stratagene), instead of the standard Ampli*Taq* Gold (Paper II). In addition, in Paper III I explain the mathematical and statistical methodologies used to construct FI and how to adapt the ranking index for any STR-based forensic DNA typing system.

Ex*Taq* Hot Start and PicoMaxx High Fidelity exhibited complementary inhibitor-tolerance properties, i.e. one polymerase remained active in the presence of substances that inactivated the other, and vice versa (Paper IV). In routine casework, it would be beneficial to use one universal PCR master mix, suitable for a broad range of sample types and background materials. To investigate whether the complementarity between polymerases could improve STR DNA analysis of PCR-inhibitory crime scene samples, a DNA polymerase blend comprising these two polymerases was developed and evaluated. The performance of the blend is discussed not only in terms of complementarity, but also in terms of synergy (Paper IV).

2 Preparation of PCR-ready DNA extracts from forensic samples

Blood, semen and saliva are the main tissue types subjected to forensic DNA analysis. Forensic light sources can be used to locate biological stains that are invisible to the naked eye, taking advantage of the fluorescent properties of some tissues (Fiedler et al., 2008; Vandenberg and van Oorschot, 2006). Presumptive testing for tissue-specific proteins may be used to verify the presence of a certain tissue type (Sensabaugh, 1978; Tobe et al., 2007) (Table 1), possibly improving the evidential value of a DNA profile, and/or indicating whether the stain contains enough cellular material for successful DNA analysis. Analysis of mRNA has recently been introduced for tissue determinations (Juusola and Ballantyne, 2005), offering high specificity and the possibility of screening for several tissues in one reverese transcription PCR reaction.

The first steps of pre-PCR processing (Rådström et al., 2004) serve to obtain heterogeneous crime scene stains and convert them into homogeneous extracts, high in DNA and low in PCR-inhibitory compounds. The objective is to generate extracts that are compatible with the PCR chemistry, i.e. the DNA polymerase and buffer composition, ensuring high-quality amplification with constant PCR kinetics. The general processing of crime scene evidence prior to PCR amplification, i.e. sampling and DNA extraction/purification is described below, with a specific focus on saliva screening of trace swabs (Paper I).

Table 1. Methods available for amylase testing of presumed saliva crime scene stains.

Product/method	Description	Reference
Fluorescence spectroscopy	Fluorescence spectroscopy	Soukos et al., 2000
Forensic light sources	Forensic light sources (400-700 nm) used for non-specific screening of surfaces	Fiedler et al., 2008
Indirect ELISA	Test with monoclonal antibodies, with saliva dissolved in microtiter plates	Quarino et al., 2005
mRNA markers	Genetic analysis using reverse transcription qPCR	Juusola and Ballantyne, 2005
Neo amylase	Colorimetric test based on enzymatic activity, with saliva dissolved in solution	Tsutsumi et al., 1991
Phadebas Forensic Press test	Screening of surfaces using impregnated paper, targeting the enzymatic activity	Hedman et al., 2008b
Trace swab screening using Phadebas	Direct testing of crime scene trace swabs, targeting the enzymatic activity	Paper I
Red Starch	Screening of surfaces using impregnated paper, targeting the enzymatic activity	Martin et al., 2006
RSID saliva	Saliva dissolved in membrane test strips, using monoclonal antibodies	Technical information sheet: RSID, 2006
SALIgAE	Colorimetric test based on enzymatic activity, with saliva dissolved in solution	Myers and Adkins, 2008
Starch-iodine assay	Colorimetric test based on enzymatic activity, with saliva dissolved in solution	Myers and Adkins, 2008

Adapted from "A Crime Scene Screening Tool for Saliva", Master's Dissertation by Erik Dalin, Linköping University, 2009. Used with permission.

2.1 Sampling

The objective of crime scene sampling is to maximise sample uptake and minimise the uptake of extraneous material that may interfere with PCR amplification. Sampling may be either direct or indirect. In direct sampling, a piece of the material carrying the target cells is submitted to treatment. Examples are cigarette butts, using the paper covering the filter, chewing gum, denim and other types of fabrics, and soil. Generally, direct sampling provides a high DNA yield, but often

leads to problematic levels of PCR inhibitors (Bourke et al., 1999; Tsai and Olson, 1992a) (Papers II and IV).

The most common indirect crime scene sampling method is swabbing. Swabbing using moistened cotton swabs is extensively used in forensic DNA analysis. Water is a common moistener, with alternatives such as physiological saline, ethanol and DNA extraction buffer (Anslinger et al., 2005; von Wurmb-Schwark et al., 2006) (Paper I).

Swabbing using cotton swabs has been used for diagnostic methods for many years (Patterson, 1971), and the methodology was not developed for sensitive PCR-based DNA analysis. The high absorption capacity of cotton may impede the release of cells, possibly causing false-negative results for low-template samples. The double-swab technique (Snijders et al., 1984) can improve sample uptake of saliva from human skin and contact traces on various surfaces (Pang and Cheung, 2007; Sweet et al., 1997). There, the evidence is first swabbed with a moistened cotton swab, and then with a second dry swab in order to soak up excess fluid. The material from the two swabs is then pooled prior to DNA extraction.

Nylon flocked swabs have been shown to improve the yield of male DNA in post-coital vaginal sampling compared with cotton swabs (Benschop et al., 2010), and may provide an alternative for specific types of samples. These swabs have thousands of nylon fibres protruding from the tip of the swab. The target cells remain on the surface of the fibres, enabling sampling of the outermost cells and easy release during DNA extraction. However, the poor absorption of nylon flocked swabs may limit cell uptake (unpublished data).

Tape lifting is gaining popularity, and has been successfully applied for sampling from diverse items such as clothes, human skin, hand guns and shoe insoles (Barash et al., 2010; Bright and Petricevic, 2004; Gunnarsson et al., 2010; Hall and Fairley, 2004; Li and Harris, 2003). A piece of adhesive tape is pressed against the object a number of times. The cells adhere to the tape, which is subsequently placed in a tube for DNA extraction.

2.2 Saliva screening of crime scene trace swabs

Blood and semen are generally sampled following visual inspection of surfaces or fabrics; in the case of semen often using forensic light sources. Visual inspection does not confirm the presence of a certain type of cell or tissue, but increases the chance of obtaining samples that provide usable DNA profiles. Saliva, however, is

often sampled on objects where its presence is expected rather than visually confirmed, e.g. bottles, cans and foods. It is usually not possible to tell whether or not a bottle has been drunk from, and if so, if the amount of saliva is sufficient for successful DNA analysis. Testing trace swabs for the presence of saliva, e.g. determining the amylase activity, would provide a method of reducing the proportion of samples giving negative DNA results.

Amylase is one of the principal salivary enzymes, and amylase activity testing has been applied in forensic science for over thirty years (Willott, 1974; Willott and Griffiths, 1980). Amylase levels vary considerably between individuals; differing by more than one order of magnitude (see Table 1 in Paper I). This may in part be explained by different amylase gene copy numbers (Perry et al., 2007). Amylase levels may also vary within individuals over time (Gutowski and Henthorn, 1983; Whitehead and Kipps, 1975). The amylase activity decreases substantially during the first hour of drying, but is then reasonably stable over time at room temperature (Tsutsumi et al., 1991) with detectable levels remaining after several months (Auvdel, 1986). Stains dried for two weeks at room temperature showed amylase levels indistinguishable from stains dried for 24 hours (Paper I).

Apart from human saliva, amylase is found in other bodily fluids such as faeces, semen, sweat and urine (Auvdel, 1986; Whitehead and Kipps, 1975; Willott, 1974) as well as in the saliva of various other mammals (Ohya et al., 1986). Faeces and saliva from other mammals may contain levels similar to those in human saliva, and vaginal secretions and semen may produce false-positive results when screening for saliva in underwear (Olsén et al., 2011). Amylase testing is thus indicative of, but does not confirm, the presence of human saliva.

No direct correlation was found between amylase activity and DNA content in saliva (Paper I). When Spearman's rank correlation test was applied to amylase activity and DNA concentrations in fresh saliva from ten males a weak positive correlation was indicated, but this was not significant ($r=0.26$). This is not surprising, as amylase is an extracellular enzyme and the DNA content depends on the individual's propensity to shed buccal cells. Moreover, salivary DNA concentrations varied considerably between the individuals, from 2.7 to 38 ng/µL of saliva.

The Phadebas Forensic Press test (Magle Life Sciences), a filter paper sprayed with Phadebas starch microspheres, may be used to detect saliva on surfaces, outperforming various light sources on denim and painted wood (Hedman et al.,

2008b). The microspheres are broken down by amylase, releasing a blue dye (www.phadebas.com/technical_info, accessed 2011-08-04).

In this work, a method utilising the Phadebas Forensic Press test was developed to provide quick, cost-effective screening of crime scene trace swabs (Table 1) (Paper I). Directly after sampling of a presumed saliva stain, the swab is briefly pressed against the Phadebas paper. The paper is then moistened with a drop of physiological saline. For samples with intermediate to high amylase activities (290 to 840 kU/L), dried stains (24 hours at room temperature) containing 0.5 µL of saliva were detected within five minutes. For samples with low amylase activity (38 kU/L), colour changes were visible within 20 minutes for 2 µL of dried saliva, but not for 0.5 µL. Adding more saline after drying enabled the detection of two of the four 0.5 µL stains.

All the analysed samples producing usable DNA profiles also showed positive amylase results, indicating that the detection limit is low enough for the purpose of detecting DNA-positive samples. Stains containing 2 µL of dried saliva from a person with intermediate DNA content (17 ng/µL), generally produced complete DNA profiles (see Tables 3 and 4 in Paper I). However, the inter-individual differences in amylase activity and DNA content, and the fact that the two are not directly correlated, should be taken into consideration when evaluating the screening results.

Applying the screening tool did not affect the DNA concentration or purity (see Table 3 in Paper I). During testing, only a minute amount of fluid is transferred from the swab to the Phadebas paper, and the strong adherence of the cells to the cotton leads to minimal cell loss. Colour changes were generally easily discernible from unreacted Phadebas spheres. However, the interpretation of the intensity of the colour change required subjective judgment. In order to avoid interpretation bias in casework, reference scales showing standardised colour changes should be distributed with the standard operating procedure (see Figure 1 in Paper I). The developed crime scene trace swab screening tool does not require laboratory equipment and can be used at crime scenes. It can be applied to identify saliva stains suitable for DNA analysis when the number of analyses is limited due to cost, e.g. in high-volume crime.

2.3 DNA extraction and purification

The purpose of sample treatment is to make the crime scene samples suitable for PCR amplification by releasing DNA into solution, increasing target DNA density and removing or neutralising PCR inhibitors. Sample treatment may involve cell separation, cell lysis and DNA purification. Direct cell lysis followed by DNA purification is arguably the most common approach within the forensic community. From a pre-PCR processing point of view, it is important that the chemicals used in DNA extraction and purification are compatible with the DNA polymerase-buffer system, and that any extraneous compounds remaining in the extracts do not impair amplification.

Several rather expensive commercial DNA extraction kits are available, with manual or automated sample handling. Most of the less costly methods are based on either Chelex 100 resin (Bio-Rad) or phenol-chloroform. Phenol-chloroform purification is a powerful method for removing organically soluble PCR inhibitors (Hochmeister et al., 1991b). Since phenol is highly toxic and corrosive, and may have a negative effect on the subsequent PCR, it is being replaced by other methods. Chelex is a chelating polymer that binds polyvalent metal ions that may otherwise catalyse DNA degradation at high temperatures and low ion contents (Walsh et al., 1991). Chelex extraction is usually performed in a single tube, avoiding sample transfer, thus minimising nucleic acid loss and lowering the risk of contamination and sample mix-ups. Cells are pelleted early in the process, and water-soluble PCR inhibitors are removed. However, disrupted cells and free DNA may be lost, possibly lowering the yield. Samples are heated to 56°C to achieve lysis, and then boiled to denature and degrade proteins. Care should be taken not to include Chelex beads in the PCR as they will chelate vital Mg^{2+} ions. Combined with an appropriate DNA polymerase-buffer system, the quick and simple Chelex method may give high-quality results; however, the extracts are generally not pure and amplification may be impaired when less inhibitor-tolerant DNA polymerases are used (Hedman et al., 2011b; Hedman et al., 2011c) (Paper II and IV). Chelex extraction is often combined with proteinase K treatment. Proteinase K mediates cell lysis, and degrades proteins that would otherwise interfere with PCR amplification (McHale et al., 1991).

