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ORIGINAL ARTICLE

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An easily automated, closed-tube forensic DNA extraction procedure using a thermostable proteinase

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Abstract Most standard procedures for extracting DNA from forensic substrates involve manipulations that expose the sample to potential contamination and which reduce yields. Furthermore, most methods require centrifugation and/or solvent extraction steps that render them difficult to automate. We describe a simple closed-tube DNA extraction procedure using a proteinase from the thermophilic *Bacillus* species EA1 that produces good DNA yields from a wide range of forensic substrates. The reaction is controlled by a temperature shift regime programmed into a thermal cycler and so eliminates the need for solvent extraction or column purification. The new method is ideally suited to forensic samples where exposure to extraneous contaminating DNA must be avoided. In addition, The simplicity of the procedure makes it suitable for automation.

Keywords Forensic science · DNA extraction · EA1 proteinase · Thermostable proteinase

Introduction

The introduction of the polymerase chain reaction (PCR) has led to a rapid development of sophisticated systems for DNA-based analysis of forensic samples. In parallel to this research, procedures have been formulated for extracting DNA from a wide range of substrates commonly found at crime scenes. Whilst many of these methods are highly effective at producing good quality DNA, few can be easily automated and many of the steps involved expose the sample to potential contamination by the technician.

In published work carried out by our group in the early 1990s, a thermostable proteinase isolated from the bacterium *Thermus* sp. Rt41a (marketed as PreTaq, Pacific Enzymes Ltd) was shown to be effective for extracting DNA from blood [1] and was a more suitable enzyme than proteinase K for preparing DNA for pulse-field gel electrophoresis [2]. One limitation of this enzyme is that like proteinase K, it is sufficiently stable to resist full inactivation at high temperatures. Rt41A proteinase works well when DNA yields are high and dilutions are required prior to PCR amplification (as is the case with fresh blood). However, trace samples typically require larger quantities of DNA extract to be used in subsequent amplification steps and when this is the case, low levels of residual proteinase activity can be sufficient to inhibit (degrade) *Taq* DNA polymerase. As a result of this limitation, Rt41A proteinase and proteinase K are less well suited for DNA extraction from samples containing small amounts of DNA unless a solvent extraction step is included in the method.

A pilot study using a selection of alternative thermostable proteinases demonstrated that some were effective in extraction of DNA from both blood and fish fins (a model substrate for difficult, cartilaginous tissue [3]). One proteinase isolated from the thermophilic *Bacillus* sp. EA1 (hereafter referred to as EA1) has a temperature stability profile ideally suited for extraction of trace DNA [4, 5, 6]. This enzyme was selected for further study.

The primary goal of the research described in this paper was to demonstrate the effectiveness of EA1 proteinase for DNA extraction from typical forensic samples. The procedure used in this study is based on that previously described for PreTaq [1]. The extraction procedure involves heating a buffered suspension of tissue to 75°C for 15 min in the presence of the enzyme. A subsequent incubation for 15 min at 94°C heat-kills the enzyme. The main advantage of using a proteinase that is optimally active at high

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temperatures is that DNA is released from the cellular material at temperatures where contaminating nucleases are inactive. Nucleases are therefore rapidly hydrolysed by the proteinase before any degradation of the DNA occurs.

We describe the validation of a DNA extraction method using EA1 proteinase on typical forensic case stains. For each sample type, a basic procedure has been developed and investigated with reference to yield and ability to obtain useful DNA profiles.

Materials and methods

Sample collection and preparation

Venous blood samples were collected from four volunteers by a registered nurse. The same volunteers (instructed on appropriate collection methods) also supplied hair and saliva samples.

For each individual experiment, four replicates and a reagent control were processed. All samples were extracted in sterile 1.5 ml microcentrifuge tubes and centrifugation steps were carried out in a microfuge at 16,000 *g* (13,000 rpm). When more than one volunteer was involved, sample sets were made of equal numbers of samples from each person. Where double-swabbing was used to collect samples, swabs were pooled and treated as a single sample. Sample collection from historic case exhibits was carried out according to the technique originally used. In this way, we minimized extraneous variables when comparing the quality of DNA profiles.

Blood, buccal swabs and hair roots

For the experiment, 5 µl of liquid blood, half of a buccal swab and a single anagen or telogen hair root approximately 5 mm long [7] were used. Degraded bloodstains were created by exposing 5 µl of fresh blood on white cotton cloth to ultra violet radiation (254 nm wavelength) from a distance of 70 cm for 60 min. Alternatively, 5 µl bloodstains were made on both well-moistened swabs and dry swabs, and sealed in glass tubes. These samples were then stored at 22°C or 55°C for 4 days. To produce bloodstains on substrates likely to contain inhibitors, 5 µl samples of liquid blood were deposited on grey wool, black denim, fresh grass and untreated wood and allowed to dry.

