# Research Reports

## Improved forensic DNA analysis through the use of alternative DNA polymerases and statistical modeling of DNA profiles

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DNA evidence, linking perpetrators to crime scenes, is central to many legal proceedings. However, DNA samples from crime scenes often contain PCR-inhibitory substances, which may generate blank or incomplete DNA profiles. Extensive DNA purification can be required to rid the sample of these inhibitors, although these procedures increase the risk of DNA loss. Most forensic laboratories use commercial DNA amplification kits (e.g., Amp*Fl*STR SGM Plus) with the DNA polymerase Ampli*Taq* Gold as the gold standard. Here, we show that alternative DNA polymerase–buffer systems can improve the quality of forensic DNA analysis and efficiently circumvent PCR inhibition in crime scene samples, without additional sample preparation. DNA profiles from 20 of 32 totally or partially inhibited crime scene saliva samples were significantly improved using Bio-X-Act Short, Ex*Taq* Hot Start, or PicoMaxx High Fidelity instead of Ampli*Taq* Gold. A statistical model for unbiased quality control of forensic DNA profiles was developed to quantify the results. Our study demonstrates the importance of adjusting the chemistry of the PCR to enhance forensic DNA analysis and diagnostic PCR, providing an alternative to laborious sample preparation protocols.

### Introduction

Physical evidence, especially DNA, is increasingly important in the investigation of criminal cases and in the court of law in today's society. Diagnostic DNA analysis, including forensic applications, is often limited by components that interfere with the amplification, so-called PCR inhibitors (1). Several substances have been identified as PCR inhibitors, and some have been characterized with respect to their PCR-inhibitory mechanism(s) (2). The problem of inhibition is especially prominent in forensic DNA analysis, due to the nature of the sampling environment at crime scenes (3–5). The consequences of PCR inhibition in forensic casework may be severe. Failure to produce a DNA profile from a crime scene stain may leave a case unsolved or a person wrongly accused. As an illustration, a police force in the UK reported that 57% of swabs from bottles and cans failed to produce acceptable DNA profiles (6), which could be explained by a lack of sufficient amounts of DNA or the presence of PCR inhibitors. The common approach to overcoming PCR inhibition is extensive DNA purification (3–5). In special cases, isolation of single cells using laser-capture microdissection can be used (7). Simple dilution of the extract may be applied (8,9), but only if the DNA concentration is sufficiently high.

It has previously been observed that thermostable DNA polymerases of different origins may have different abilities to withstand the effects of various PCR inhibitors (10). For example, DNA polymerases from *Thermus aquaticus* (*Taq*) are more sensitive to the PCR inhibitors in human blood [i.e., hemoglobin, immunoglobulin G, and lactoferrin (11,12)] than DNA polymerases from *Thermus thermophilus* (*Tth*). Protein engineering has recently been used to improve the tolerance of *Taq* to PCR inhibitors in blood and soil in an attempt to develop a better *Taq* DNA polymerase for diagnostic PCR (13). A derivative of *Taq*, Ampli*Taq* Gold (Applied Biosystems, Foster City, CA, USA), is currently the standard DNA polymerase in several forensic DNA typing kits in use worldwide, including the widely used kits Amp*Fl*STR SGM Plus (Applied Biosystems) and PowerPlex 16 (Promega, Madison, WI, USA).

The objective of this study was to systematically investigate the potential of several DNA polymerase–buffer systems not currently used in forensic DNA analysis. The success rate of casework DNA samples was also investigated to evaluate the impact of PCR inhibition in routine analysis at the Swedish National Laboratory of Forensic Science (SKL). We present an approach to improve the quality of forensic DNA analysis and at the same time circumvent PCR inhibition in crime scene DNA samples. Modifying the PCR chemistry by employing alternative DNA polymerases and PCR facilitators was found