Filtration and dilution are two quick and simple sample treatment methods that can be applied following extraction. In filtration, the extract, together with an aqueous buffer, is passed through a filter with pores that allow the smaller inhibitor molecules to pass through, while retaining the larger DNA molecules. For example, Microcon filter tubes (Millipore) have been used to purify forensic DNA extracts

from cigarette butts (Watanabe et al., 2003). However, filtration and other DNA purification strategies will inevitably lead to DNA loss, e.g. due to irreversible binding of DNA to surfaces and filters. Dilution, i.e. simply adding water or a buffer to the DNA template, has been successfully applied to circumvent inhibition by humic substances (Tsai and Olson, 1992a; b) and urine (Mahony et al., 1998), but this obviously lowers the amount of available target DNA.

The purity of the generated DNA extracts can be estimated using optical density measurements, and the amplifiability tested using qPCR prior to STR DNA typing. However, the results of optical density testing are not correlated to the success of PCR (Gryson et al., 2007; Roussel et al., 2005), since the optical density mainly determines the protein content, while other substances can interfere with PCR. Quantitative PCR provides a better estimate of purity, but requires that the assay is correlated with the following multiplex STR assay regarding DNA polymerase-buffer system, fragment length and the ratio of template-to-reaction volume (see Chapter 3.2). From a pre-PCR processing perspective, the purity of the DNA extract is actually defined by the DNA polymerase-buffer system of the STR assay. As long as the DNA polymerase is able to amplify the target efficiently, the extract is sufficiently pure, even if it is considered "dirty" by visual inspection or according to other measurements. Thus, customising the DNA polymerase-buffer system to the samples at hand is a good first measure when aiming to reduce the effects of PCR inhibition (Hedman et al., 2011c) (Papers II and IV).

3 Mathematical modelling of PCR

Professor Kjell Kleppe described the principle of a PCR-like process in 1969 and published a paper describing the technique in 1971 (Kleppe et al., 1971). He envisioned how DNA amplification could be carried out in a cyclic manner using temperature changes to enable denaturation and annealing of primers to primer sites, and the addition of DNA polymerase to finalise primer extension. However, he was unable to make the process work in practice. The invention of PCR is credited to Kary Mullis (Mullis, 1987), who together with colleagues published the first paper describing an application of the method in 1985 (Saiki et al., 1985).

Using PCR, millions of copies of a specified region of the DNA can be produced within hours. This enables sensitive and rapid diagnostic analysis in various scientific fields, such as archaeology, clinical diagnostics, environmental studies, food and feed testing, and forensics (Hedman and Rådström, 2011).

3.1 The kinetics of PCR

The kinetics of PCR is determined by the efficiencies of the integrated chemical reactions, i.e. DNA denaturation, primer annealing, binding of DNA polymerase to the primer-target complex and extension of primers (Booth et al., 2010). The kinetic behaviour is affected by the amount and purity of the target DNA, the quality and concentration of primers and nucleotides, the cation content (specifically Mg^{2+}), the pH, and the processivity and inhibitor-tolerance of the DNA polymerase. Understanding PCR kinetics is therefore important for successful pre-PCR processing, e.g. when optimising new PCR assays or customising an assay for certain types of samples. In order to study PCR kinetics,

the amplicon increase must be continuously monitored throughout the reaction. In qPCR, the amplicons produced are detected through fluorescent detection dyes such as SYBR Green I binding to dsDNA (Wittwer et al., 1997), or by using fluorescently labelled probes such as hydrolysis (*Taq*Man) probes (Heid et al., 1996). The intensity of released fluorescence is proportional to the number of generated amplicons (Heid et al., 1996). The fluorescence is measured once in each PCR cycle, and the intensity is plotted in a graph giving an amplification curve that reflects the reaction kinetics (Higuchi et al., 1993) (Figure 2).

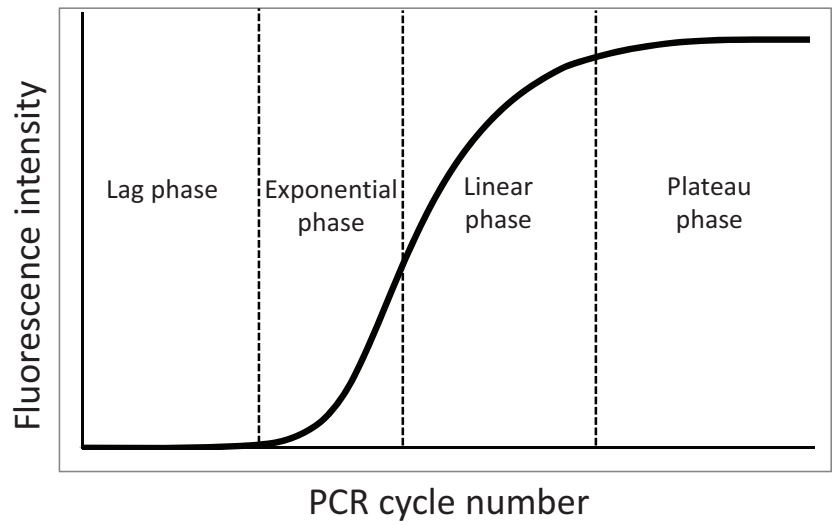

Figure 2. The kinetics of PCR. The increase in fluorescence, reflecting the growth of amplicons in qPCR, ideally has a sigmoidal shape, exhibiting a lag phase, an exponential or near-exponential phase, a linear phase, and a plateau phase.

The qPCR amplification curve should ideally have a sigmoidal shape (Figure 2), with a lag phase, an exponential or near-exponential phase, a linear phase showing reduced amplification efficiency, and finally, a plateau phase. Amplification is initiated in the first few cycles, and the lag phase merely reflects the fact that the emitted fluorescence is below the detection limit of the qPCR instrument. In other words, there is no actual "lag" in the process. Thus, the kinetics of the first few cycles can not be studied directly. However, the first cycles are vital for the yield and specificity of the reaction, as they involve a screening process where the

efficiency of specific primer annealing determines whether the desired reaction will be favoured over any non-specific reactions (Ruano et al., 1991).

The maximum theoretical efficiency of amplification is 1.0, i.e. the number of copies is doubled in each cycle (Mullis and Faloona, 1987). In reality, the efficiency is lower, and is not constant during thermal cycling. Instead, it decreases steadily from its peak value, in the exponential phase, to 0 when the reaction reaches the plateau phase (Liu and Saint, 2002a). The rate limiting mechanism changes during the course of PCR (Booth et al., 2010). In the first few cycles, all reagents are in large excess compared with the number of target molecules, maximising the chance of primers finding their sites and DNA polymerase molecules attaching to the primer-target complex. At this stage, the number of template molecules is the limiting factor. During the course of the PCR, the ratio between template and DNA polymerase molecules changes from about $1:10^6$ to 1:1 or less, and the amount of polymerase becomes the limiting factor (Ruano et al., 1991). Depending on the thermal stability of the DNA polymerase, the activity can be reduced during cycling. For natural *Taq* polymerase, half of the initial activity remains after about one hour at the denaturation temperature (94-95°C) (Pavlov et al., 2004), indicating that reduction in activity has a minor effect on PCR kinetics when using high-quality DNA polymerases.

The plateau phase is often falsely attributed to the depletion of reagents. In fact, after 30 cycles of PCR, well over 90% of primers and nucleotides are not consumed (Ruano et al., 1991), and once the plateau is reached, amplification can not be restarted by adding fresh reagents (Morrison and Gannon, 1994). The kinetics of PCR in the later cycles is thus not only controlled by the levels of the reagents, but by the changes in the proportions between the reagents and template as well as diffusion limitations. Product inhibition is probably the most important explanation of the plateau phase. Self-annealing of the produced amplicons probably inhibits amplification by non-specific binding of the resulting short dsDNA fragments to the polymerase (Kainz, 2000), and possibly to a smaller extent by blocking primer annealing (Morrison and Gannon, 1994).

PCR kinetics can be described by modelling the reaction amplification efficiency (AE) using the qPCR amplification curve. Several methods for calculation of AE have been proposed, generally employing exponential or sigmoidal curve fitting to parts of the amplification curve (Liu and Saint, 2002b; Ramakers et al., 2003; Tichopad et al., 2003). After log-transforming the curve, AE can be calculated from the slope of the straight line describing the exponential phase. Sigmoidal models are preferable as they follow the PCR kinetics more closely, improving the precision of

the model fitting (Tichopad and Bar, 2009). AE describes the kinetic behaviour of one single reaction, and is affected by the DNA concentration, pipetting errors and the presence of PCR inhibitors. The presence of inhibitors affecting the DNA polymerase activity leads to a flattening of the amplification curve (Higuchi et al., 1993) (Figure 3) and a thus lower AE.

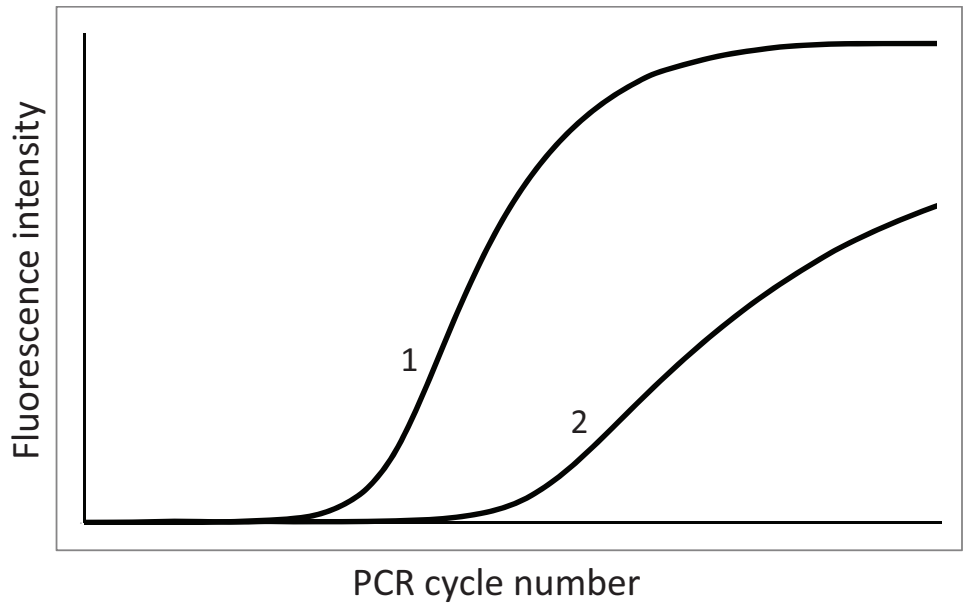

Figure 3. Amplification curves illustrating an ideal reaction (1), and a reaction with the same amount of DNA affected by PCR inhibitors (2). The presence of compounds affecting the DNA polymerase lowers the efficiency of amplification, generally resulting in a flatter amplification curve, starting at a higher cycle number.

The term amplification efficiency (or PCR efficiency) is also used in the literature to describe values calculated from the slopes of qPCR standard curves generated by several reactions (Arezi et al., 2003; Meijerink et al., 2001). The two strategies of calculating amplification efficiency may give substantially different results (Ramakers et al., 2003). In order to distinguish between these different values, I propose the notation AE_R for single reaction efficiency, and AE_A for assay or standard curve derived efficiency. For pure DNA, AE_A describes how well the assay performs using different amounts of DNA, and it may be used to evaluate primers or to optimise the Mg^{2+} concentration. The calculation of AE_A and the ways in

which it can be used to study PCR performance and inhibition are described in Chapter 3.2.

AE_R calculations generally imply that the "exponential" part of the amplification curve is defined by the user, leading to bias. If too few data points are chosen for the calculations, the variation will increase, and if too many are used, there is a risk of including data outside the exponential phase (Kontanis and Reed, 2006; Ramakers et al., 2003). Different AE_R calculation methods may also give different results from the same amplification curves (Tichopad et al., 2010).

For quantification purposes, fractional PCR cycle numbers are calculated from qPCR amplification curves. This fractional cycle value, called quantification cycle (C_q) (Bustin et al., 2009), should occur somewhere in the exponential phase, where the fluorescence intensity is proportional to the amount of input DNA (Heid et al., 1996; Wittwer et al., 2001). The C_q value may be derived using either fluorescence thresholds (e.g. in the qPCR instruments from Applied Biosystems), or using the second derivative maximum method (in the LightCycler instruments from Roche). In the threshold approach, C_q is defined as the intersection between a threshold, either set arbitrarily or 10 standard deviations above the background level (Heid et al., 1996), and the amplification curve, as illustrated in Figure 4. If C_q values from different runs are to be compared, the same threshold level must be applied.

When using the second derivative maximum method a polynomial equation is fitted to the amplification plot (Wittwer et al., 2001). The value of C_q is defined as the fractional cycle number at which the second derivative of the polynomial function has its maximum, i.e. the point where the "acceleration" of fluorescence intensity is highest, also illustrated in Figure 4.