Mock case trace samples

These were prepared as follows:

1. The method of Sweet et al. [8] was used to double-swab beer bottle necks after consumption of the contents by volunteers.
2. Strips of paper 1 cm wide were taken from the ends of smoked cigarette butts. These were each divided into four equal portions.
3. An 8×1 cm strip of fabric from the inner collar of a worn T-shirt was cut into eight equal sections.
4. The steering wheel of a vehicle with a primary driver of known DNA profile was double-swabbed. The driver accounted for 90% of vehicle usage.
5. The rim of a teacup was double-swabbed following consumption of a beverage by a volunteer.
6. The interior sole regions of woolen socks were tape-lifted using a 2×5 cm section of adhesive tape per sock. The socks had been worn during a normal working day.
7. Greasy marks on a window, left by a volunteer pressing their forehead and nose against the glass were double-swabbed.
8. Latex gloves were worn by a volunteer while carrying out light household duties for 10 min. These were then sampled by double-swabbing the interior.
9. An apple core was double-swabbed after the apple had been eaten by a volunteer.
10. The surface of a teaspoon used by a volunteer to consume food, was double-swabbed.

Historic cases

Ten previously profiled bloodstains were re-sampled by taking either a 0.5 cm^2 portion from each sample or half of the remaining swab head material (one quarter of the full swab head). In addition, 10 previously DNA-profiled articles of clothing from cases with no suspect were sampled as follows:

1. The interiors of five woolen hats ('beanies') were tape-lifted by applying, lifting and reapplying a single section of adhesive tape (3×2 cm) until the entire interior surface of the hat had been covered.
2. A 2 cm^2 section of the lining from the back of the palm area of a glove was sampled.
3. A 2 cm^2 portion of fabric was sampled from the periphery of the mouth hole of a home made balaclava. Initial analysis had used approximately 16 cm^2 from the mouth area.
4. A stain of 0.5 cm^2, suggested by preliminary tests to be blood, was sampled from a sock.
5. An area of 2 cm^2 was sampled from a light yellow stain on a handkerchief.
6. A 2 cm^2 strip was taken from the perimeter of a napkin. The previously sampled area was approximately 10 cm^2.

Sample extraction

Chelex extraction was performed essentially as described by Walsh et al. [9] with minor variations for different tissue types. Blood and saliva samples were suspended in 1 ml of sterile H_2O in a microcentrifuge tube. The tube was vortexed and left at 22°C for 20 min. The tube was then centrifuged for 3 min and the supernatant discarded. Using a large bore pipette, 170 µl of 5% Chelex was added and the beads dispersed. The sample was then incubated at 55°C for 20 min, boiled for 8 min, vortexed and centrifuged for 3 min. Hair roots were incubated at 55°C for 4 h in a microcentrifuge tube containing 50 µl of 5% Chelex, 2 µl of 10 mg/ml proteinase K with and without 7 µl of 60 mg/ml dithiothreitol (DTT). The sample was then boiled and centrifuged as above.

Organic solvent extraction

A standard method described by Sambrook and Fritsch [10] was used for all trace samples. Samples were incubated with 1 ml of extraction buffer (10 mM Tris-base, 10 mM EDTA and 100 mM NaCl, pH 8.0) for 20 min at 22°C and then centrifuged at 16,000 *g* in a microfuge for 3 min, 810 µl of the supernatant was removed, the pellet re-suspended and 10 µl of proteinase K (10 mg/ml) added. After incubating at 55°C for 20 min, an equal volume (200 µl) of pH-equilibrated phenol/chloroform (1:1) was added. Samples were vortexed briefly, and centrifuged for 3 min. The aqueous phase was transferred into a new microcentrifuge tube and 1 µl glycogen, 20 µl of 3 M sodium acetate and 2.5 volumes ice-cold ethanol were added. After incubating at –20°C for 30 min, samples were centrifuged for 10 min at 16,000 *g* to collect the precipitated DNA. The ethanol was decanted, pellets washed in 70% ethanol to remove the sodium acetate, dried and resuspended in sterile distilled water to a final volume equal to that obtained using thermophilic proteinase extraction. Trace samples were resuspended in 500 µl of sterile distilled water.

EA1 proteinase extraction

Essentially, the same procedure was used for all sample types. On ice, 200 µl of 0.2 M Hepes buffer (pH 7 at 75°C) and 2 U EA1 proteinase (1 Unit is ΔOD_{420}=1 per hour incubation with azocasein, pH 6.5, at 75°C) was added to the samples. For the trace samples

and bloodstains on fabric, 400 µl reaction volumes were used. Tubes were incubated at 75°C for 15 min, followed by a further 15 min incubation at 94°C. Tubes were then vortexed briefly and centrifuged for 3 min to sediment any solids.

Sample profiling

Attempts were made at quantifying DNA yield using the Applied Biosystems Quantiblot Human DNA quantification system. This approach was found to be insufficiently sensitive to be useful. Because the volume of DNA extract used in amplification reactions was held constant in comparative experiments, total peak height provided a valid estimation of relative concentration (Applied Biosystems AmpFLSTR SGM Plus PCR amplification kit user's manual and references [11, 12, 13]). This measurement was calculated by highlighting all genuine alleles in each electrophoretogram and summing the peak height values. Volumes of DNA extract added to the PCR were as follows: blood, hair and saliva 0.75 µl, trace samples 10 µl, historic case samples 5 µl. Based on quantification estimates, two of the latter samples were re-amplified using 10 µl.

Samples were DNA profiled using the Applied Biosystems AmpFLSTR SGM Plus system as recommended by the manufacturers with the exception that half reaction volumes were used, 28 PCR cycles were carried out in an Applied Biosystems 9700 thermal cycler. Profiles were visualized by electrophoresis on 0.2 mm thick, 5% denaturing polyacrylamide slab gels run on an Applied Biosystems Prism 377 DNA sequencer with ABI Prism 377 collection software. Profile analysis was performed using Genotyper and GeneScan software.