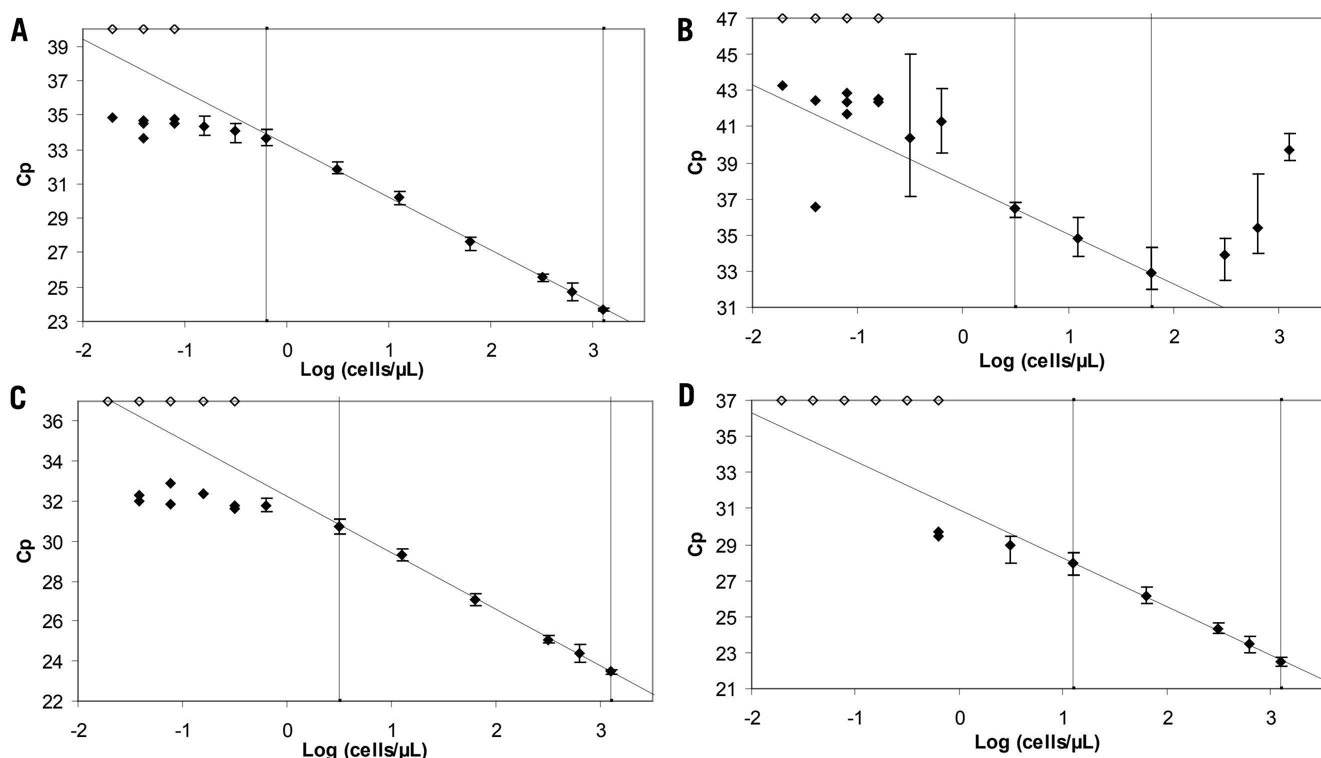


**Figure 1. Standard curves illustrating real-time PCR analysis of mock crime scene saliva samples using four different DNA polymerases.** (A) Bio-X-Act Short. (B) Ampli*Taq* Gold. (C) *Taq*. (D) *Tth*. Filled symbols with error bars show the average value of crossing points (Cp) for four replicates, and the error bars show the range of the measurements. Filled symbols without error bars show the results of single measurements of concentrations giving both positive and negative results. Unfilled symbols represent measurements in which amplification failed. The dynamic range of amplification is indicated by dotted vertical lines.

to be a successful approach to generate high-quality DNA profiles without using additional sample preparation. The results were evaluated on two different instrumental platforms, and a statistical model for unbiased quality control of forensic DNA profiles was developed to quantify the results.

## Materials and methods

The amplification efficiency (AE), dynamic range of amplification, and detection limit of nine DNA polymerase–buffer systems were evaluated on mock crime scene saliva samples using a standardized forensic singleplex real-time PCR assay. The three best-performing systems were then assessed under routine conditions at SKL. Short tandem repeat (STR) analysis was performed on 32 inhibited real crime scene saliva samples with low/medium DNA concentrations which had previously failed to produce complete forensic DNA profiles, to assess possible improvements compared with the standard method using Ampli*Taq* Gold. Finally, a statistical tool was developed to assess the quality of the forensic DNA profiles (i.e., capillary electrophoresis electropherograms) in an unbiased way.

### Success rate of routine DNA profiling of crime scene saliva stains

We investigated the success of DNA typing of 1936 crime scene saliva samples (with DNA concentrations of 0.025–0.25 ng/μL) from volume crimes analyzed at SKL during 2007. DNA extraction was performed using Chelex beads (Bio-Rad Laboratories, Hercules, CA, USA) (14) with the addition of Centricon (Millipore, Billerica, MA, USA) purification (15) for visibly dirty samples. Ten microliters of DNA template was used in 25 μL Amp*Fl*STR SGM Plus PCR reactions. Mastermix preparation and PCR programming was performed according to the manufacturer's recommendations (Amp*Fl*STR SGM Plus PCR Amplification Kit User's Manual).

### Preparation and analysis of standardized mock crime scene saliva samples

Samples of saliva corresponding to 250,000; 125,000; 62,500; 12,500; 2500; 625; 125; 62; 32; 16; 8; and 4 epithelial cells were put into two series of tubes containing a cotton swab (SelefaTrade, Spånga, Sweden) used to swab 4 cm² of clean, sterilized window glass. Cells were counted using a Bürker chamber (Hawksley, Sussex, UK). Sterile saline was used for dilution and moistening of the swabs. DNA was extracted using Chelex (14). One negative extraction control was added for each extraction batch. The extraction volume was 200 μL. A singleplex real-time PCR assay amplifying a 156-bp gene fragment was used (16,17). The polymerases investigated were Ampli*Taq* Gold (modified *Taq*), Bio-X-Act Short (undisclosed blend of enzymes) (Bioline, London, UK), Ex*Taq* Hot Start (modified *Taq*) (Takara Bio Inc., Shiga, Japan), KAPA2G Robust (*Taq* mutant) (KAPA Biosystems, Cape Town, South Africa), Omni*Taq* (*Taq* mutant) (DNA Polymerase Technologies, St. Louis, MO, USA), PicoMaxx High Fidelity (a mixture of recombinant *Taq* and cloned *Pyrococcus furiosus* polymerase) (Stratagene, La Jolla, CA, USA), r*Tth* (recombinant *Tth*) (Applied Biosystems), *Taq*, and *Tth* (both, Roche Diagnostics, Mannheim, Germany).