When analysing ideal reactions with constant AE_R, the threshold approach and the second derivative maximum method provide comparable C_q values. However, if AE_R is reduced, e.g. due to the presence of inhibitors, care should be taken when evaluating the C_q values. Using the threshold approach, a flatter amplification plot gives rise to an elevated value of C_q as more cycles will be needed to reach the threshold fluorescence. On the other hand, using the second derivative maximum approach, it was noted that a flatter amplification curve leads to underestimation of the C_q, compared to sharper curves with a similar initial exponential phase (Paper II).

C_q is a general notation (Bustin et al., 2009). In qPCR instrument software the fractional cycle number has other notations, e.g. the threshold cycle (C_t) or crossing

point (C_p). In Paper II, the notation C_p is used, but C_q will be used throughout this summary.

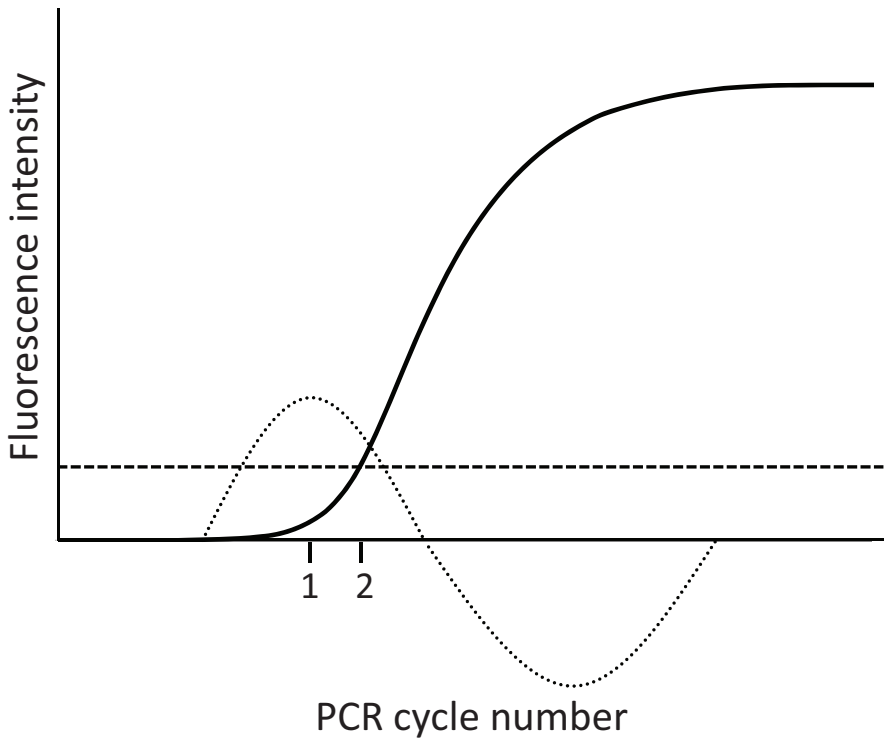

Figure 4. Illustration of the two most commonly used methods of calculating the value of C_q from the qPCR amplification curve. The dotted curve represents the second derivative of an equation describing the amplification curve, and the value of C_q is defined as the value on the x-axis at which the second derivative is a maximum (1). The dashed line shows the fluorescence threshold, and the value of C_q is defined as the value on the x-axis at which the amplification curve intersects the threshold (2).

3.2 Quantitative PCR

In forensic DNA analysis, qPCR is routinely used to determine the quantity and quality of DNA prior to STR DNA typing (Andreasson et al., 2002; Green et al., 2005). Depending on the resulting DNA concentrations, samples may be diluted to avoid DNA overload, or excluded from subsequent STR DNA typing. Autosomal, X-Y chromosomal or mitochondrial DNA may be targeted, and analysis of fragments of different lengths may provide information on DNA degradation (Hudlow et al., 2008; Niederstätter et al., 2007; Swango et al., 2006). An internal amplification control (IAC), i.e. an "alien" DNA fragment of known concentration, should be included in the qPCR to monitor the quality of the reaction and to avoid false-negative results (Hoorfar et al., 2003; Reiss and Rutz, 1999). The presence of PCR inhibitors or other problems related to PCR chemistry may result in either complete failure to amplify the IAC, or an elevated value of C_q. Samples showing impaired amplification of the IAC may, for example, be purified prior to STR DNA typing.

For absolute quantification, dilution series of pure DNA with known concentrations are analysed in order to obtain a standard curve (Figure 5). The standard curve is then used to calculate the DNA concentrations of the samples. For correct DNA quantification, it is important that the reaction kinetics of the sample is the same as that of the DNA used to obtain the standard curve. Concentrations could be seriously underestimated if the AE_R of the sample is lower than that of the DNA standard (Cankar et al., 2006). Quantification could also be compromised if immortalised cell line DNA is used for standard curve generation, and the target gene of the cell line contains a higher level of mutations than normal cell DNA (Nielsen et al., 2008). Mutations in primer or probe annealing sites would lead to lowered AE_R for the cell line standard DNA, and subsequent overestimation of the sample DNA concentrations.

Tichopad et al. (2010) proposed a bivariate kinetic outlier detection (KOD) method to evaluate the kinetics of individual reactions by comparing them with ideal standard reactions, in which first and second derivative maxima of a fitted sigmoidal model are calculated (Tichopad and Bar, 2009; Tichopad et al., 2010). The two values are then combined to give one χ^2 distributed value. High values imply that the reaction kinetics is significantly different from the kinetics of pure reactions. The method can be used to identify kinetic outliers in quantification,

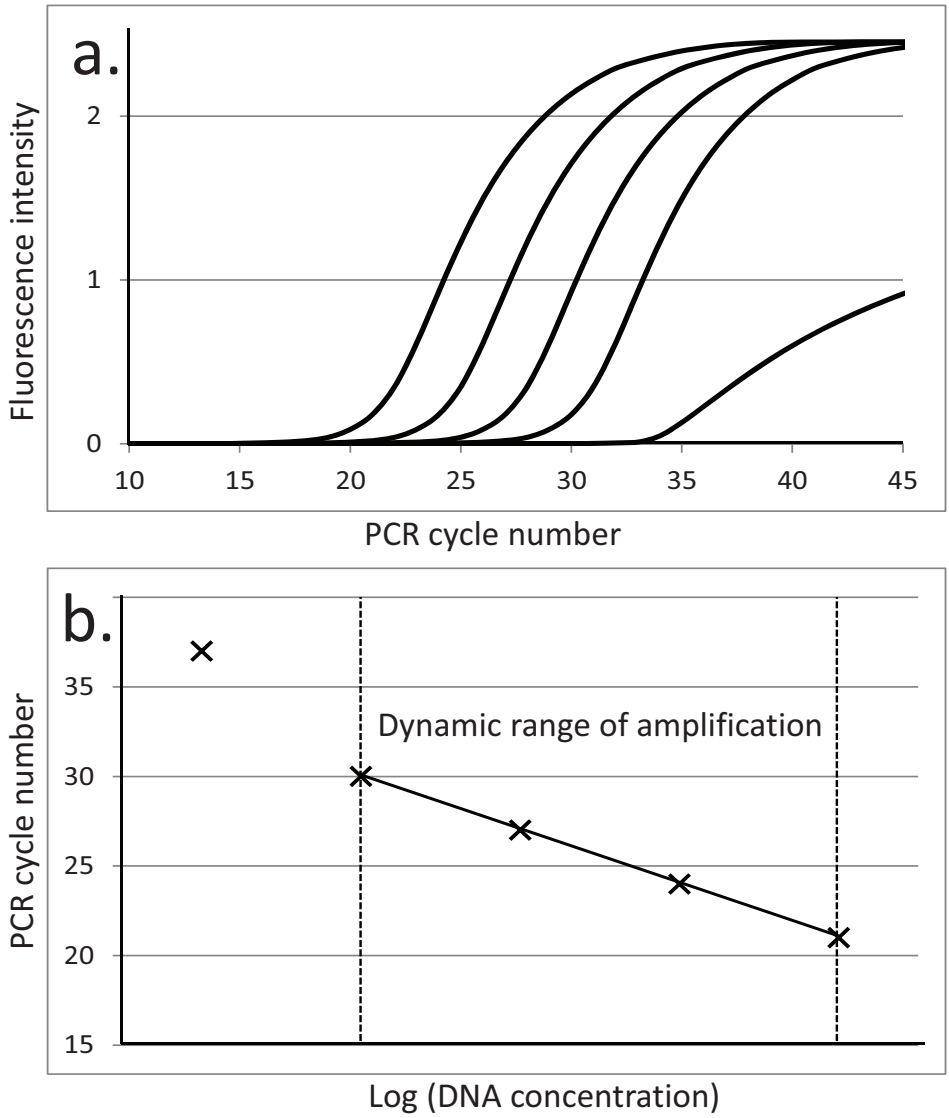

Figure 5. Generation of a standard curve for DNA quantification. a) A dilution series of pure DNA is analysed, with increasing C_q values given for reduced DNA concentrations. The amplification curve on the far right shows impaired amplification efficiency and is considered a kinetic outlier. b) A standard curve is generated by the C_q values of the dilution series, forming a straight line within the dynamic range of amplification (i.e. the concentration range within which quantification can be performed). Concentrations are calculated from the standard curve using the equation $conc=10^{((Cq-m)/slope)}$, where m is the intercept. AE_A is calculated from the slope of the standard curve, using the formula $AE_A=10^{(-1/slope)}-1$. A slope of -3.32 gives AE_A 1.0, generally described as exponential or ideal amplification. An AE_A of 1.0 implies that if the ratio of input DNA concentrations in two samples is 1:2, then the difference in C_q will be one cycle.

i.e. samples that may not be properly quantified using the standard curve, or to estimate the level of inhibition in a reaction. The KOD method has been shown to be more reliable in identifying reactions affected by PCR inhibitors than various AE_R calculation methods (Tichopad et al., 2010).

Inhibitory compounds that have a direct effect on DNA, lowering the amount of available template, may impair amplification and elevate C_q values without affecting the AE_R or being flagged as kinetic outliers (Opel et al., 2010). Thus, an IAC is needed in the qPCR assay to ensure that all inhibitory effects are detected. When designing an appropriate IAC for monitoring of PCR inhibitors, several criteria must be considered.

The IAC primer sites may be equivalent to those of the target (Maaroufi et al., 2006), or they may be different, creating a need for separate primer pairs (Hartman et al., 2005). In the first case there is competition for the primers, affecting the AE_R of both the template and the IAC. Incorporating a second primer pair may increase the risk of primer-dimer formation and non-specific primer annealing.

If the IAC concentration is too high, the amplification of the target may be negatively affected. The appropriate number of IAC molecules depends on the assay, but generally low numbers are preferable, e.g. from around 20 to 100 copies per reaction (Maaroufi et al., 2006; Rodriguez-Lazaro et al., 2005; Rosenstraus et al., 1998). Since the amplification of target DNA competes with IAC amplification, even when different primer pairs are used, the value of C_q for the IAC may be elevated when levels of target DNA are high, generating a false inhibitory effect (Hudlow et al., 2008).

The IAC must be at least as sensitive to PCR inhibitors as the target. The size of the IAC is important in this respect, since shorter fragments are generally more readily amplified in the presence of inhibitors than longer ones (Eckhart et al., 2000). An IAC amplicon longer than the target is therefore recommended (Maaroufi et al., 2006).

When applying qPCR prior to STR DNA typing, the ratio of the template-to-reaction volume of both assays should preferably be identical. In one study, the IAC of the quantification kit Quantifiler Human (Applied Biosystems) was found to be more sensitive to haematin inhibition than the AmpFlSTR Identifiler typing kit (Applied Biosystems) (Green et al., 2005), indicating that inhibitor concentrations affecting AmpFlSTR Identifiler DNA profiling would first be noted in the initial qPCR analysis. However, in Quantifiler Human qPCR, the template-to-reaction volume ratio is 2:25, compared to 10:25 in AmpFlSTR Identifiler STR

amplification (Applied Biosystems, 2003; 2006). A sample that appears pure according to the Quantifiler Human IAC may therefore be inhibitory in AmpFlSTR Identifiler or comparable systems, simply because a greater amount of the sample is used. In the studies described in Papers II and IV, only 18 of 74 crime scene samples that were clearly inhibitory in AmpFlSTR SGM Plus analysis affected the C_q values of the Quantifiler Human IAC. This can be partly explained by the different template-to-reaction volume ratios (10:25 for AmpFlSTR SGM Plus). The Quantifiler Human target and IAC fragment are both around 60 bp long, while AmpFlSTR SGM Plus amplicons range from approximately 100 to 350 bp (Applied Biosystems, 1999), indicating that the qPCR and STR DNA typing assays are not correlated. To ensure similar PCR inhibition effects in the qPCR and STR assays, they should preferably have identical DNA polymerase-buffer systems. In the present work (Paper II), the qPCR assay used in the model system was adapted for STR analysis by using a PCR fragment of a similar length to that of the STR markers (156 bp) (Niederstätter et al., 2007), by applying identical template-to-reaction volume ratios and by using the same DNA polymerase-buffer systems.