Results and discussion

Blood

EA1 proteinase is Ca^{2+} dependent but is unaffected by a concentration of citrate below 5 mM and EDTA below 2 mM. Carry-over of these reagents from blood storage media falls well below these concentrations and so EA1 is compatible with standard blood storage procedures (data not shown).

Most DNA extraction techniques designed to prepare DNA from blood for multiplex STR amplification include an initial pre-wash step [9]. Pre-washing blood samples serves two purposes:

1. Red blood cells lyse releasing heme into the wash solution. Heme is known to cause inhibition of the PCR [14].
2. A pre-wash leaches water-soluble inhibitors away from the sample. Centrifugation can then be used to separate the white blood cells from the inhibitors in the supernatant.

Most forensic case bloodstains have been exposed to some level of environmental abuse (desiccation, heat, sunlight or humidity) and these physical stresses result in damage to the white blood cells. Thus, DNA may be lost in a pre-wash and any procedure that avoids this step is desirable, particularly for samples containing small amounts of biological material.

EA1 proteinase extraction (without a pre-wash) of fresh blood yielded DNA sufficiently free of inhibition to produce high quality profiles, despite the samples being red in coloration (Table 1). A similar result was found by Klintschar and Neuhuber when using an alkaline extraction procedure [15]. Table 1 demonstrates the yield reduction if a pre-wash step is used. Because EA1 proteinase produces DNA that is unaffected by heme contamination, the method is a significant improvement on previous procedures. Peak heights when using 2 U of enzyme are approximately twice the values obtained using Chelex or solvent extraction.

EA1 proteinase extraction out-performed Chelex for bloodstains on most of the substrates known to release inhibitors of *Taq* DNA polymerase (Fig. 1: graph). Samples deposited on white cotton (positive control) performed equally well for both systems, with a slightly lower total peak height when Chelex was used. This loss was again most likely due to the pre-wash step. With black denim both extraction systems performed poorly – peaks were uneven, amplitude low and several profiles were incomplete (data not shown). This result is not unexpected because black denim is known to release dyes inhibitory to *Taq* DNA polymerase. However, DNA profiles obtained from samples associated with wool, wood and grass were substantially better with the new extraction method. The most significant improvement was seen for bloodstains on dyed wool. When extracted using EA1 proteinase, all replicate samples produced full profiles with even peaks that were comparable with those produced from blood on white cotton. In contrast, samples extracted using Chelex had low peaks (often barely above the baseline) and gave only partial profiles (Fig. 1).

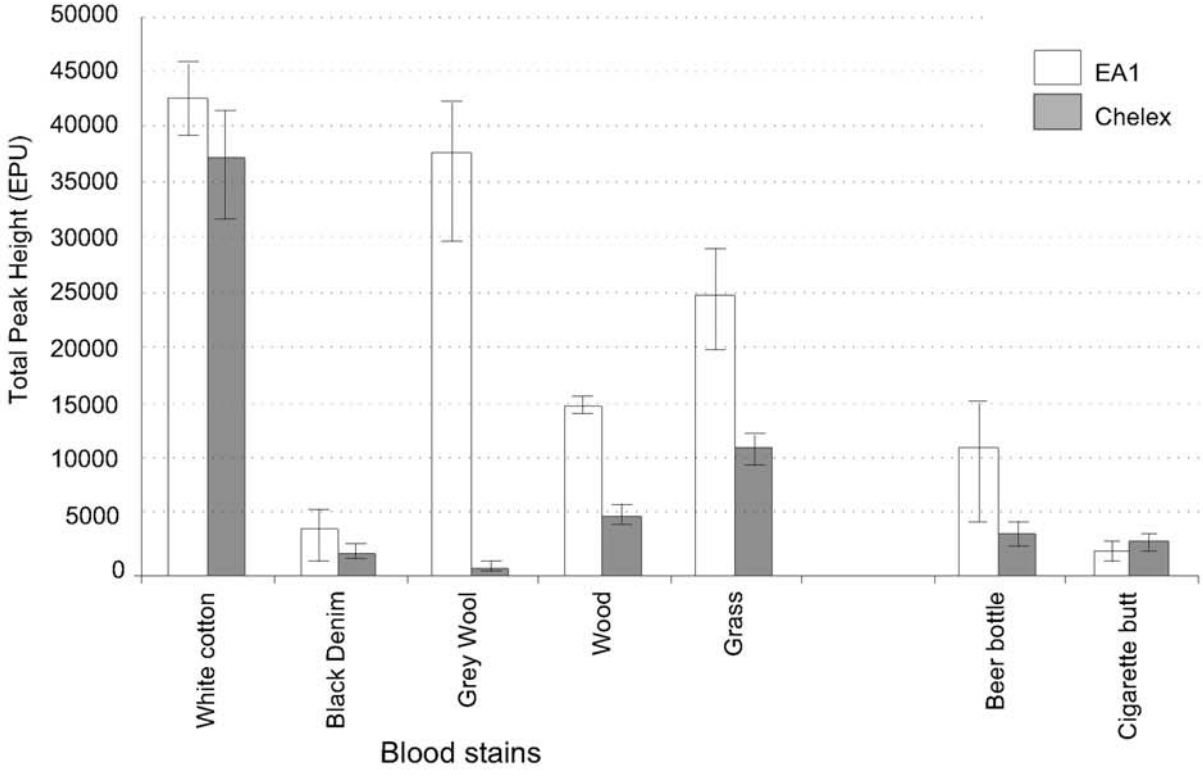
Table 1 Comparison of the STR profile peak heights obtained from DNA extracted from fresh blood using standard methods or EA1 proteinase