Each reaction contained 1 U polymerase, 1× polymerase-specific PCR buffer, 0.2 mM dNTP (Roche Diagnostics), 3.5 mM $MgCl_2$ (in total), 0.5 μg/μL BSA (Roche Diagnostics), 0.3 μM forward primer (RB1 80 F), 0.3 μM reverse primer (RB1 235 R), and 0.2 μM *Taq*Man MGB probe (RB1 212 MGB, Fam-labeled). Autoclaved MilliQ water (Millipore) was added to a total master mix volume of 12 μL for each reaction. Eight microliters of DNA template was added,

giving a final reaction volume of 20 µL. Negative amplification controls were used. Primers were purchased from MWG Biotech AG (Ebersberg, Germany) and the *Taq*Man MGB probe from Applied Biosystems. A LightCycler 2.0 (Roche Diagnostics) was used for thermal cycling and detection, using the following PCR program: 95°C for 1 min; 50 cycles of 95°C for 0 s, 60°C for 20 s, and 72°C for 20 s; and 40°C for 30 s. For Bio-X-Act Short, the extension temperature was 68°C. For Ampli*Taq* Gold and PicoMaxx High Fidelity, the initial 95°C step was lengthened to 10 min.

Results are given as crossing points (Cp)—that is, the fractional cycle number at which the second derivative of the amplification curve is a maximum. The two saliva dilution series were amplified in duplicate, giving four results for each level of dilution. Standard curves were prepared by plotting Cp values against the logarithm of the cell concentration (cells/µL) in the DNA template. The slope of the standard curve within the dynamic range of amplification was estimated using the regression tool in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The slope was used to calculate the PCR efficiency using the equation $E = 10^{(-1/\text{slope})}-1$. A slope of –3.32 gives the ideal efficiency of 1.0. The amplification efficiency was calculated for each of the four data sets. Two detection limits were defined: 100%, the lowest dilution giving positive results for all four replicates; and 50%, the lowest dilution giving positive results for two of the four replicates.

**Table 1. Performance of nine commercially available DNA polymerase–buffer systems on mock crime scene saliva samples**

| DNA polymerase–buffer system | Mean amplification efficiency ± SD | Dynamic range of amplification (log units) | Detection limit, 100% (cell equivalents/µL) | Detection limit, 50% (cell equivalents/µL) |
|---|---|---|---|---|
| Ampli*Taq* Gold | 1.46 ± 0.67 | 1.3 | 0.31 | 0.04 |
| Bio-X-Act Short | 1.12 ± 0.06 | 3.3 | 0.16 | 0.04 |
| Ex*Taq* Hot Start | 0.99 ± 0.05 | 2.6 | 0.31 | 0.16 |
| KAPA2G Robust | 1.08 ± 0.11 | 2.0 | 0.63 | 0.63 |
| Omni*Taq* | 0.95 ± 0.04 | 2.6 | 0.63 | 0.04 |
| PicoMaxx High Fidelity | 0.93 ± 0.05 | 3.3 | 0.31 | 0.04 |
| r*Tth* | 1.40 ± 0.10 | 2.0 | 3.1 | 0.63 |
| *Taq* | 1.26 ± 0.10 | 2.6 | 0.63 | 0.04 |
| *Tth* | 1.38 ± 0.23 | 2.0 | 3.1 | 0.63 |

Real-time PCR analysis was used to investigate the amplification efficiency, dynamic range of amplification, and detection limit.

## STR analysis of inhibited crime scene saliva samples

Thirty-two crime scene saliva samples, previously giving no or incomplete DNA profiles, were amplified in duplicate using the Amp*Fl*STR SGM Plus primer set with each of the three best-performing DNA polymerase–buffer systems from the first study (i.e., Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity), and Ampli*Taq* Gold as reference. Sixteen samples were extracts from cigarette butts, and 16 were extracts from cans, bottles, or foodstuffs collected with cotton swabs. For Ampli*Taq* Gold, the reaction mix was prepared according to the manufacturer's recommendations (Amp*Fl*STR SGM Plus PCR Amplification Kit User's Manual), and 10 µL DNA template was used with 15 µL mastermix in the PCR. For Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity, reaction mixes were prepared using 2.5 U polymerase, 1× polymerase-specific PCR buffer, 0.2 mM dNTP, 0.25 µg/µL BSA, and 5.5 µL Amp*Fl*STR SGM Plus primer mix. For Bio-X-Act Short, 1 mM $MgCl_2$ was added (Ex*Taq* Hot Start and PicoMaxx High Fidelity buffers contain $MgCl_2$). Autoclaved MilliQ water was added, giving a final master mix volume of 16.5 µL, of which 15 µL was used together with 10 µL DNA template in each reaction. Thermal cycling was performed on a GeneAmp PCR System 9700 (Applied Biosystems) using the standard PCR program (Amp*Fl*STR SGM Plus PCR Amplification Kit User's Manual), with the exception of Bio-X-Act Short, for which the following program was used: 94°C for 5 min; 28 cycles of 94°C for 60 s, 59°C for 60 s, and 68°C for 60 s; 68°C for 45 min; and store at 10°C. An ABI 3130*xl* Genetic Analyzer (Applied Biosystems) was used for fragment separation, and DNA profiles were evaluated using GeneMapper ID software, Version 3.1 (Applied Biosystems).

## A statistical quality model for forensic DNA profiles

The tool developed to assess the quality of forensic DNA profiles is based on principal component analysis (PCA) (18) using three factors: (*i*) the total peak height (TPH) of the capillary electrophoresis electropherograms [i.e., the sum of the heights of the observed STR peaks given in relative fluorescent units (rfu)], (*ii*) the mean local balance (MLB) (i.e., the mean of intra-locus balances or discrepancies between peak heights within a heterozygous STR marker), and (*iii*) the Shannon entropy (SH) (19) (i.e., discrepancies, or inter-locus balance, between the sum of the peak heights between the markers). The higher the value of the TPH, the higher the quality of the forensic DNA profile, provided fluorescence saturation is avoided by not overloading sample. The intra-locus balance for a marker is defined as the ratio given by dividing the height of the lower peak in a heterozygous marker by that of the higher, giving a marker-specific ratio varying from 0 to 1. For a homozygous marker, the measure is defined as 1, while for a false homozygous marker it is defined as the lowest balance obtained within the calibration set of electropherograms. High values of this measure therefore imply good balance. The MLB is a global measure of local balance, obtained by calculating the mean of these measures for all the markers analyzed. In this way, the MLB is consistent with the TPH in that the higher the value, the higher the quality. The Shannon entropy was defined as

$$\mathrm{SH} = -\sum_{i=1}^{M} p_i \cdot \ln(p_i), \qquad \text{[Eq. 1]}$$

where $p_i$ is the proportion of summed peak heights in STR marker $i$ of the TPH, and $M$ is the number of STR markers investigated (in this case 10). SH varies between 0 and $\ln(M)$, where 0 is obtained when only one marker has observable peaks, and $\ln(M)$ is obtained when the sums of the peak heights in all markers are equal. Here, 10 STR markers are used, giving 2.30 as the highest possible value for SH.