Standard curve AE_A calculations may be used to estimate the level of PCR inhibition by spiking a sample background with different amounts of "alien" DNA (Cankar et al., 2006; Volkmann et al., 2007). Thus, the level of PCR inhibitors is kept constant in all DNA dilutions. Lower concentrations of DNA are expected to be more severely affected by inhibitors (Roussel et al., 2005), leading to elevated values of C_q and values of AE_A significantly below 1.0. However, if all the samples analysed are affected similarly, inhibitory effects may be disguised using this method, as only the differences in C_q are taken into account, not the actual reaction kinetics.

Another means of estimating inhibition using AE_A calculations is to dilute a PCR-inhibitory sample directly and create a standard curve based on the target DNA. The inhibitors are also diluted, meaning that the samples with the lowest DNA concentrations are the purest. Since the presence of PCR inhibitors generally lowers AE_R and elevates C_q, the difference in C_q between adjacent dilutions may be reduced. Thus, the standard curve obtained may be flatter than theoretically possible, giving a value of AE_A above 1.0 (Paper II).

In this work, AE_A calculations were used for quantitative screening to identify DNA polymerases suitable for forensic DNA analysis. Mock crime scene samples were prepared from dilution series of saliva to create a model system consisting of samples similar to those encountered in casework (Paper II). Analyses were performed using qPCR, and standard curves were obtained based on the input cell

levels. The samples contained a background from the saliva itself, and from the sampling and extraction procedures, together with a controlled amount of DNA. The samples with most DNA also contained the highest amount of PCR-inhibitory compounds from saliva. The weakest samples, containing just a few DNA molecules, were purer. A DNA polymerase that has low tolerance to the sample background would thus generate AE_A values above the theoretical maximum of 1.0 (see Table 1 in Paper II).

In this set-up, a long dynamic range of amplification (Figure 5) (see Figure 1 in Paper II) implies that the polymerase in question is tolerant to the saliva compounds in the extracts, and also has a low general detection limit. Thus, the model system does not only estimate PCR inhibitor-tolerance. It provides information on the resistance of the DNA polymerase to inhibitory compounds, as well as the general sensitivity and efficiency of amplification, both of which are vital properties of DNA polymerases used in forensic casework.

3.3 Multiplex STR analysis

In forensic DNA analysis, multiplex PCR is used to simultaneously analyse several STR markers in one reaction. Primer pairs for each marker are added to the PCR master mix, requiring careful primer design, and optimisation of primer and Mg^{2+} concentrations and annealing temperature, in order to ensure even and efficient amplification for all markers and avoid primer-dimer formation (Henegariu et al., 1997). Commercial STR multiplexes are developed by global companies, with input from the forensic community. The ten STR marker kit AmpFlSTR SGM Plus was used in the studies presented in Papers I, II and IV.

Following multiplex PCR amplification of STR markers, an EPG is obtained for each sample (see Figure 2 in Paper II), using capillary electrophoresis separation of DNA fragments and detection using fluorescently labelled primers (Butler et al., 2004). Small peaks indicate that the amount of input DNA is low, that the DNA is partly degraded, and/or that PCR inhibitors are present. Low amounts of DNA, degraded DNA and PCR inhibition may also cause imbalance between peaks. The extreme case of imbalance is called drop-out, i.e. an expected allelic peak is missing in the EPG. Imbalances are also seen in samples containing DNA from more than one individual.

In multiplex PCR, inhibitory effects are more complex compared to single fragment amplification. The presence of inhibitors generally suppresses amplification, leading to overall lower allelic peaks in the EPGs. The greatest

negative effect is observed for the longer amplicons (Funes-Huacca et al., 2011) (Papers II and IV), resulting in imbalances between alleles of different lengths (see Figure 2 in Paper II). Additionally, substances binding to DNA in a sequence-specific manner may lead to marker-specific effects that are independent of amplicon size (Funes-Huacca et al., 2011). When determining the reaction conditions for a multiplex PCR assay, a compromise may be necessary between the included markers. Inhibitory effects can therefore be more pronounced for markers with larger differences between optimal and actual annealing temperatures and reagent concentrations. For example, inhibitors that lower the melting temperature of dsDNA, e.g. by chelating Mg^{2+} ions, are most problematic for markers whose annealing temperature is already close to the melting temperature of the primer, since their annealing efficiency will be more severely impaired by the inhibitors.

The evaluation of DNA profiles in order to establish their evidential value and determine the contributions from different individuals in mixed profiles is an important task for forensic scientists. A number of statistical tools and expert software systems have been developed to streamline analysis and improve the quality of DNA profile interpretation, by reducing the manual evaluation of DNA profiles in routine analysis (Bill and Knox, 2005; Hedman et al., 2008a; Power et al., 2008), providing a quantitative approach to the interpretation of mixed DNA profiles (Bill et al., 2005; Cowell et al., 2007; Haned et al., 2011) and handling the risk of encountering artefact peaks and allelic drop-out (Balding and Buckleton, 2009; Gill et al., 2007; Gill et al., 2008; Tvedebrink et al., 2009).

3.4 The forensic DNA profile index

Assessment of the analytical quality of DNA profiles is important when comparing the performance of different DNA analysis protocols in validation studies or research. In several studies where forensic DNA analysis methods have been compared, DNA profile quality was assessed by manual examination based on empirical knowledge, and/or by comparing the peak heights or areas of the allelic peaks in EPGs (Abaz et al., 2002; Castella et al., 2006; Forster et al., 2008; Li and Harris, 2003; Moss et al., 2003). Manual examination suffers from bias, and if only peak intensities are compared, the question of balance is not taken into account. Zahra et al. (2011) developed a set of IACs for STR typing to monitor analytical success. The two IACs were 90 and 410 bp long, flanking the STR alleles. The longer fragment is more sensitive to haem and humic acid than the shorter one, and the ratio between their respective peak heights can be used as a measure of the inhibitory effect (Zahra et al., 2011). However, IACs add complexity to the assay,

and the effect of the inhibitors on these fragments may be different from those on the target DNA (Huggett et al., 2008). A direct method of determining the quality of the reaction from the allelic peaks in the EPG arising from the target DNA is therefore preferable.

The total sum of peak heights (TPH) is a good measure of DNA profile quality, since it is directly correlated to the number of amplicons generated. Impaired amplification is seen as lower peak heights or drop-outs, lowering TPH. However, TPH must be complemented with measures of balance within and between STR markers to provide a complete description of DNA profile quality. A common measure of balance within markers is the heterozygote balance, i.e. the height ratio between the lowest and the highest allelic peak in a heterozygous marker (Promega, 2006). Calculating the mean of heteozygote balances for all loci in a DNA profile provides a global measure of intra-locus balance, i.e. mean local balance (MLB). Inter-loci balance can be described by calculating the standard deviation of each marker's relative contribution to TPH (Debernardi et al., 2011) or by calculating the Shannon entropy (Shannon, 1948) (Papers II and III). Shannon entropy (SH) was first used in information theory, and is also used to describe biodiversity (Foody and Cutler, 2003). The measure is maximised when all markers have identical peak heights.

The three measures discussed above, TPH, MLB and SH, may be used individually to describe the quality of a DNA profile. When studying several EPGs, such as Figure 2 in Paper II and Figure 1 in Paper III, it is obvious to a skilled forensic scientist that the three measures are correlated to each other. Greater peak heights generally lead to better intra- and inter-loci balances, although the presence of PCR inhibitors complicates the process, leading to preferred amplification of certain alleles and markers. Combining the three measures into one value would provide a more complete measure of DNA profile quality, simplifying interpretation and taking the relationship between the three measures into account. Data reduction is needed to accomplish this.

Principal component analysis (PCA) is a mathematical tool that can be used to reduce the number of variables in a data set (Johnson and Wichern, 2002). The resulting principal components (PCs) are uncorrelated, and the first PC describes more of the variation in the data set than any other PC. PCA is frequently applied in biology, e.g. in gene expression studies to reduce the regulation patterns of thousands of genes to a few PCs (Ringnér, 2008), and in genome-wide association studies to locate and evaluate forensically relevant phenotypic markers (Liu et al., 2010).

In this work, PCA was applied to the measures TPH, MLB and SH to create a ranking index called the forensic DNA profile index (FI) (Figure 6) (Papers II and III). The methods used may be applied to adapt FI for any STR DNA profiling system.

Standardisation was necessary before applying PCA since the three measures differ in magnitude. A calibration set of EPGs with qualities representative of casework analysis was used for this purpose. Following PCA, only the first PC was retained, since the second and third PCs both had eigenvalues below 1 (Johnson and Wichern, 2002). This implies that the first PC describes the essential part of the variation, and discarding the other PCs does not lead to any significant loss of information. As a result of standardisation, the values of the retained PC varied around zero. The PC was translated to provide a well-defined zero for the ranking index, simplifying its interpretation.

The retained PC describes the behaviour of the three original measures and their correlation. However, there is no guarantee that differences in PC values coincide with expert opinions on what constitutes a high-quality DNA profile. When applying the ranking index to EPGs from the study described in Paper II, it was found that the PC was greatly affected by insignificant changes in MLB, and less affected by substantial differences in TPH. Therefore, the PC was validated against another ranking system, based on empirical knowledge of DNA profile quality. Profile quality was graded according to the opinions of experienced reporting officers, where TPH was given a stronger weight than the balance values, but still taking the latter into account (see Table 1 in Paper III). Following validation, the PC coefficients were updated, providing an FI that better reflected the expert opinions on EPG quality (Papers II and III).

The FI enables quantitative quality comparisons between EPGs, allowing statistical tests to be performed on the values to verify the significance of differences. In this work, ANOVA (Montgomery, 2009) was applied to the logarithm of FI (to make it more normally distributed). I analysed two replicates of a set of samples for a range of DNA polymerase-buffer systems (Paper II and IV). Geometric means of FI (FIgm) were calculated (back-transformed logarithmic means), followed by ratios between FIgm values for different DNA polymerase-buffer systems. The FIgm ratios for pairs of DNA polymerase-buffer systems must be above a certain least significant ratio (LSR) for the difference in DNA profile quality to be considered significant (Papers II and IV). The LSR depends on the number of polymerase-buffer systems compared, the number of replicates and the significance level.