Extraction method	Profile description	Mean total peak height
Chelex	Full/even	23061
Organic solvent extraction	Full/even	35589
EA1 (1 U) no prewash	Full/even	21483
EA1 (1 U) + prewash	Full/even	9410
EA1 (2 U) no prewash	Full/even	50529
EA1 (5 U) no prewash	Full/even	56528

The mean total peak height was calculated from four replicate experiments.
Units are electrophorectic units as used by the GeneScan software.

Saliva

Results for saliva on beer bottles and cigarette butts are also summarized on the graph in Fig. 1. Amplification using 5 µl of DNA extract from swabbed bottle-necks gave full profiles for all EA1 proteinase-extracted samples and three out of four of the Chelex samples (data not shown). Average total peak height was greater for profiles generated using EA1 (mean total peak heights – Chelex: 3878, EA1:11367). With cigarette butts, EA1 extraction gave good yields of DNA as quantified using Quantiblot, but no DNA profiles were obtained. Chelex achieved mean total peak heights of 3102 whereas the peaks of EA1 were



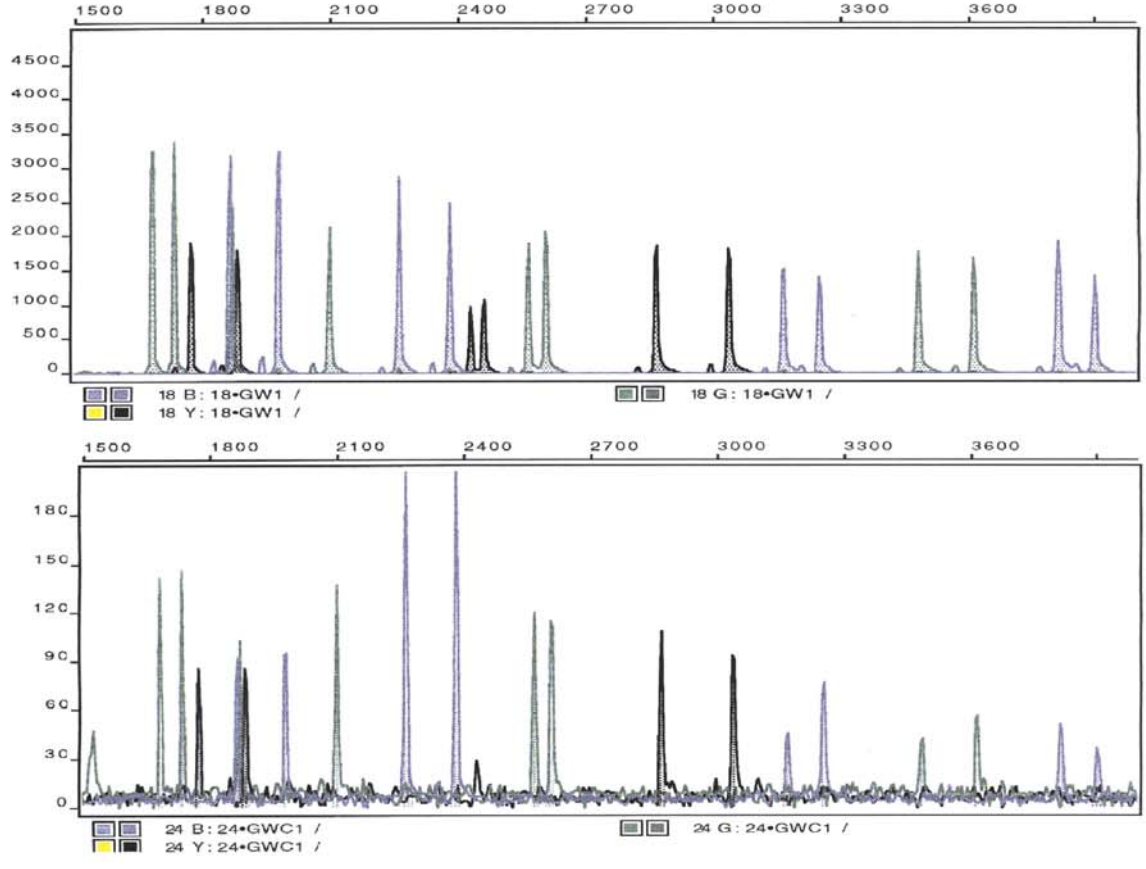


Fig. 1 *Graph* Total peak heights of DNA profiles generated from amplification of DNA extracted from stains on different substrates. Values are the means of four replicates and the error bars are standard errors of the mean. Units are electrophorectic units (EPU) as used by the GeneScan software. *Profiles* Dyed wool (grey) using EA1 proteinase (*top profile*) and the best profile from the Chelex-extracted samples *(bottom profile*). Note the difference in peak height as recorded on the Y axis. Profiles were generated using the Applied Biosystems AmpFLSTR SGM Plus system