The measures TPH, MLB, and SH can be used separately or combined to form a univariate measure, according to the linear combination

$$\mathrm{I} = a_1 \cdot \mathrm{TPH} + a_2 \cdot \mathrm{MLB} + a_3 \cdot \mathrm{SH}, \qquad \text{[Eq. 2]}$$

where $a_1$, $a_2$, and $a_3$ are chosen constants. PCA was used as a data reduction method, and the first principal component was proven to provide sufficient discrimination between higher- and lower-quality electropherograms/DNA profiles (i.e., the reduction in eigenvalues between the successive principal components was such that the first component would suffice). This was shown by carrying out PCA on a

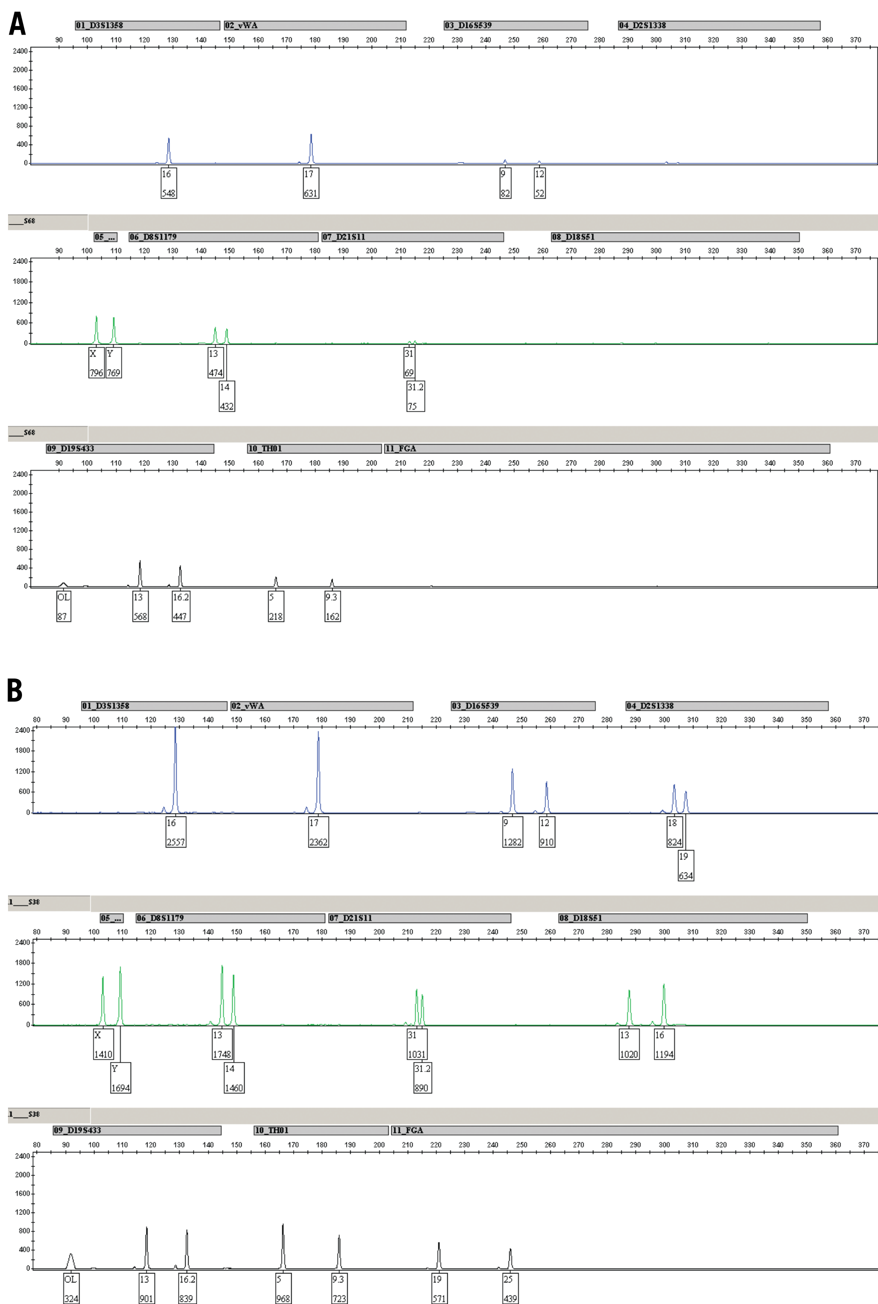


**Figure 2. Electropherograms showing improved PCR amplification using an alternative DNA polymerase.** A crime scene cigarette butt (Sample 5 in Table 2) was analyzed using (A) standard Ampli*Taq* Gold DNA polymerase and (B) PicoMaxx High Fidelity. Ten short tandem repeat (STR) loci are presented together with the gender marker amelogenin. The heights of the allelic peaks are given in relative fluorescence units (rfu). The electropherogram resulting from Ampli*Taq* Gold amplification shows that shorter STR markers were amplified, whereas amplification of longer STR markers failed, a phenomenon which indicates PCR inhibition. With PicoMaxx High Fidelity, all 10 STR loci contain distinct allelic peaks, with peak heights well above the baseline.