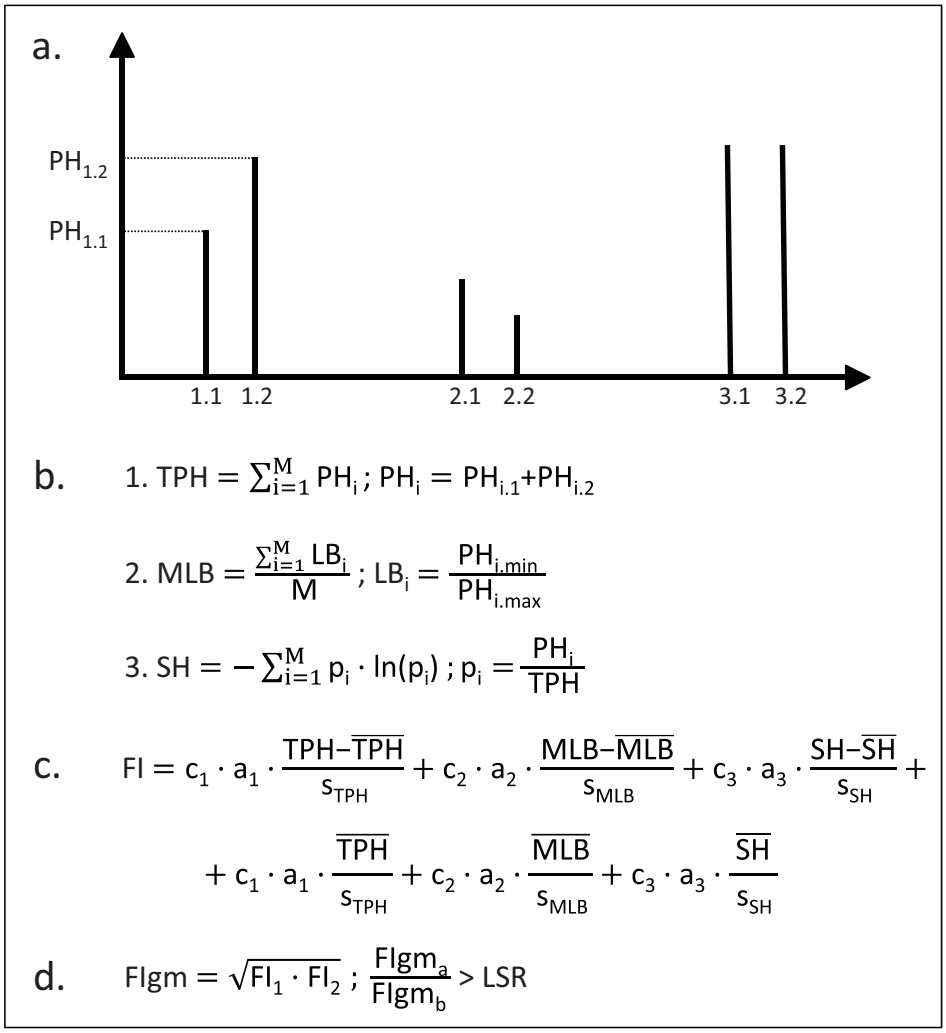

Figure 6. Mathematical description of FI. a) An EPG consists of fluorescence peaks representing alleles, with peak heights (PH) given in RFU. Each marker has either one peak (homozygote) or two peaks (heterozygote). b) TPH describes the intensity of the EPG; MLB describes heterozygote balance; and SH describes balance between markers. c) FI was constructed by standardising TPH, MLB and SH using sample means and standard deviations from a calibration set of representative EPGs; performing PCA on the standardised variables; keeping the first PC, with coefficients a_1, a_2 and a_3; validating the PC against an EPG grading system based on expert opinions on EPG quality, adjusting PC coefficients with the factors c_1, c_2 and c_3; and translating the score using sample means and standard deviations. d) FIgm is calculated from FI values of replicate analyses. The ratio between FIgm values for different analytical methods (indexed "a" and "b") is used for ANOVA testing of the significance of DNA profile quality differences. Method "a" generates DNA profiles of significantly higher quality compared to method "b" provided that the ratio is greater than the appropriate LSR.

4 PCR-inhibitory forensic samples

Some substances have been identified as PCR inhibitors, and completely or partly characterised regarding their molecular PCR-inhibitory mechanism(s) (Table 2). Any substance interfering with one or more of the reactions involved in PCR, thereby impairing reaction kinetics and the detection limit, is considered a PCR inhibitor (Rådström et al., 2004) (Figure 7). Categorising PCR inhibitors is therefore a difficult task. The exact mechanisms of most inhibitors are unknown, and the common classification is thus based on the affected targets, leading to three main groups of inhibitors: (1) DNA polymerase inhibitors, (2) nucleotide/nucleic acid inhibitors and (3) fluorescence inhibitors (qPCR specific).

PCR-inhibitory effects and mechanisms can be investigated by adding a single molecular PCR inhibitor to pure DNA in the reaction tube. Humic acid and haematin are the inhibitory molecules of choice for the validation of forensic DNA profiling kits by manufacturers (Applied Biosystems, 2009; Tucker et al., 2011). However, even if a PCR assay is tolerant to these substances when used separately, the success rate and detection limit may be seriously affected by the PCR-inhibitory compounds often present in complex crime scene samples (Hedman et al., 2011b).

Crime scene samples with heterogeneous backgrounds may contain mixtures of various PCR-inhibitory compounds, providing a more realistic inhibitor-tolerance test, but not ideal for studying PCR inhibition mechanisms. In this work, DNA extracts from chewing gum, cigarette butts and moist snuff generated severe inhibitory effects using certain DNA polymerases, whereas other DNA polymerases were virtually unaffected (Papers II and IV).

Table 2. Overview of PCR-inhibitory compounds and ions.

Type of inhibitor	Compound or ion	Source	Mechanism(s)[a]	Reference(s)
DNA polymerase inhibitors	Al^{3+} ions	Sampling using Al-shafted swabs	Alters ion composition	Wadowsky et al., 1994
	Bile salts	Faeces	Direct effect on polymerase	Abu Al-Soud et al., 2005; Lantz et al., 1997
	Ca^{2+} ions	Bone, milk	Competition with cofactor Mg^{2+}, alters ion composition	Bickley et al., 1996; Opel et al., 2010
	EDTA	Anticoagulant	Chelation of Mg^{2+}	Rossen et al., 1992
	Free radicals	UV treatment of PCR tubes	React with polymerase	Burgess and Hall, 1999; Fox et al., 2007; Tamariz et al., 2006
	Fulvic acid	Soil	Binds to polymerase	Kreader, 1996
	Gum	Chewing gum	Effect on polymerase activity	Paper IV
	Haem/haematin/ haemoglobin	Blood	Release of iron ions, competition with template, lowers polymerase processivity	Abu Al-Soud and Rådström, 2001; Akane et al., 1994; Opel et al., 2010
	Lactoferrin	Blood	Release of iron ions	Abu Al-Soud and Rådström, 2001
	Myoglobin	Muscle tissue	Release of iron ions	Belec et al., 1998
	Phenol	Soil, DNA purification	Denaturation of polymerase, binding to polymerase	Katcher and Schwartz, 1994
	Phytic acid	Faeces	Chelation of Mg^{2+} and alters ion content if present as salt	Thornton and Passen, 2004
	Polysaccharides	Faeces	Bind to polymerase	Monteiro et al., 1997
	Proteases (plasmin)	Milk	Degradation of polymerase	Powell et al., 1994
	Tannic acid	Leather, plants, soil	Interaction with polymerase, chelation of Mg^{2+} ions	Kreader, 1996; Opel et al., 2010
	Tobacco	Moist snuff	Effect on polymerase activity	Paper IV
	Urea	Urine	Prevents non-covalent bonding, acts directly on polymerase and/or prevents primer annealing	Khan et al., 1991; Mahony et al., 1998

Nucleic acid inhibitors	Bilirubin	Faeces	Competition with template	Abu Al-Soud and Rådström, 2001; Kreader, 1996
	Cellulose	Cigarette filter paper, sampling filters, wood	Binds to DNA, effect on polymerase activity	Bej et al., 1991; Lee and Cooper, 1995; Watanabe et al., 2003; Paper II and IV
	Collagen	Bone	Lowers polymerase processivity by binding to DNA, alteration of ion composition by binding cations	Opel et al., 2010; Scholz et al., 1998
	Ethanol	DNA extraction	Precipitation of DNA	Rossen et al., 1992
	Formaldehyde	Preservative	Interference with DNA and DNA polymerase	Johnson et al., 1995
	Heparin	Anticoagulant	Binds to DNA, competition with template and/or interaction with polymerase	Abu Al-Soud and Rådström, 2001; Satsangi et al., 1994
	Immunoglobulin G	Blood	Formation of a complex with single-stranded DNA	Abu Al-Soud et al., 2000
	Isopropanol	DNA extraction	Precipitation of DNA	Rossen et al., 1992
	Melanin	Skin, hair	Binds to DNA, binds to polymerase	Eckhart et al., 2000; Opel et al., 2010
	SYBR Green I	Detection dye	Binds to dsDNA with high affinity, binds to single-stranded DNA (primers), affects polymerase activity	Arezi et al., 2003; Gudnason et al., 2007; Zipper et al., 2004
Fluorescence inhibitors	Humic acids	Soil, bone	Quenches fluorescence, binds to DNA polymerase and to nucleic acids	Kreader, 1996; Opel et al., 2010; Sutlovic et al., 2008; Tsai and Olson, 1992a; b
	Indigo dye	Denim fabric	Hinders qPCR detection through strong colouring, interferes with SYBR Green I-DNA interactions	Opel et al., 2010
	Polymeric surfaces	Miniaturised real-time PCR instruments	Binding of detection dye	Gonzalez et al., 2007

a) Presumed mechanism(s). Inhibitors affecting the ion composition will affect the DNA polymerase (cofactor) and annealing properties of primers and probe system through nucleotides/nucleic acids and/or the qPCR fluorescence detection.

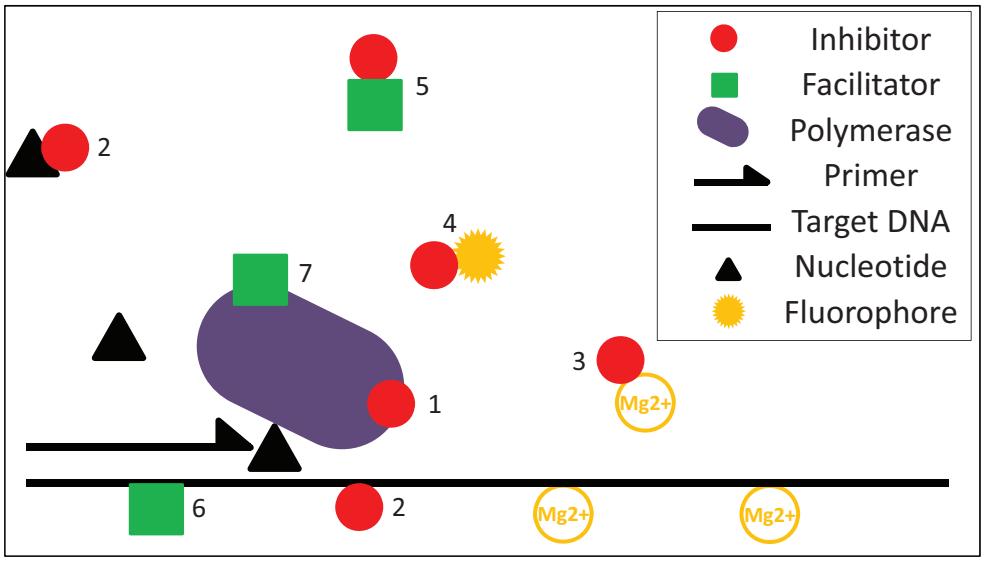

Figure 7. The complexity of PCR inhibition. A single PCR-inhibitory compound may affect the reaction in several ways, and different inhibitors can have different effects. Inhibitors may (1) bind to or degrade the DNA polymerase, (2) bind to nucleotides, primers or the DNA template, (3) chelate cations and/or (4) prevent the release of fluorescence from detection dyes. PCR facilitators (see Chapter 4.4) may counteract inhibition by (5) serving as a target for inhibitors or bind to inhibitors, (6) stabilising single-stranded DNA and/or (7) improving the activity of the DNA polymerase.

This indicates that these extracts primarily affect the DNA polymerase itself, not the nucleic acids (Table 2). For a substance that binds DNA, making it inaccessible for amplification, changing the type of DNA polymerase would arguably not affect amplification success in the same magnitude. Preliminary results show similar inhibitory effects for filter paper from "smoked" and "un-smoked" cigarettes, indicating that the cellulose in the paper could be the principal inhibitory compound in these samples, rather than tobacco residues (unpublished data). Gum and tobacco are probable inhibitory agents from chewing gum and moist snuff, respectively.

4.1 Thermostable DNA polymerases

High-quality thermostable DNA polymerases are a vital component of PCR (Mullis et al., 1990). Any compound that interferes with their enzymatic activity will reduce the formation of specific PCR products (Abu Al-Soud and Rådström, 1998; Akane et al., 1994) (Papers II and IV). The DNA polymerase obtained from *Thermus aquaticus* (*Taq*) and its commercial derivatives are most widely used because of their high thermostability and good processivity (Pavlov et al., 2004). Polymerases from a range of other organisms are also commercially available, e.g. *Thermus thermophilus* (*Tth*), *Thermus flavus* (*Tfl*) and *Pyrococcus furiosus* (*Pfu*); differing in catalytic properties (e.g. 3′-5′ and 5′-3′ exonuclease activity), extension rates, and fidelity (Cline et al., 1996; Pavlov et al., 2004). DNA polymerases from different sources also differ in their tolerance to various PCR inhibitors (Abu Al-Soud and Rådström, 1998). The choice of DNA polymerase influences the performance of all types of PCR assays, including qPCR (Wolffs et al., 2004) and multiplex STR analysis (Moretti et al., 1998). However, this fact is often overlooked, and most end-user laboratories and researchers simply use the DNA polymerase that is provided with the commercial kits they purchase.