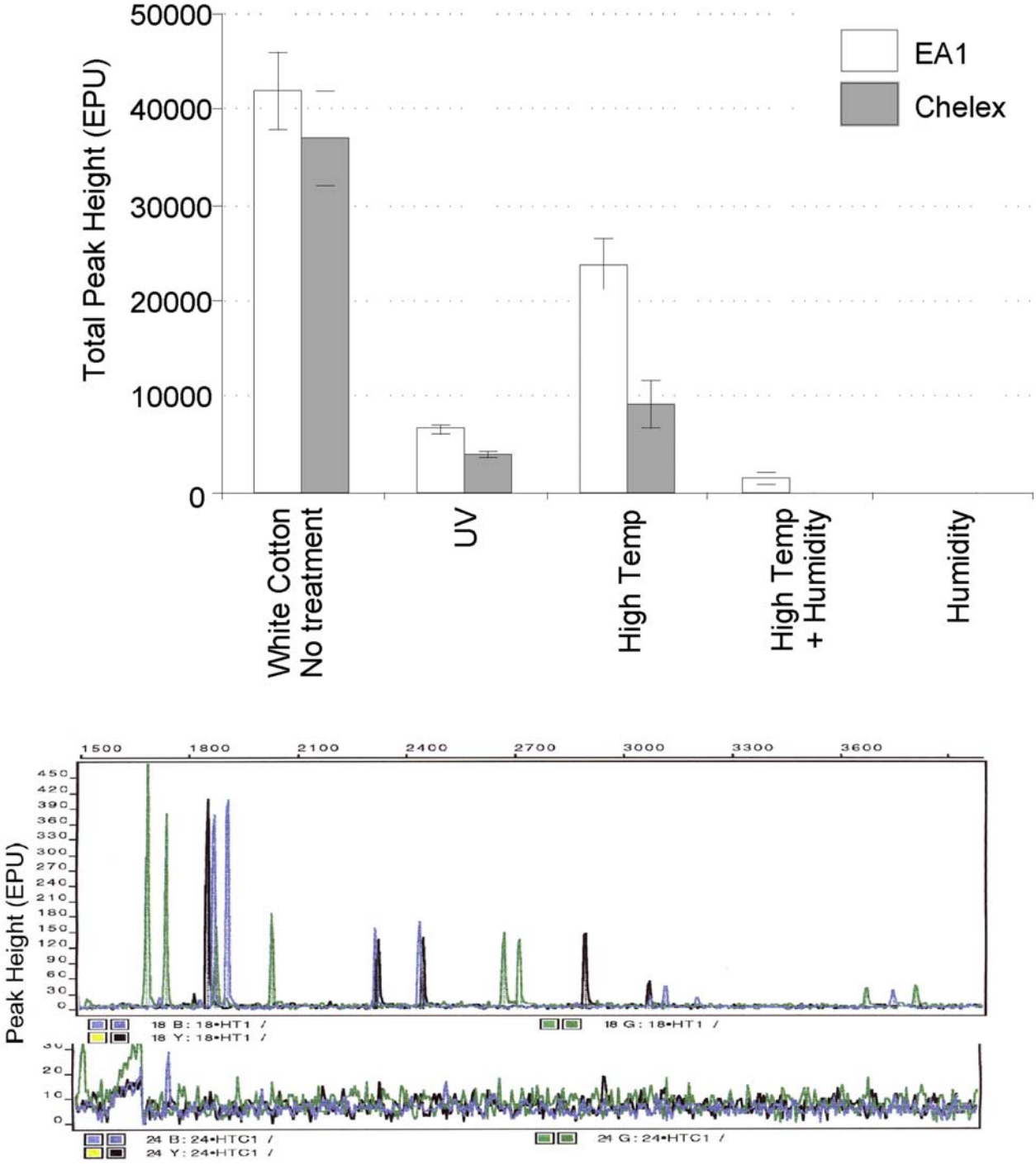


Fig. 2 *Graph* Comparison of the mean DNA profile peak heights for degraded blood samples following extraction using EA1 or Chelex. The mean (from four replicates) and standard error for each sample set is shown. Units are electrophorectic units (EPU) as used by the GeneScan software. *Profiles* Typical DNA profiles achieved following extraction of DNA treated with high temperature and humidity. *Upper profile* was from DNA extracted using EA1 proteinase and the *lower* using Chelex. Profiles were generated using the Applied Biosystems AmpFLSTR SGM Plus system

obscured by background noise. Full DNA profiles with even peaks were produced from the Chelex extraction. Inhibition of *Taq* DNA polymerase by nicotine and other aromatic compounds is a common problem with DNA extracted from cigarette butts. Inhibition is the most probable explanation for the failure of samples prepared using EA1 proteinase.