calibration set consisting of 446 representative DNA samples showing high-quality DNA profiles. Each of the original variables, TPH, MLB, and SH, was standardized (by subtracting the sample mean and dividing by the standard deviation for that sample) before PCA was applied. The standardized variables are denoted tph, mlb, and sh. The coefficients of these components were all found to be positive, which confirms that they form a basis for a final measure. At this stage, the coefficients only reflect the correlations between the original measures TPH, MLB, and SH within the calibration set. To enhance the discriminating power of this measure, we applied a manual grading scale from 1–20. This scale was based on the knowledge of SKL's experienced reporting officers of the relationship between TPH and a high quality electropherogram/DNA profile, combined with a measure of how close MLB and SH are to their respective maxima. Each profile in the calibration set was graded using this scale, and cross-validation (20) was applied to "shrink" the coefficients obtained for the retained principal component (PC) so that the component becomes an optimal linear predictor of the scores on the grading scale. More specifically, if $a_1$, $a_2$, and $a_3$ are the coefficients of the first principal component obtained from the calibration set, and $g$ denotes the score on the grading scale for a profile, the modified version ($PC_s$) becomes

$$PC_s = c_1 \cdot a_1 \cdot \mathrm{tph} + c_2 \cdot a_2 \cdot \mathrm{mlb} + c_3 \cdot a_3 \cdot \mathrm{sh},$$

[Eq. 3]

where $c_1$, $c_2$, and $c_3$ are constants chosen so that the square sum of leave-one-out cross-validation prediction errors obtained from a linear prediction model $\hat{g} = b_0 + b_1 \cdot PC_s$ applied to all profiles is minimized ($b_0$ and $b_1$ being the least-squares estimated parameters of a linear regression model).

As $PC_s$ is calculated using standardized values of TPH, MLB, and SH, its values will vary around zero. To make the measure easier to interpret and to obtain a well-defined zero, the values of $PC_s$ are transformed by adding the expression

$$a_1 \cdot b_1 \cdot \frac{\overline{\mathrm{TPH}}}{s_{\mathrm{TPH}}} + a_2 \cdot b_2 \cdot \frac{\overline{\mathrm{MLB}}}{s_{\mathrm{MLB}}} + a_3 \cdot b_3 \cdot \frac{\overline{\mathrm{SH}}}{s_{\mathrm{SH}}},$$

[Eq. 4]

where $\overline{\mathrm{TPH}}$, $\overline{\mathrm{MLB}}$, and $\overline{\mathrm{SH}}$ are the sample means and $s_{\mathrm{TPH}}$, $s_{\mathrm{MLB}}$, and $s_{\mathrm{SH}}$ are the sample standard deviations of the three original measures in the calibration set. We call the resulting transformed measure the forensic DNA profile index (FI). With the calibration set used in this study FI was found to be

$$\mathrm{FI} \approx 2.4035 \cdot \mathrm{tph} + 0.0122 \cdot \mathrm{mlb} + 0.0565 \cdot \mathrm{sh} + 4.1235.$$

[Eq. 5]

The principal component was used instead of only the manual grading scale, since the scale could be biased and the intra-relationships between the three measures are not included in the manual scale. Using the principal component includes the relationships between the three measures, and the shrinking is a compromise between a measure based on data and one based on common knowledge. Terms describing this

procedure are "data-driven" or "model-assisted" grading.

In the current study, different DNA polymerase–buffer systems were compared with respect to forensic DNA profile quality using analysis of variance of the FI, as defined above. The measurements were performed in such a way that a design can be identified, although not balanced. The default case is two replicates for each analysis of each sample. With the existing data, we can compare the population means of the FI and make pair-wise comparisons using Ampli*Taq* Gold as the reference system. Upon investigating the data further, it was found to be beneficial to apply a logarithmic transformation of the values of FI to make them more normally and homoscedastically distributed. The resulting least significant difference between sample means is then exponentiated so that it can be interpreted on the original scale, and the result is a least significant ratio between two sample means.

We investigated the interpretation of the values of FI from a sample using two replicate measurements with each of the DNA polymerase–buffer systems. It is clear that a larger ratio between sample means from such a set of measurements means that the two polymerase systems differ and, in particular, that one system is better than the other. To find an approximation of this ratio, we used the samples for which the FI was obtained (at least once) for each of the polymerase systems studied. A one-way analysis of variance was performed on each of these samples (logarithmic values) and a pooled sample variance was calculated. This estimated variance was then used to calculate the least difference in the means of logarithms of profile indices required to be able to state that a particular system has a higher population FI than the control system. Finally, this least significant difference is transformed into a least significant ratio (LSR) of the geometric means of the original profile indices (FIgm). More specifically, if $x_1$ and $x_2$ are the two replicate FI values obtained with the polymerase system being investigated, and $y_1$ and $y_2$ are the corresponding indices obtained with the reference system, the following inequality should hold if the chemistry of the polymerase system investigated is significantly better than that of the reference system

$$\mathrm{LSR} \leq \frac{\sqrt{x_1 \cdot x_2}}{\sqrt{y_1 \cdot y_2}} , \qquad \text{[Eq. 6]}$$

where LSR is the estimated least significant ratio at a specified level of significance. We suggest a family significance level of 10% for the three systems compared with Ampli*Taq* Gold, resulting in an LSR of 1.985.