Thermostable DNA polymerases have high optimal operating temperatures, e.g. 72°C for *Taq* polymerase, but show some activity at room temperature. To avoid the extension of non-specifically annealed primers and primer-dimers before starting thermal cycling, DNA polymerases may be temporarily inactivated. Since the amplification of non-specific fragments would complicate the interpretation of forensic DNA profiles, these hot-start DNA polymerases are generally applied. Temporary inactivation of DNA polymerases can be achieved either by binding a heat-sensitive antibody to the active site of the polymerase (Scalice et al., 1994) or by covalently binding a blocking molecule to lysine residues within and outside the active site (Birch et al., 1998). Blocking the lysine residues outside the active site may help inactivate the enzyme by steric hindrance of the substrate or by inducing conformational changes. Ex*Taq* Hot Start, PicoMaxx High Fidelity and various other DNA polymerases utilise an antibody-mediated hot-start, whereas Ampli*Taq* Gold is reversibly blocked by covalent bonding. Antibodies are released quite quickly at the denaturation temperature. The covalently bound blocking molecule is removed as an effect of the lowering of the pH of the PCR buffer at high temperatures, and activation takes about ten minutes at the denaturation temperature (Birch, 1996; Birch et al., 1998). Hot-start DNA polymerases provide higher amplicon yields than natural variants (Kebelmann-Betzing et al., 1998; Moretti et al., 1998), and also exhibit improved inhibitor-tolerance (Baar et al.,

2011); partly explained by the continuous release of active polymerase throughout PCR cycling.

Increasing the amount of DNA polymerase is one way of titrating out polymerase inhibitors, and has been shown to relieve inhibition caused by humic acid (Sutlovic et al., 2005). However, the success of this approach is limited, and depends on the amounts and types of inhibitors present. When analysing crime scene samples of different kinds, doubling the amount of Ampli*Taq* Gold improved amplification for moist snuff, but no or minor improvements were seen for chewing gum and cigarette butts (see Table 1 in Paper IV), indicating that inhibitors in the latter had a stronger negative effect on the DNA polymerase activity. Increasing the amount of DNA polymerase further was not beneficial, possibly due to increased glycerol concentrations in the reactions.

Several DNA polymerases originating from *Taq* are more susceptible to inhibitors found in blood, bone, foods, faeces and soil, than *Tth*-based polymerases (Abu Al-Soud and Rådström, 1998; Belec et al., 1998; Eilert and Foran, 2009; Katcher and Schwartz, 1994). Moreover, notable inhibitor-tolerance differences have been found between *Taq*-based polymerases (Eilert and Foran, 2009; Kermekchiev et al., 2009) (Papers II and IV).

Ampli*Taq* Gold is the DNA polymerase of choice in forensics worldwide since the introduction of STR profiling kits such as AmpFlSTR SGM Plus and PowerPlex 16, and is an integral part of the new AmpFlSTR NGM amplification kit. However, the resistance of Ampli*Taq* Gold to inhibitors from various sources is inferior to that of several alternative DNA polymerases (Abu Al-Soud and Rådström, 1998; Belec et al., 1998; Eilert and Foran, 2009; Kermekchiev et al., 2009). Customising the DNA polymerase-buffer system to suit the complex crime scene samples is one way of improving the analytical success. Using pre-PCR processing, I compared the performance of Ampli*Taq* Gold to 15 DNA polymerases not previously used in forensic DNA analysis. A qPCR model system for quantitative screening was developed for this purpose, and eight DNA polymerases were more thoroughly investigated. Standardised mock crime scene samples were analysed to establish AE_A, the dynamic range of amplification and the detection limit of the DNA polymerases, providing quantitative information on both polymerase tolererance to mock crime scene extracts and sensitivity (Paper II).

Bio-X-Act Short, Ex*Taq* Hot Start and PicoMaxx High Fidelity performed best, giving AE_A values from 0.93 to 1.12, a dynamic range of amplification from 2.6 to 3.3 log units and detection limits of 0.16 to 0.31 cell equivalents/µL (see Table 1 in

Paper II). These polymerases were virtually unaffected by the complex DNA extracts, and their amplification curves retained the sigmoidal shape and an amplitude well above the background, even for samples close to the detection limit.

Ampli*Taq* Gold enabled sensitive qPCR detection, with a detection limit of 0.31 cell equivalents/µL (see Table 1 in Paper II). However, it had the shortest dynamic range of amplification of all the polymerases tested: 1.3 log units compared with 3.3 log units for Bio-X-Act Short and PicoMaxx High Fidelity. The AE_A was 1.46, and Ampli*Taq* Gold gave higher values of C_q for the samples containing the highest levels of saliva, indicating that this polymerase can not tolerate high amounts of background compounds from these extracts (see Figure 1 in Paper II).

Tth and r*Tth*, which have shown high resistance to PCR inhibitors in other studies (Abu Al-Soud and Rådström, 1998; Belec et al., 1998; Katcher and Schwartz, 1994), gave poor results in the qPCR analysis of mock crime scene saliva samples (see Table 1 in Paper II). Their mean AE_A values were 1.38 (*Tth*) and 1.40 (r*Tth*), indicating that the polymerases were negatively affected by the extracts. The detection limit for both *Tth* and r*Tth* was 3.1 cell equivalents/µL, whereas the other polymerases had detection limits ranging from 0.16 to 0.63 cell equivalents/µL. Clearly, DNA polymerases performing well in certain assays and for certain types of problematic samples may not be suitable for PCR of other kinds of samples in other assays.

Bio-X-Act Short, Ex*Taq* Hot Start and PicoMaxx High Fidelity were applied in multiplex STR analysis of PCR-inhibitory forensic samples. The quality of the resulting DNA profiles was quantitatively assessed using FI, the ranking index developed in this work. The significance of differences in FI between the standard Ampli*Taq* Gold and the alternative DNA polymerases was established using ANOVA (10% level). All three of the alternative polymerases showed better amplification than Ampli*Taq* Gold, giving DNA profiles of significantly improved quality for 20 of 32 samples (Paper II).

Despite providing the most efficient amplification in both qPCR and the multiplex STR system, Bio-X-Act Short may not be appropriate for casework, due to its lack of hot-start properties. In order to avoid non-specific products, Bio-X-Act Short master mix must be prepared on ice and the reactions set up quickly, which is not practical in high-throughput casework analysis. Ex*Taq* Hot Start and PicoMaxx Hot Start showed complementary inhibitor-resisting properties for various crime scene samples. For example, Ex*Taq* Hot Start performed significantly better for moist snuff, and PicoMaxx High Fidelity for chewing gum and cigarette butts

(Paper IV). Thus, Ex*Taq* Hot Start is resistant to inhibitors in raw tobacco, whereas PicoMaxx High Fidelity tolerates higher levels of cellulose in the filter paper, as well as possible tobacco residues.

4.2 Polymerase engineering

During recent years, new DNA polymerase variants have been developed with the objective of improving PCR inhibitor-tolerance. Using a protein engineering approach, random mutations have been introduced into natural *Taq* and its N-terminally truncated version Klen*Taq* (Kermekchiev and Barnes, 2008; Kermekchiev et al., 2009). Subsequent screening for inhibitor-resistant properties led to the development of DNA polymerases with improved tolerance to blood and soil components, one bearing the commercial name Omni*Taq*. However, when applied in qPCR analysis of mock crime scene saliva samples in the present work, Omni*Taq* gave a dynamic range of amplification and detection limit identical to those of natural *Taq* (dynamic range: 2.6 log units, detection limit: 0.63 cell equivalents/µL for both polymerases) (see Table 1 in Paper II). This indicates that although Omni*Taq* is tolerant to various inhibitory compounds, it does not provide any improvements in terms of sensitivity and general amplification efficiency.

Molecular breeding and compartmentalised self-replication have been used in a previous study to form chimeric DNA polymerases with elements from various *Thermus* polymerases, e.g. *Taq*, *Tth*, *T. oshimai* and *T. brockianus* (Baar et al., 2011). One of these engineered DNA polymerases, called 2D9, contained 81 mutations compared to natural *Taq*, and showed considerable tolerance to inhibitors present in soil and bone. Since inhibitors such as these are likely to form colloids, it was suggested that 2D9 may interact less with colloids. However, 2D9 was not successful in the analysis of whole blood, indicating that it is not suitable for broad-range PCR-inhibitory crime scene samples.

Different polymerases clearly have different abilities to provide efficient amplification in various environments. Although highly resistant to blood, Omni*Taq* did not perform well with mock crime scene saliva samples, and its sensitivity and processivity were not as good as those of Bio-X-Act Short, Ex*Taq* Hot Start and PicoMaxx High Fidelity (Paper II). 2D9, on the other hand, tolerates humic acids present in bone and soil, but not blood inhibitors.

The different mechanisms and targets of PCR-inhibitory compounds may be part of the reason why none of the individual DNA polymerases discussed above can

provide universally robust and sensitive amplification. Nucleic acid inhibitors reducing the amount of available DNA call for DNA polymerases with high processivity, fast extension rates and low detection limits rather than high tolerance to extraneous substances. For inhibitory compounds affecting the DNA polymerase highly tolerant enzymes are needed, and a higher detection limit may be acceptable. The problem is that in crime scene samples a number of different inhibitors may be present, and the amount of DNA is generally low. Applying one specific DNA polymerase, such as Ex*Taq* Hot Start, Omni*Taq*, PicoMaxx High Fidelity or 2D9, may provide high-quality DNA profiles for certain problematic samples, but not for others. A different approach is needed to customise the DNA polymerase-buffer system for a broad range of PCR-inhibitory samples. Manipulating the PCR buffer, e.g. by adding facilitating substances, may be one possibility, as described in Chapter 4.4.

4.3 Blending DNA polymerases

Blending DNA polymerases is one way of achieving desirable properties in a PCR assay. This has been done previously to improve the fidelity of *Taq* polymerase assays, by adding a small amount of a polymerase with proofreading capacity (3′-5′ exonuclease activity), e.g. *Pfu* polymerase (Barnes, 1994; Cheng et al., 1994). In the present work, it was hypothesized that combining two complementary, inhibitor-tolerant DNA polymerases would provide a DNA polymerase-buffer system with more general inhibitor-resistant properties (Paper IV). Pure DNA and inhibitory crime scene samples were analysed using standard Ampli*Taq* Gold, Ex*Taq* Hot Start, PicoMaxx High Fidelity and a 1:1 blend of Ex*Taq* Hot Start and PicoMaxx High Fidelity. The same amount of DNA polymerase, 2.5 U, was used in all reactions to avoid possible inhibitor-relieving effects from using different amounts of active polymerase.

FI values were calculated from the resulting EPGs, allowing FIgms to be determined from replicate analyses of each sample with each DNA polymerase-buffer system (Figure 6). ANOVA was applied to ratios between FIgm values for different DNA polymerase-buffer systems to establish the significance of differences in the quality of the DNA profiles obtained.

The DNA polymerase blend amplified pure DNA with higher efficiency than Ampli*Taq* Gold, as reflected by their respective FIgm values of 9.62 and 7.11 (FIgm ratio =1.35, LSR=1.22, 10% significance level) (Paper IV). This may be due to higher processivity and extension rate. When used separately, Ex*Taq* Hot Start

and PicoMaxx High Fidelity provided DNA profiles of quality indistinguishable from that of Ampli*Taq* Gold.

Additionally, the DNA polymerase blend produced DNA profiles with significantly improved quality for 34 of 42 PCR-inhibitory crime scene samples, outperforming Ampli*Taq* Gold (FIgm ratios over LSR=1.62, 10% significance level) (Paper IV). When used separately, Ex*Taq* Hot Start and PicoMaxx High Fidelity improved the DNA profile quality for 26 and 23 samples, respectively. The blend performed at least as well as the best-performing individual polymerase for all the samples analysed.

The complementarity of Ex*Taq* Hot Start and PicoMaxx High Fidelity partly explains the superior performance of the DNA polymerase blend. For six especially problematic crime scene samples, the blend provided DNA profiles of significantly higher quality than both Ex*Taq* Hot Start and PicoMaxx High Fidelity used separately (Paper IV). This can not be explained by complementary effects, it rather suggests that there is synergy between the polymerases. It should also be noted that the blend consisted of 1.25 U of each polymerase, whereas 2.5 U of the individual polymerases was applied when Ex*Taq* Hot Start and PicoMaxx High Fidelity were used separately.

Two cigarette butts and two chewing gum samples that were successfully amplified using PicoMaxx High Fidelity and the blend, but generated blank EPGs using Ex*Taq* Hot Start, were re-analysed using 1.25 U PicoMaxx High Fidelity. The blend performed better than 1.25 U of PicoMaxx High Fidelity for all samples (FIgm ratios between 1.40 and 2.10, see Table S2 in Paper IV), indicating that Ex*Taq* Hot Start, although inactive when used on its own, showed some activity in the blend.