Degraded blood samples

EA1 proteinase-extracted DNA produced profiles with higher peak heights than the equivalent Chelex extraction when bloodstains had been subjected to typical environmental stresses (Fig. 2). Samples treated with ultraviolet

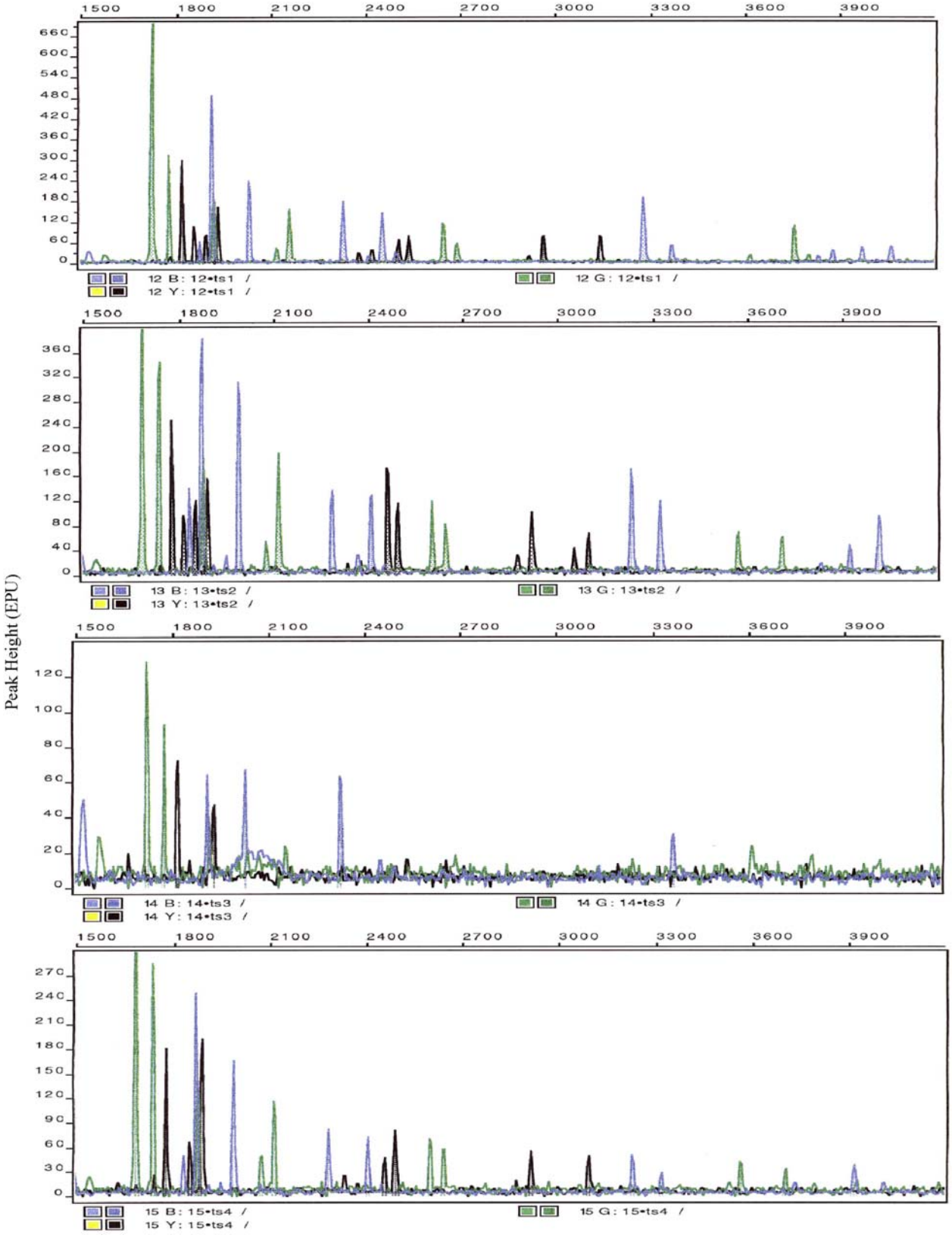


Fig. 3 DNA profiles generated from four samples of fabric from around the collar of a worn T-shirt. No profiles were obtained when organic solvent-based DNA extraction was used. Profiles were generated using the Applied Biosystems AmpFLSTR SGM Plus system