FI cannot be calculated for blank electropherograms/DNA profiles. Therefore, it was defined as 0.05 in these cases. This is the lowest value obtainable, given by a profile with only one detected peak at the detection limit (50 rfu).

## Results

### Success rate of routine DNA profiling of crime scene saliva stains

Twelve percent (232 of 1936) of the investigated saliva crime scene samples produced blank electropherograms/DNA profiles using standard Ampli*Taq* Gold DNA polymerase. 19.2 percent of samples from cigarette butts (174 of 907 samples) and 2.6% of samples from swabs from bottles, cans, and foodstuffs (22 of 843 samples) produced blank DNA profiles.

### Analysis of standardized mock crime scene saliva samples using a forensic singleplex real-time PCR assay

Five of the nine DNA polymerases (Bio-X-Act Short, Ex*Taq* Hot Start, KAPA2G Robust, Omni*Taq*, and PicoMaxx High Fidelity) produced average amplification efficiencies around the ideal 1.0 (0.93–1.12) (Table 1). The other four

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**Table 2. Results from DNA analysis of six crime scene saliva samples containing inhibitors using four different DNA polymerases**

| Sample | Substrate | DNA conc. (ng/µL) | DNA polymerase | TPH | MLB | SH | FI | Geometric mean of FI (FIgm) | Ratio of FIgm to FIgm for Ampli*Taq* Gold | Significant improvement (Ratio> LSR = 1.985) | Number of STR markers with true allelic peaks >50 rfu (0–10) | Manual profile quality ranking (1–8) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Cigarette butt | 0.059 | Ampli*Taq* Gold | 1345 | 0.57 | 1.94 | 0.69 | 0.72 | 1.00 | — | 6 | 8 |
| | | | | 1639 | 0.56 | 2.03 | 0.75 | | | | 7 | 7 |
| | | | Bio-X-Act Short | 8104 | 0.75 | 2.23 | 1.69 | 1.61 | 2.23 | YES | 10 | 1 |
| | | | | 6961 | 0.80 | 2.19 | 1.53 | | | | 10 | 2 |
| | | | Ex*Taq* HS | 3690 | 0.83 | 2.22 | 1.10 | 1.09 | 1.52 | NO | 10 | 4 |
| | | | | 3680 | 0.66 | 2.25 | 1.09 | | | | 10 | 3 |
| | | | PicoMaxx HF | 2797 | 0.75 | 2.20 | 0.97 | 0.96 | 1.33 | NO | 10 | 5 |
| | | | | 2749 | 0.61 | 2.20 | 0.95 | | | | 9 | 6 |
| 2 | Cigarette butt | 0.13 | Ampli*Taq* Gold | 2305 | 0.57 | 1.79 | 0.79 | 0.82 | 1.00 | — | 7 | 8 |
| | | | | 2803 | 0.64 | 1.81 | 0.86 | | | | 7 | 7 |
| | | | Bio-X-Act Short | 16087 | 0.77 | 2.23 | 2.77 | 2.79 | 3.38 | YES | 10 | 1 |
| | | | | 16230 | 0.76 | 2.25 | 2.80 | | | | 10 | 2 |
| | | | Ex*Taq* HS | 8220 | 0.77 | 2.24 | 1.71 | 1.72 | 2.08 | YES | 10 | 4 |
| | | | | 8278 | 0.83 | 2.24 | 1.73 | | | | 10 | 3 |
| | | | PicoMaxx HF | 8104 | 0.83 | 2.23 | 1.70 | 1.64 | 1.99 | YES | 10 | 5 |
| | | | | 7307 | 0.80 | 2.22 | 1.59 | | | | 10 | 6 |
| 5 | Cigarette butt | 0.13 | Ampli*Taq* Gold | 3758 | 0.55 | 1.75 | 0.97 | 0.97 | 1.00 | — | 7 | 8 |
| | | | | 5447 | 0.66 | 1.84 | 1.23 | | | | 8 | 7 |
| | | | Bio-X-Act Short | 33153 | 0.86 | 2.26 | 5.10 | 5.67 | 5.83 | YES | 10 | 2 |
| | | | | 41971 | 0.91 | 2.28 | 6.30 | | | | 10 | 1 |
| | | | Ex*Taq* HS | 15836 | 0.82 | 2.26 | 2.75 | 2.85 | 2.93 | YES | 10 | 6 |
| | | | | 17239 | 0.88 | 2.25 | 2.95 | | | | 10 | 5 |
| | | | PicoMaxx HF | 20353 | 0.84 | 2.26 | 3.37 | 3.37 | 3.46 | YES | 10 | 3 |
| | | | | 18759 | 0.89 | 2.27 | 3.16 | | | | 10 | 4 |
| 18 | Swab, aluminum can (Red Bull) | 0.038 | Ampli*Taq* Gold | 1008 | 0.49 | 1.50 | 0.54 | 0.50 | 1.00 | — | 4 | 8 |
| | | | | 1020 | 0.49 | 1.33 | 0.50 | | | | 4 | 7 |
| | | | Bio-X-Act Short | 4567 | 0.83 | 2.25 | 1.23 | 1.14 | 2.29 | YES | 10 | 1 |
| | | | | 3380 | 0.82 | 2.21 | 1.05 | | | | 10 | 2 |
| | | | Ex*Taq* HS | 2820 | 0.64 | 2.25 | 0.97 | 0.97 | 1.95 | NO | 9 | 6 |
| | | | | 2861 | 0.67 | 2.23 | 0.97 | | | | 10 | 5 |
| | | | PicoMaxx HF | 2790 | 0.61 | 2.15 | 0.94 | 0.98 | 1.98 | NO | 8 | 4 |
| | | | | 3207 | 0.77 | 2.21 | 1.03 | | | | 9 | 3 |
| 19 | Swab, PET bottle | 0.031 | Ampli*Taq* Gold | 0 | 0.40 | N/A | 0.05 | 0.05 | 1.00 | — | 0 | Blank |
| | | | | 0 | 0.40 | N/A | 0.05 | | | | 0 | Blank |
| | | | Bio-X-Act Short | 614 | 0.46 | 1.45 | 0.47 | 0.51 | 10.22 | YES | 3 | 2 |
| | | | | 1008 | 0.46 | 1.61 | 0.56 | | | | 3 | 1 |
| | | | Ex*Taq* HS | 0 | 0.40 | N/A | 0.05 | 0.13 | 2.50 | YES | 0 | Blank |
| | | | | 263 | 0.42 | 1.01 | 0.31 | | | | 1 | 4 |
| | | | PicoMaxx HF | 788 | 0.45 | 1.06 | 0.40 | 0.32 | 6.47 | YES | 2 | 3 |
| | | | | 677 | 0.44 | 0.54 | 0.26 | | | | 2 | 5 |
| 20 | Swab, aluminum can (beer) | 0.083 | Ampli*Taq* Gold | 2263 | 0.62 | 1.90 | 0.81 | 0.71 | 1.00 | — | 7 | 7 |
| | | | | 1530 | 0.56 | 1.50 | 0.61 | | | | 5 | 8 |
| | | | Bio-X-Act Short | 10999 | 0.75 | 2.28 | 2.09 | 1.92 | 2.73 | YES | 10 | 1 |
| | | | | 8552 | 0.77 | 2.27 | 1.76 | | | | 10 | 2 |
| | | | Ex*Taq* HS | 8440 | 0.85 | 2.26 | 1.75 | 1.68 | 2.39 | YES | 10 | 3 |
| | | | | 7405 | 0.83 | 2.27 | 1.61 | | | | 10 | 4 |
| | | | PicoMaxx HF | 6503 | 0.86 | 2.24 | 1.49 | 1.44 | 2.04 | YES | 10 | 5 |
| | | | | 5728 | 0.88 | 2.25 | 1.39 | | | | 10 | 6 |