These results show that there is some kind of synergy between the two DNA polymerase-buffer systems when applied in the blend. Not only do the DNA polymerases function together; they amplify each other's performance. It has been reported that enzymes with different catalytic properties may be synergistic *in vivo*, enhancing the overall catalytic effect. For example, synergy between enzymes from *Aspergillus* has been found to improve the catalysis of cell wall polysaccharides (de Vries et al., 2000), and rumen enzymes have been found to exhibit synergistic effects with enzymes from *Trichoderma longibrachiatum*, enhancing feed digestion in cattle (Morgavi et al., 2000).

There may be several explanations of the synergy observed between DNA polymerases *in vitro*, i.e. in the PCR environment. No synergy was detected when

analysing pure DNA, indicating that the positive interaction indeed reflects improved performance on PCR-inhibitory crime scene samples. For example, one polymerase may initiate the reaction in the first few cycles, enabling the amplification of a low number of initial DNA molecules in the presence of inhibitory compounds. When a critical mass has been reached, the other polymerase, possibly with higher processivity and extension rate but lower tolerance to the inhibitors present, may be able to produce large numbers of amplicons. Using only the first polymerase would provide successful amplification but lower yield than the blend. If only the second polymerase is applied, amplification may not be possible.

The PicoMaxx High Fidelity system contains an accessory protein, purified from *Pfu*, which increases the processivity of *Pfu* DNA polymerase by binding the polymerase to the DNA more tightly (Hogrefe, 2002). The protein also affects *Pwo* DNA polymerases, but showed no effect for the *Taq* polymerases tested. However, Ex*Taq* Hot Start was not tested in that study, and thus, some of the synergistic effects of the Ex*Taq* Hot Start/PicoMaxx High Fidelity blend may arise from the accessory protein, by increasing the Ex*Taq* Hot Start processivity.

4.4 PCR buffer composition

Differences in AE_R and PCR inhibitor-tolerance between DNA polymerases can be explained to some extent by differences in the PCR buffers used, i.e. the pH and ion content and the presence of PCR facilitators (Abu Al-Soud and Rådström, 2000; Knutsson et al., 2002; Wolffs et al., 2004) (Table 3). For most *Taq* polymerases, a Tris buffer with pH 8.3 (measured at room temperature) is recommended. Elevating the pH of the Tris buffer to 9.0 or more has been shown to relieve inhibition caused by leukocytes (Nishimura and Nakayama, 1999). The reaction pH decreases at the elevated temperatures used for PCR, and using a buffer with a higher initial pH may provide a better environment for the DNA polymerase at the working temperature. However, as Ampli*Taq* Gold is activated at a pH below 7 (Birch et al., 1998), elevating the pH of the buffer may hinder activation. In the present work, Ampli*Taq* Gold failed to amplify pure DNA using a Tris buffer of pH 8.8 (unpublished data), indicating that the polymerase was not activated due to the higher pH. Replacing the Tris buffer with a zwitterionic buffer such as tricine may improve inhibitor-tolerance, as previously shown for direct amplification of whole blood (Yang et al., 2009).

4. PCR-inhibitory forensic samples

Table 3. PCR facilitators used to alleviate inhibition.

Type of facilitator [a]	Facilitator	Concentration	Source(s) of inhibition	Mechanism(s)[b]	Reference(s)
Protein	BSA	0.1-1.28 g/L	Bile salts, faeces, $FeCl_3$, fulvic acid, haemoglobin, humic acid, immunoglobulin G, lactoferrin, meat, melanin, proteases, saliva stains, tannic acid, waste water sludge	Binding of inhibitory compounds such as haem, fatty acids, melanin and phenol; competitive target for proteases	Abu Al-Soud et al., 2000; Abu Al-Soud and Rådström, 2000; 2001; Abu Al-Soud et al., 2005; Andreasson and Allen, 2003; Eckhart et al., 2000; Juen and Traugott, 2006; Kreader, 1996; Powell et al., 1994; Wang et al., 2007; Papers II and IV
	Casein	0.01% (w/v)	Bile salts, humic material, phenols	Binding of inhibitory compounds	Abu Al-Soud et al., 2005; Arbeli and Fuentes, 2007; De Boer et al., 1995
	Gp32[c]	0.02-0.15 g/L	Faeces, $FeCl_3$, fulvic acid, haemoglobin, humic acid, lactoferrin, meat, tannic acid	Enabling annealing by stabilising single-stranded DNA; binding to inhibitory compounds	Abu Al-Soud et al., 2000; Abu Al-Soud and Rådström, 2000; Chang et al., 2008; Kreader, 1996; Tebbe and Vahjen, 1993
	Lima bean trypsin inhibitor	0.02 g/L	Blood	Inhibition of protease activity	Abu Al-Soud and Rådström, 1998
	Phytase	50 U/mL	Phytic acid in faeces	Catalysing the hydrolysis of phytic acid	Thornton and Passen, 2004
	Protease inhibitor	1x	Faeces	Inhibition of protease activity	Abu Al-Soud and Rådström, 2000
Non-ionic detergent	NP-40 (Igepal)	0.8%	Blood	Improves cell lysis when whole blood is used in PCR	Zhang et al., 2010
	Tween 20	0.1-0.5 (w/v)	Faeces, phenolic compounds, plant polysaccharides	Reduction of false primer extension terminations	Abu Al-Soud and Rådström, 2000; Demeke and Adams, 1992; Innis et al., 1988; Simon et al., 1996
Organic solvent	DMSO	2-10%	GC-rich fragments	Destabilising nucleotide base pairing, lowering of DNA melting temperature	Pomp and Medrano, 1991; Sidhu et al., 1996

	Formamide	0.01% (w/v)	Bile salts, facilitate amplification for assays with insufficient thermal denaturation	Destabilising nucleotide base pairing, lowering of DNA melting temperature	Abu Al-Soud et al., 2005; Comey et al., 1991
	Propanediol	1 M	Haemoglobin	Destabilising nucleotide base pairing, lowering of DNA melting temperature	Horakova et al., 2011)
Biologically compatible solute	Betaine	5.9-11.7% (w/v)	Haemoglobin	Improves thermostability of DNA polymerase	Abu Al-Soud et al., 2000; Abu Al-Soud and Rådström, 2000
	L-carnitine	0.06-0.5 M	Blood	Protects reaction from osmotic stress	Zhang et al., 2010
	Sorbitol	2-30% (w/v)	Blood	Neutralises inhibitors	Yang et al., 2009
	Trehalose	2-30% (w/v)	Blood, haemoglobin, tobacco	Lowering of DNA melting temperature, improves thermal stability of DNA polymerase, neutralises inhibitors	Horakova et al., 2011; Spiess et al., 2004; Yang et al., 2009; Zhang et al., 2010; This thesis
Polymer	PEG 400	5-15%	Blood, faeces, polysaccharides	Stabilises DNA polymerase	Abu Al-Soud and Rådström, 2000; Demeke and Adams, 1992; Pomp and Medrano, 1991
Polyamine	Spermidine	0.5-4 mM	Blood, faeces	Binding to DNA, may stimulate DNA polymerase activity	Kato and Nishimura, 2002; Yang et al., 2009

a) Classification according to Abu Al-Soud, 2000
b) Presumed mechanism(s)
c) T4 bacteriophage gene 32 product

Increasing the amount of Mg^{2+} ions is a possible way of counteracting inhibition arising from chelating agents, DNA intercalating dyes or Ca^{2+} ions (Bickley et al., 1996; Nath et al., 2000). Apart from optimising the core reagents, PCR facilitators may be added to the buffer. PCR facilitators are substances that are not vital for amplification under ideal conditions, but may have a beneficial effect when analysing problematic samples. Various facilitators have been used to increase the specificity and fidelity of PCR, and several substances also have the capacity to relieve PCR inhibition (Figure 7 and Table 3).

Recently, manufacturers have started adding PCR facilitators to the buffers supplied with commercial DNA polymerases. Several buffers contain bovine serum albumin (BSA) and Tween 20, or have undisclosed contents, possibly containing various PCR facilitators. This is not always considered when comparing the amplification performance of DNA polymerases. The effect of PCR facilitators is concentration dependent, and overloading will result in inhibition of amplification (Ahokas and Erkkila, 1993; Rossen et al., 1992).

Different types of PCR facilitators may provide complementary or synergistic effects. Two recently developed facilitator blends have been shown to increase the tolerance to blood and soil inhibitors (Horakova et al., 2011; Zhang et al., 2010). Both blends applied the same osmoprotectant, the sugar trehalose, as a key component. Trehalose was complemented with the detergent NP-40 and L-carnitine, and propanediol, respectively. However, positive interactions between PCR facilitators depend on their nature, and should not always be expected (Abu Al-Soud and Rådström, 2000). On the contrary, combining facilitators may induce inhibition (Ahokas and Erkkila, 1993).

Trehalose may improve the thermal stability of DNA polymerases and have an inhibitor-neutralising effect, and is a promising agent for reducing inhibition in crime scene samples. In preliminary studies investigating the effects of various standardised PCR-inhibitory sample backgrounds, the addition of trehalose enabled amplification in the presence of moist snuff extract (Figure 8). Without trehalose, the amplification was completely inhibited. Using a Tris buffer with a pH of 8.8 instead of the standard pH 8.3 appeared to slightly improve the positive effect of trehalose.

BSA is the most commonly used PCR facilitator (Table 3). BSA is a blood-tissue transport protein that binds fatty acids (lipids) and other organic molecules. Its excellent binding capacity, and the fact that it does not affect the DNA polymerase, makes it suitable for relieving various types of PCR inhibition.

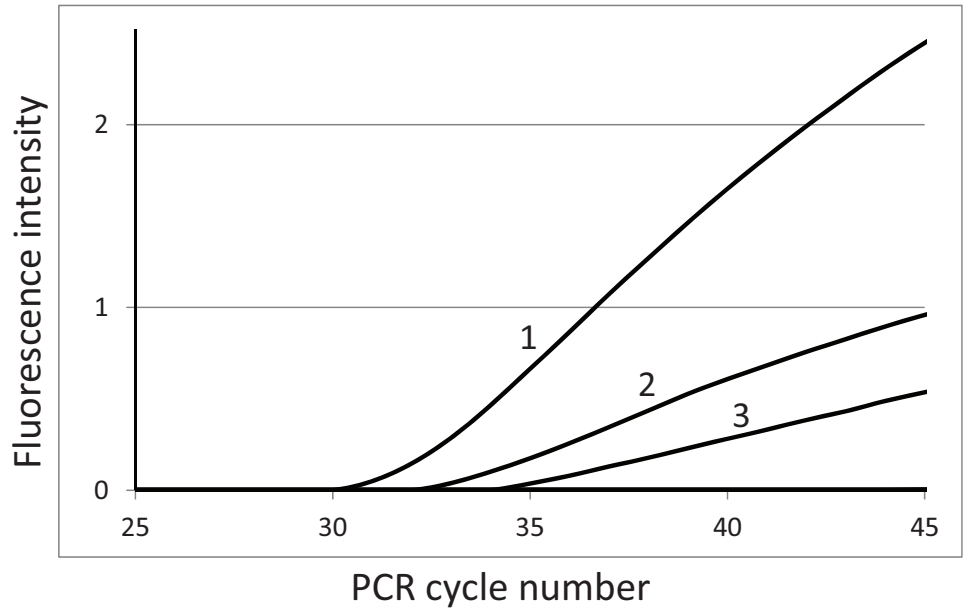

Figure 8. Illustration of the PCR inhibitor-relieving properties of trehalose. The amplification curves were generated using the qPCR assay described in Paper II with *Taq* DNA polymerase and 0.2 ng DNA. In reactions containing moist snuff extract and no trehalose (Tris buffers at pH 8.3 and pH 8.8) no amplification was noted due to complete inhibition. Curve 1 was obtained from a pure reaction without inhibitors or trehalose, curve 2 from a sample containing moist snuff extract, 0.2 M trehalose and a Tris buffer of pH 8.8, curve 3 from a sample containing moist snuff extract, 0.2 M trehalose and a Tris buffer of pH 8.3. All analyses were run in triplicates.

It has been suggested that BSA binds inhibitory compounds such as haem, phenols and melanin (Abu Al-Soud and Rådström, 2000; Eckhart et al., 2000; Kreader, 1996), thereby protecting the polymerase. BSA may also act as a competitive target for proteases (Powell et al., 1994).

A range of different BSA concentrations have been used to counteract inhibition, and the optimal concentration depends on the assay and the nature of the sample (Table 3). BSA has been shown to outperform other facilitators such as dimethyl sulphoxide (DMSO), polyethylene glycol (PEG) and Tween 20 for removing inhibitory effects from blood, faeces and meat samples (Abu Al-Soud and Rådström, 2000).