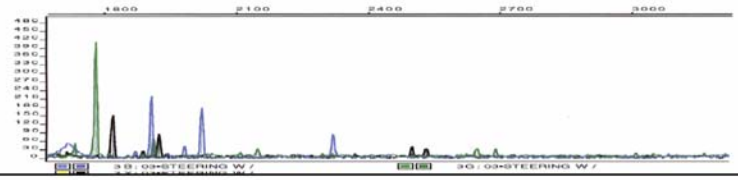
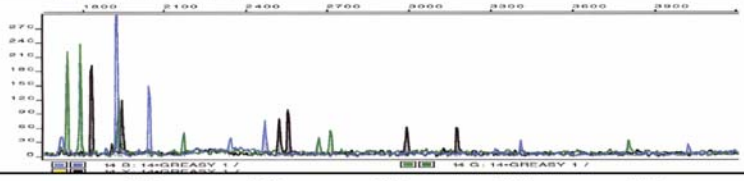
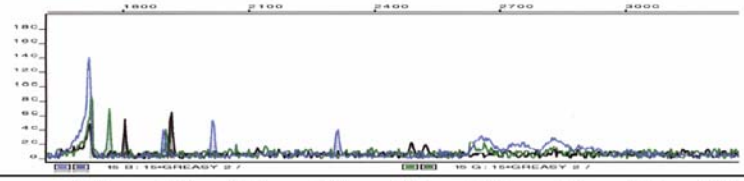
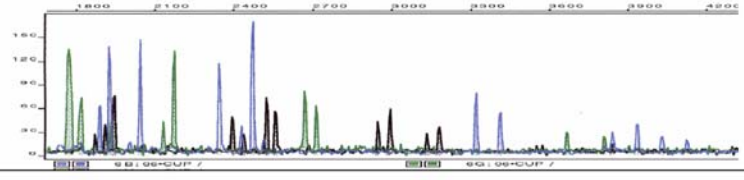
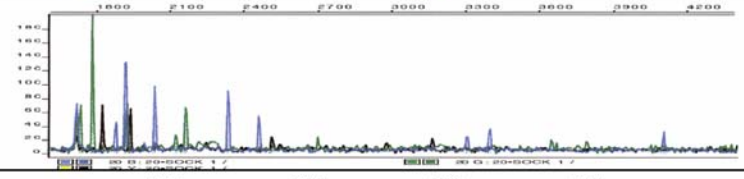
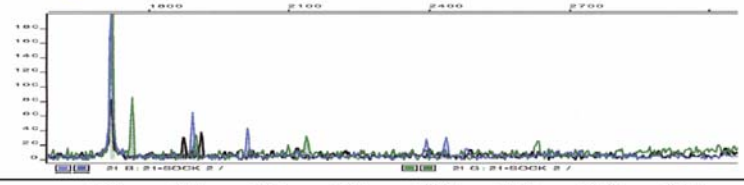
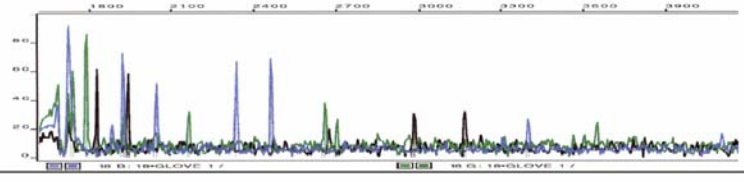
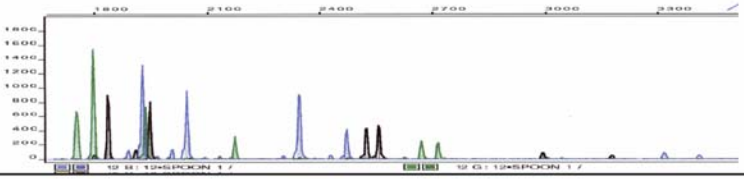
Sample	Profile
Car steering wheel Partial profile, consistent with secondary driver of vehicle. Low-level mixture.	
Greasy mark from window (1) Full profile of contributor. No evidence of mixture	
Greasy mark from window (2) Partial profile of contributor. No evidence of mixture	
Tea cup Full profile of contributor. Secondary profile consistent with cohabiter	
Sock (1) Partial profile of contributor. No evidence of mixture	
Sock (2) Low-level partial profile of contributor. No evidence of mixture	
Latex glove Partial profile of contributor. No evidence of mixture	
Apple core **Spoon** Full profile of contributor. Possibly low level secondary profile	Amelogenin only 

Fig. 4 Descriptions of DNA profiles obtained using the EA1 proteinase extraction method on mock trace samples

light gave a significant reduction of peak heights across all loci, presumably the result of single-stranded breaks and cross-linkage of DNA strands. DNA profiles obtained from swabs exposed to prolonged, elevated temperatures had a slight reduction in peak height, but were otherwise high quality and even. Samples subjected to high humidity developed a green coloration and were odorous. These samples failed to produce any DNA profiles when extracted using Chelex, but partial profiles were obtained for all replicates when processed using EA1 proteinase (Fig. 2).

Hair

Hair samples required 90 min of treatment with EA1 proteinase before visible damage to the shafts could be seen. All anagen hair root extractions gave acceptable yields of DNA but these varied from hair to hair (data not shown). The amount of nuclear DNA in hair roots and the attached cellular material can vary greatly, and so differences in DNA yield are not unusual. The DNA profile quality obtained from the EA1 proteinase extract of anagen hair roots was as good as that achieved with Chelex (mean peak heights – Chelex: 40972, EA1:52512, no enzyme: 7240). However, no profiles were obtained from telogen hairs and using Chelex with the addition of DTT did not significantly increase yield. This is in accord with others [16].

Buccal swabs

DNA samples from all extraction methods produced full, 'reportable' profiles (mean peak heights – Chelex: 57064, EA1:63196, no enzyme: 7452). Those produced from samples extracted with EA1 proteinase had a moderately 'inhibited' appearance, characteristic of excess template in the PCR (Applied Biosystems AMPFlSTR SGM Plus PCR amplification kit user's manual). The difference in quality of DNA profiles generated by the enzyme-based method and Chelex was minimal considering the variable amount of cellular material transferred to a buccal swab.

Trace samples

Overall, the results on mock case samples containing trace amounts of DNA demonstrate that the new extraction procedure can be applied to a diverse range of sample types. In some cases, profiles were obtained when using the EA1 method but failed with current protocols. For example, EA1-extracted DNA from the T-shirt collar gave full DNA profiles consistent with the donor for three out of the four replicate experiments and a partial DNA profile for the fourth (Fig. 3). No DNA profiles were obtained using the organic solvent-based extraction procedure. The T-shirt profiles were consistent with the donor but all demonstrated a presence of a low level of DNA from a second person. Investigation identified the second source of DNA as the donor's partner, who handled the shirt briefly prior to sampling.