The standard polymerase, Ampli*Taq* Gold, was compared with Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity. Three profile quality measures, the total peak height (TPH), the mean local balance (MLB), and the Shannon entropy (SH), were used to calculate the forensic DNA profile index (FI).

(Ampli*Taq* Gold, r*Tth*, *Taq*, and *Tth)* gave higher values, from 1.26 to 1.46, and also showed higher standard deviations. The linear ranges of amplification differed considerably between polymerases, from 3.3 log units for Bio-X-Act Short and PicoMaxx High Fidelity, down to 1.3 log units for Ampli*Taq* Gold (Figure 1, A and B). The detection limit for the least sensitive polymerases, r*Tth* and *Tth*, was almost 20× higher than the limit for the most sensitive polymerase, Bio-X-Act Short (Table 1; Figure 1, A and D). Based on these results, Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity were deemed the most robust. All the analyzed negative amplification controls were blank.

### STR analysis of inhibited crime scene saliva samples

The statistical assessment tool described in the "Materials and methods" section was applied to the 32 inhibited crime scene samples. The forensic DNA profile index (FI) geometric means were used for pair-wise comparisons of the alternative polymerases to Ampli*Taq* Gold. Twenty out of 32 inhibited crime scene saliva samples showed statistically significant electropherogram/DNA profile improvements with at least one alternative polymerase. Table 2 provides examples, while the complete list of results can be found in Supplementary Table 1. Samples from cigarette butts showed improvements in 11 of 16 cases, and those from cotton swabs showed improvements in 9 of 16 cases. Figure 2 shows an example in which the profile was clearly improved from partial to complete when PicoMaxx High Fidelity was used instead of Ampli*Taq* Gold. The respective FI values for this sample were 0.97 for Ampli*Taq* Gold and 3.37 for PicoMaxx High Fidelity. The alternative polymerase–buffer systems Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity provided

improved forensic DNA profiles in 20, 11, and 14 samples, respectively. No statistically significant differences were found between the three alternative polymerases. When the DNA profiles were ranked manually by an experienced reporting officer, 28 samples were categorized as showing higher quality profiles with two or three of the alternative polymerases, compared with Ampli*Taq* Gold.

## Discussion

The success rate of routine DNA analysis at SKL shows the need for a PCR that is more robust to inhibitors. Using Chelex extraction, a widely used method in forensic DNA laboratories (14,21), 12% of crime scene saliva samples that contained detectable amounts of DNA (0.025–0.25 ng/μL) still produced blank electropherograms/DNA profiles. The reason for only studying samples in the concentration range of 0.025–0.25 ng/μL is that these samples are usually analyzed undiluted, and it is thus possible that PCR inhibitors will have considerable effects.

When analyzing real crime scene trace samples, their content of inhibitory components is generally unknown. However, knowledge about the origin of the sample can provide information about its possible inhibitor content. Aluminum cans may release $Al^{3+}$ ions, which can inhibit PCR (22), while tobacco contains about 4000 chemical compounds, several of which may have inhibitory effects, such as formaldehyde (23) and phenols (24). Cigarette filter and paper may also have a negative effect since components of wood are known to inhibit PCR (25).

In forensic DNA analysis, commercial DNA typing kits including primers, DNA polymerase, and buffer are used almost exclusively throughout the world. Amp*Fl*STR SGM Plus and PowerPlex 16 are two of the most commonly used kits. Such DNA typing kits simplify the standardization and comparison of DNA profiles across borders, and enable relatively straightforward validation for each laboratory, compared with the use of in-house assays. However, the complete chemical content of the kits is not disclosed, making PCR chemistry a "black box." Due to the wide use of analysis kits, modifying PCR chemistry to reduce the effects of inhibition has become rare in the field of diagnostic PCR (2). In forensics, only some minor DNA typing studies with alternative polymerases have been performed, and mainly on pure standard DNA (26).