4.5 Casework analysis using a DNA polymerase blend

In November 2010, the Ex*Taq* Hot Start/PicoMaxx High Fidelity blend described in Paper IV was introduced into routine analysis of crime scene samples at SKL, following a decrease in quality of the standard AmpFlSTR SGM Plus method. At about the same time, a police force in Connecticut, USA, reported similar problems with the AmpFlSTR Identifiler kit, which also contains Ampli*Taq* Gold DNA polymerase (http://www.nbcconnecticut.com/news/local/Faulty-DNA-Kits-Could-Mean-Retesting-Hundreds-of-Cases-104597249.html, accessed 2011-09-08). When using commercial DNA typing kits, in-house quality control of new lots is vital to ensure that the supplied DNA polymerase-buffer system is compatible with the samples analysed at the laboratory (Albinsson et al., 2011). Different forensic laboratories use different DNA extraction and purification methods, affecting the content and purity of the DNA extracts. A DNA typing kit lot that has passed the manufacturer's quality control may therefore not be suitble for robust analysis at every laboratory.

When applying a new DNA polymerase-buffer system in casework, it is vital to validate allele call concordance with the previously used DNA polymerase. In PCR, the primers initiate amplification and determine which DNA fragments will be amplified. Apart from primer design and concentration, specificity is to some extent controlled by the ion content of the buffer, especially Mg^{2+} concentration, and the hot-start capacity of the DNA polymerases. Mg^{2+} ions increase the affinity between the primers and DNA. Thus, increasing the amount of Mg^{2+} ions increases the amplicon yield but decreases the specificity. Ex*Taq* Hot Start and PicoMaxx High Fidelity are hot-start DNA polymerases, minimising the risk of generating non-specific products. In in-house validation of the Ex*Taq* Hot Start/PicoMaxx High Fidelity blend, 70 reference samples were analysed, showing 100% concordance with the Ampli*Taq* Gold system, with no artefact peaks (Hedman et al., 2011c).

In-house validation showed that column DNA purification and dilution slightly improved STR analysis using standard Ampli*Taq* Gold, but had the reverse effect for the customised DNA polymerase blend (Hedman et al., 2011c) (Table 4). Irrespective of sample treatment and input amount of blood, the blend performed significantly better than Ampli*Taq* Gold, as shown by large FIgm ratios (triplicate analysis, LSR=1.48, 10% level). The blend obviously tolerated the extraneous compounds in the samples, producing high-quality DNA profiles for crude Chelex extracts, and purification decreased DNA profile quality by reducing the amount of available DNA.

Table 4. Evaluation of the effects of three sample treatment methods using AmpFlSTR SGM Plus with standard Ampli*Taq* Gold or an Ex*Taq* Hot Start/PicoMaxx High Fidelity blend.

DNA polymerase-buffer system	Amount of blood (µL)	Chelex extraction (FIgm[a])	Chelex + dilution 1:2[b] (FIgm)	Chelex + Microsep column purification[c] (FIgm)
Ampli*Taq* Gold	0.1	0.05	0.8	0.9
	0.25	0.05	0.08	1.6
	0.5	0.10	0.9	2.7
	1	0.05	0.5	5.0
Ex*Taq* Hot Start/PicoMaxx High Fidelity blend	0.1	4.8	1.8	3.2
	0.25	10.9	5.6	2.5
	0.5	13.7	10.3	4.4
	1	13.1	13.3	8.8

a) DNA profile quality expressed as geometric means of FI (FIgm, triplicate analysis). FIgm values below 1 indicate partial or negative DNA profiles; FIgm values between 1 and 2 indicate complete DNA profiles with low peak heights; FIgm values above 2 indicate complete DNA profiles with peak heights well above threshold levels

b) DNA extracts diluted in deionised water prior to PCR

c) DNA extracts purified using Microsep columns (Pall), with TE buffer. The customised DNA polymerase blend produced complete DNA profiles for all analysed samples (FI values over 1), whereas column purification was necessary for analytical success using standard Ampli*Taq* Gold.

The success rate of casework samples at SKL was considerably improved by introducing the Ex*Taq* Hot Start/PicoMaxx High Fidelity blend in routine analysis (Table 5). Compared to previous, "normally" performing AmpFlSTR SGM Plus lots, the proportion of complete DNA profiles generated from impure samples was increased from 38% to 87% for saliva, and from 69% to 94% for blood.

Table 5. Assessment of routine DNA analysis of crime scene stain extracts using different DNA polymerase-buffer systems.

DNA polymerase-buffer system	Sample type	Complete DNA profiles[b] (%)	Partial DNA profiles[c] (%)	Blank/negative DNA profiles (%)
Ampli*Taq* Gold, lot 1[a]	Blood (n=411)	69	26	4
	Saliva (n=430)	38	47	15
Ampli*Taq* Gold, lot 2[a]	Blood (n=314)	7	53	40
	Saliva (n=413)	4	43	53
Ex*Taq* Hot Start/PicoMaxx High Fidelity blend[a]	Blood (n=229)	94	2	4
	Saliva (n=212)	87	8	5

a) The AmpFlSTR SGM Plus primer set was used in all analyses. DNA concentrations were 0.025-0.15 ng/µL. Ampli*Taq* Gold lot 1 is a "normally" performing lot, and lot 2 is the one that performed poorly.
b) Complete DNA profiles are defined as having 10 complete STR markers with heterozygote peak heights above 200 RFU
c) Partial profiles have at least one labelled allelic peak, i.e. above 50 RFU

Extensive DNA purification may not be the first method of choice in efforts to improve the analytical success of PCR-inhibitory crime scene samples, since it increases the workload, reduces the DNA yield and involves an elevated risk of contamination. The pre-PCR processing approach described in this thesis proved to be a powerful way of relieving PCR-inhibitory effects, without introducing complex sample processing steps or risking loss of valuable evidence material. The polymerase blend is presently applied at SKL for analysis of low-template DNA samples, i.e. problematic crime scene samples with low amounts of target DNA.

5 Conclusions

The following conclusions can be drawn from the work presented in this thesis:

- The developed screening tool for saliva on trace swabs is sensitive, and can be used to identify samples likely to produce usable DNA profiles, e.g. in high-volume crime, where the number of DNA analyses is limited due to cost. Since there is no direct correlation between salivary amylase activity and DNA content, the screening tool should not be used to remove samples from DNA analysis if biological evidence is scarce.

- The developed ranking index, FI, enables unbiased, quantitative comparisons of the quality of EPGs. Statistical testing can be performed on FIgm values to determine the significance of differences in quality. FI can be applied to any STR DNA typing system using the validation and calibration methodology described in this thesis. Choosing a representative calibration set of EPGs and defining a relevant quality scale for validation are key parameters when customising FI for a new STR assay.

- The inhibitor-tolerance of forensic DNA analysis was improved by using pre-PCR processing to customise the DNA polymerase-buffer system to the chemical content of the samples. In this work it was shown that the DNA polymerases Bio-X-Act Short, Ex*Taq* Hot Start and PicoMaxx High Fidelity are more tolerant to various PCR-inhibitory substances present in forensic samples than the commonly used polymerase Ampli*Taq* Gold. Moreover, Ex*Taq* Hot Start and PicoMaxx High Fidelity have complementary abilities to generate efficient amplification in the presence of different PCR-inhibitory compounds. For example, Ex*Taq* Hot Start is better in the analysis of moist snuff samples, while PicoMaxx High Fidelity is better suited for the analysis of chewing gum and cigarette butts.

- Blending complementary inhibitor-tolerant DNA polymerases was found to further improve resistance to PCR inhibitors. A customised blend of Ex*Taq* Hot Start and PicoMaxx High Fidelity showed resistance to a broader range of inhibitory crime scene samples than either of the polymerases when used separately. The improved tolerance of the DNA polymerase blend is partly explained by their complementarity, and partly by synergy. Introducing the customised DNA polymerase blend in casework improved the success rate of crime scene sample analysis at SKL.

6 Future perspectives

STR markers have been applied in forensic casework for almost two decades and are likely to form the basis of routine forensic DNA analysis in the foreseeable future thanks to their good discrimination power. In addition, a change of system would mean that the DNA of millions of samples recorded as STR DNA profiles in national DNA databases worldwide would have to be re-analysed. In order to further increase the value of forensic DNA evidence, future research and development should be focused on sensitive, robust and cost-effective high-throughput analysis.

The recently released commercial STR DNA typing kits AmpFlSTR NGM and PowerPlex ESI/ESX (Promega) show improved amplification of problematic crime scene samples compared to AmpFlSTR SGM Plus (Hedman et al., 2011b). However, their detection limits are affected by the presence of inhibitory compounds. The use of pre-PCR processing to customise the chemistry of the PCR to the content of crime scene samples may improve the amplification of dirty samples to levels obtained with pure samples. In this work, a blend of two DNA polymerases was used. Adding other types of DNA polymerases with tolerance to specific inhibitors may provide a means of obtaining a truly universal PCR master mix. Optimising the buffer composition is an important task, as modifying the buffer content may in itself counteract inhibition.

Applying a more inhibitor-tolerant PCR master mix will reduce the need for pure DNA extracts. Recent research has shown that if an inhibitor-resistant DNA polymerase with suitable PCR facilitators and buffer is used, biological samples can be loaded directly into the PCR tube, without prior DNA extraction. However, sample treatment will always be necessary in forensic DNA analysis to release the cells from swabs or other materials. A one-tube sample treatment process is preferable to reduce the risk of contamination and loss of target DNA when transferring samples. Simple elution followed by cell lysis may provide efficient amplification when using an optimal DNA polymerase-buffer system. However, more rigorous DNA purification may be needed for long-term storage of DNA, to avoid degradation.

As a result of the research on improving the inhibitor-tolerance of PCR, the bottleneck for successful DNA analysis is shifting from the purity of the DNA extracts to the efficiency of sampling and release of cells. So far, efforts have been focused on development and quality assurance of sensitive and highly discriminatory PCR-based identification systems, while little has been done to

develop and standardise sampling methods. Further investigations into alternatives to cotton swabs, such as nylon flocked swabs and sponge materials, are therefore necessary. In the food and feed industry there are precise international standards governing sampling for PCR-based analysis. These standards define where samples should be taken, the size of the sampling surface, how the swab should be held, how many times the area should be swabbed and which buffer must be used to moisten the swab. Standards such as these may be difficult to apply in forensic DNA analysis due to the wide variety of evidence, but increasing the level of standardisation should be considered. Optimising the elution of cells, for example, by customising the sample treatment buffer, is another vital factor. The elution buffer must be compatible with the chemistry of the DNA polymerase-buffer system.

PCR is the backbone of forensic DNA analysis. It is well-known that non-ideal reaction conditions lead to imbalanced EPGs with low peak heights and drop-outs. However, several issues regarding how the chemical reactions in PCR are affected by inhibitors and low levels of input DNA are still unknown. Attempts have been made to model forensic STR DNA typing to determine the probability of drop-outs (Gill et al., 2005; Haned et al., 2011) and to investigate the occurrence of stutter alleles (Weusten and Herbergs, 2011). However, these models are based on the assumption that the amplification efficiency is constant throughout PCR, and that the efficiency is the same for all amplicons in multiplex STR analysis. In reality the efficiency is not constant and it may differ between amplicons due to differences in length and base sequence. Gill et al. (2005) came to the conclusion that, with constant amplification efficiency, imbalances between heterozygote peaks, and the incidence of drop-outs, are the effects of transferring different numbers of the different chromosomes to the PCR. Although this may have an effect, the stochasticity of primer annealing and extension during the first few cycles of PCR arguably has a greater influence on the allele balance in EPGs. Booth et al. (2010) modelled PCR in more detail, splitting up amplification into a set of reactions and taking the varying amplification efficiency into account.

It may be possible to create a mathematical model that takes the inherent dynamics of PCR into account by complementing the concepts presented by Gill et al. (2005), Weusten and Herbergs (2011) and Booth et al. (2010) with the effects of analysing low levels of DNA and PCR inhibitors that interfere with particular reactions in PCR. This would improve our understanding of the reasons behind poor heterozygote balances and drop-out when analysing problematic crime scene samples. Developing such a model and implementing it into casework may lead to

a new way of evaluating EPGs, in which discrete peak height and peak balance thresholds are replaced by continuous scales based on the overall intensity and degree of balance of the EPG.

The findings presented in this thesis show that pre-PCR processing offers a powerful means of improving the success rate of forensic DNA analysis. Further improvements can be made by optimising sampling and sample treatment, and customising them to the PCR chemistry. Detailed modelling of PCR, involving specific inhibitory effects, may increase our knowledge on how impaired PCR kinetics affects the resulting DNA profiles.

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