Table 2 Description of DNA profiles obtained for genuine case samples

Sample type	Blood		
	Chelex extraction	EA1 extraction	
	Original profile description	Re-sampling	Profile description
Bloodlift	Full profile	As previous	Full profile
Blood on towel	Full profile	As previous	Full profile
Blood on tissue	Full profile	As previous	Full profile
Bloodlift	Full profile, low peaks	As previous	Full profile
Swab	Partial profile	Half previous	Partial profile, low peaks
Blood on glass	Full profile	As previous	Full profile
Blood on fabric	Full profile	Half previous	Full profile
Swab	Full profile	Half previous	Full profile
Bloodlift	Full profile	Half previous	Full profile
Blood on paper	Full profile, low peaks	As previous	Full profile
Sample type	Trace		
	Organic solvent extraction	EA1 extraction	
	Original profile description	Re-sampling	Profile description
Handkerchief	Partial profile, low peaks	Different location, lighter stain	More complete than previous (partial)
Beanie (grey, acrylic)	Full profile	Second time tape-lifted	Higher peaks, low level mix
Beanie (black, acrylic)	Full profile, mix	Second time tape-lifted	Slightly lower peaks, less complete
Beanie (black, 50% wool)	Full profile, low level mix	Second time tape-lifted	Lower peaks, less complete
Glove (acrylic inner)	Partial profile, low peaks, mix	Approx. 10% previous	Low peaks (below threshold)
Sock (small blood stain)	Full profile	Approx. half previous	Full profile
Beanie (black, acrylic)	Full profile, low level mix	Second time tape-lifted	Full profile, lower peaks, mix evident
Beanie (black, acrylic)	Full profile, mix	Second time tape-lifted	Full profile
Home-made balaclava	Full profile, mix	Approx. 10% previous	Full profile, higher peaks, mix evident
Napkin	Full profile	Approx. 20% previous	Partial profile

Columns two and three are referring to the original profiling where Chelex or organic solvents were used. These results are compared with de novo extraction using EA1 proteinase (columns three and four).

Figure 4 summarizes the results with EA1 from the other mock case samples. Although few samples produced DNA at levels sufficient for quantification, most produced at least partial DNA profiles of a reportable quality. The DNA profile obtained from the car steering wheel sample was primarily that of a second driver despite the main driver being the last person to drive the car and the second individual accounting for only 10% of vehicle use. The quantity of DNA deposited on the steering wheel was so low that slight variations could have a significant effect and it is possible that the secondary driver is a 'shedder' or a 'good donor' of DNA [17].

Historic cases

Table 2 compares the quality of DNA profiles obtained in the original investigation with new profiles generated from EA1 proteinase extracts. Average total peak heights were not recorded because of unavoidable sampling variation such as the advanced age of the samples subjected to the new extraction method and the scarcity of material available for the second analysis (for obvious reasons, the best regions of the sample had already been used). Despite being disadvantaged, the EA1 proteinase gave useful profiles for most cases and the new DNA profiles were consistent (in terms of allelic designation) with those previously obtained. Some were of higher quality (providing results from more loci) than those achieved using the standard protocol but this was not always the case. With the blood samples, EA1 extraction produced profiles of comparable quality with the original but in some cases the new profiles had a slightly overamplified appearance indicating excessive DNA supply to the reaction – EA1 had been more efficient than we had anticipated.

Some of the DNA profiles generated using EA1 extraction from trace samples were incomplete, suggesting insufficient template DNA. After concentration of failed extracts by ethanol precipitation, DNA profiles were generated for all samples.

Conclusions

Comparisons of the quality of the profiles obtained from most of the samples tested suggest that the thermophilic proteinase system is better or at least as effective as the currently employed Chelex and organic solvent procedures. This result is significant for three reasons:

1. Forensic scientists often face samples with marginal quantities of DNA for profiling. By removing steps in a DNA extraction method (particularly pre-washes, solvent extraction and ethanol precipitation) yields can be improved substantially.
2. All procedural steps in the EA1 proteinase method are easily automated. Any commercially available liquid-handling robot coupled with a thermal cycler can be employed for high thoughput processing.
3. The extraction method is closed-tube. Hence, it is less susceptible to contamination by extraneous DNA.

The new proteinase-based method failed on sample types known to cause inhibition (e.g. black denim and cigarette butts) and so other steps designed to remove the inhibitors must be included in the procedure for them to be effective. In addition, the enzyme was ineffective on semen and telogen hairs with or without the addition of dithiothreitol (data not shown). As the standard procedure using proteinase K worked routinely with semen, we conclude that the substrate specificity of EA1 proteinase limits its use on this type of sample. For telogen hair and hair shafts, profiles are also normally be obtained using proteinase K [18, 19]. We are currently exploring other proteinases for hair and the results of this research will be reported elsewhere.

Overall we conclude that the method using EA1 proteinase offers a reliable, simplified and in many cases superior procedure for DNA extraction from most forensic samples. It is apparent that the new method is a particularly attractive option for laboratories dealing with large volumes and those wishing to automate their DNA extraction procedures to achieve a higher throughput and greater certainty of interpretable data from forensic samples.

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