We started this study by investigating the AE, the dynamic range of amplification, and the detection limit for nine different DNA polymerases using standardized mock crime scene saliva samples in a forensic singleplex real-time PCR assay. The ideal value of AE is 1.0, which corresponds to exponential amplification. Deviating values indicate inefficient amplification, due to either non-optimal PCR conditions or the presence of PCR inhibitors. The values above 1.0 obtained for Ampli*Taq* Gold, r*Tth*, *Taq*, and *Tth* are the effect of postponed amplification (elevated Cp) due to inhibitory compounds in the saliva extracts; an effect that increases with increasing amount of saliva, and a flattening of the amplification curves for samples approaching the detection limit, giving lower Cp values than would be obtained from ideal sigmoid curves. Three of the polymerases producing AE values close to 1.0—Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity—were virtually unaffected by inhibitors in the saliva extract, and their real-time PCR amplification curves retained the sigmoid shape and an amplitude well above the baseline, even for samples close to the detection limit.

Using Ampli*Taq* Gold, the three samples with the highest saliva/cell amounts were detected at higher Cp values than the fourth strongest sample (Figure 1B). For the other polymerases, the higher the saliva amount, the lower the Cp (Figure 1, A, C, and D). The Ampli*Taq* Gold results are counterintuitive, but since a higher amount of cells also means a higher amount of PCR inhibitors, it is in fact a sign that the polymerase does not perform ideally in the presence of inhibitors present in saliva. However, the detection limit with Ampli*Taq* Gold is low, and is only surpassed by Bio-X-Act Short (Table 1). When the amount of inhibitors is small, the enzyme readily amplifies even very small amounts of DNA. The sensitivity of Ampli*Taq* Gold to inhibitors is consistent with the results found in previous studies (10,24,27).

Differences in the AE between DNA polymerases (Table 1) can be explained in part by the presence of PCR facilitators in the different buffer systems (28). BSA is a well-documented PCR facilitator known to reduce the inhibition resulting from a range of substances, such as human bone (29), phenols (30), hemoglobin (31), and proteases (32). Here, BSA was added to the PCR master mixes with all nine DNA polymerases, to make the conditions for the DNA polymerases more similar.

In the second part of the study, the alternative DNA polymerases were compared with Ampli*Taq* Gold using real crime scene saliva samples. Bio-X-Act Short provided the highest number of improved DNA profiles, but also showed somewhat uneven amplification between replicates (Table 2, Supplementary Table 1). Ex*Taq* Hot Start and PicoMaxx High Fidelity gave more reproducible results. The robustness to inhibitors from the sample matrices seemed to differ between the three polymerases, even though the differences could not be verified statistically. Ex*Taq* Hot Start performed better for swabs from cans and bottles, and PicoMaxx High Fidelity performed better for cigarette ends.

Crime scene DNA samples are routinely quantified at SKL using the Quantifiler Human DNA quantification kit (Applied Biosystems), which includes an internal amplification control indicating inhibition by an elevated Cp value. However, the Quantifiler kit only detected inhibition in 4 of 32 clearly inhibited samples in this study; the others appeared pure (results not shown). This depends to some extent on the difference in the template-to-reaction volume ratio between the assays. For the Quantifiler kit, 2 μL of template is used in 25-μL reactions (Quantifiler Kit User's Manual), whereas for Amp*Fl*STR SGM Plus, ≤5× more template is added to make up the same final reaction volume (Amp*Fl*STR SGM Plus PCR Amplification Kit User's Manual), giving a five-fold higher PCR inhibitor concentration. The real-time PCR assay used here to evaluate the polymerases on mock crime scene samples was modified by using the same template-to-reaction volume as for Amp*Fl*STR SGM Plus, to provide similar inhibitory effects.

The proposed statistical model will improve the quality control of forensic DNA profiles, and aid in the evaluation of novel forensic DNA analysis procedures. In particular, it provides a tool for objective assessment that can both save time and ensure the overall quality of DNA profiling within the laboratory. The strength of the model is that it combines the heights of allelic peaks with the balance between peaks within a STR marker, as well as the balance between markers. Peak heights alone can sometimes give a decent picture of the performance of PCR. However, since preferential amplification of some alleles cannot be completely avoided—especially in samples with low levels of DNA—peak heights need to be complemented with balance. An increase in the FI can be interpreted as an improvement of the DNA profile; however, we emphasize that statistically significant increases in FI must be based on at least two electropherograms from the same sample.

The results from real crime scene saliva samples revealed that Bio-X-Act Short, Ex*Taq* Hot Start, and PicoMaxx High Fidelity—as alternatives to Ampli*Taq*

Gold—all produced capillary electrophoresis electropherograms of improved quality with correctly labeled allelic peaks. Modifying PCR chemistry by employing alternative DNA polymerases and PCR facilitators thus proved to be a successful means of circumventing PCR inhibition in crime scene saliva samples. Moreover, a robust and sensitive amplification step will eliminate the need for complicated, time-consuming pretreatment of PCR samples, and reduce the risk of losing evidentiary DNA during sample preparation.

In conclusion, we show that the polymerase Ampli*Taq* Gold, commonly used in the forensic community, is not the optimal choice for crime scene samples showing PCR inhibition. Further studies on the mechanisms behind PCR inhibition in crime scene stains, and on the use of alternative DNA polymerase–buffer systems such as the ones described here, will benefit justice by rendering useful DNA profiles from a significantly higher percentage of forensic samples.

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## Competing interests

The authors declare no competing interests.

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