An enzyme-based DNA preparation method for application to forensic biological samples and degraded stains

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Extraction of DNA from forensic samples typically uses either an organic extraction protocol or solid phase extraction (SPE) and these methods generally involve numerous sample transfer, wash and centrifugation steps. Although SPE has been successfully adapted to the microdevice, it can be problematic because of lengthy load times and uneven packing of the solid phase. A closed-tube enzyme-based DNA preparation method has recently been developed which uses a neutral proteinase to lyse cells and degrade proteins and nucleases [14]. Following a 20 min incubation of the buccal or whole blood sample with this proteinase, DNA is polymerase chain reaction (PCR)-ready. This paper describes the optimization and quantitation of DNA yield using this method, and application to forensic biological samples, including UV- and heat-degraded whole blood samples on cotton or blue denim substrates. Results demonstrate that DNA yield can be increased from 1.42 (±0.21) ng/μL to 7.78 (±1.40) ng/μL by increasing the quantity of enzyme per reaction by 3-fold. Additionally, there is a linear relationship between the amount of starting cellular material added and the concentration of DNA in the solution, thereby allowing DNA yield estimations to be made. In addition, short tandem repeat (STR) profile results obtained using DNA prepared with the enzyme method were comparable to those obtained with a conventional SPE method, resulting in full STR profiles (16 of 16 loci) from liquid samples (buccal swab eluate and whole blood), dried buccal swabs and bloodstains and partial profiles from UV or heat-degraded bloodstains on cotton or blue denim substrates. Finally, the DNA preparation method is shown to be adaptable to glass or poly(methyl methacrylate) (PMMA) microdevices with little impact on STR peak height but providing a 20-fold reduction in incubation time (as little as 60 s), leading to a ≥1 h reduction in DNA preparation time.

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1. Introduction

Generally, DNA extraction is the first step in forensic DNA analysis for use in human identification, and is essential to generating short tandem repeat (STR) profiles from forensic biological samples. DNA must be separated from other components (proteins, nucleases and other cellular material) that would hinder and inhibit downstream analyses, such as STR PCR amplification [1]. As a result, DNA extraction from biological samples is the first and most crucial step in forensic DNA analysis. If the extraction method has a low efficiency, precious quantities of DNA could be lost. Low efficiency purification may lead to the presence of contaminants that will inhibit PCR. Although there are many different extraction protocols available, they typically fall into one of two categories: liquid or solid phase. Table 1 provides a comparison of several commonly used DNA extraction methods including phenol/chloroform [2,3], Chelex® [4,5] and solid phase extraction (SPE) [6,7]. Although each method has been validated for use in forensic laboratories, each still requires multiple sample handling steps, creating potential points for ingress of contaminants into the system.

In this respect, it would be beneficial to perform DNA purification on an automated microfluidic platform where handling and contamination can be minimized. Such a platform provides an alternative, low-volume, closed analysis method reducing overall analysis costs, sample and reagent consumption, increasing sample throughput and allowing for the development of point-of-care systems with sample-in, answer-out capability [8]. Solid phase extraction has been successfully adapted to a microdevice and provides DNA in a small, concentrated volume.
Table 1

Comparison of different DNA extraction and preparation methods. “Spins (#)” refers to the number of centrifugation steps required. “Tube open (#)” refers to the number of times the sample tube must be opened to add and/or remove reagents. “Tips (#)” refers to the number of pipette tips that are needed to complete the procedure.

<table>
<thead>
<tr>
<th>Company</th>
<th>Brand name</th>
<th>Extraction type</th>
<th>Time (min)</th>
<th>Spins (#)</th>
<th>Tube open (#)</th>
<th>Tips (#)</th>
<th>Time hands-on (min)</th>
<th>Ease of automation (1: good; 2: avg; 3: bad)</th>
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<tr>
<td>ZyGEM</td>
<td>forensicGEM&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Enzyme-based</td>
<td>22</td>
<td>0–1</td>
<td>1</td>
<td>9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Qiagen</td>
<td>QIAmp</td>
<td>Silica membrane</td>
<td>45</td>
<td>4–6</td>
<td>5–6</td>
<td>9</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>Invitrogen</td>
<td>ChargeSwitch&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Magnetic silica beads</td>
<td>60–90</td>
<td>4</td>
<td>11</td>
<td>14</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>Chlex&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Silica membrane</td>
<td>45</td>
<td>4–6</td>
<td>5–7</td>
<td>9</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>Promega</td>
<td>DNA IQ&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Chelating resin</td>
<td>40–100</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>2</td>
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<tr>
<td></td>
<td>Organic</td>
<td>Paramagnetic resin</td>
<td>30–60</td>
<td>0–1</td>
<td>6–8</td>
<td>12–14</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenol-Choloform</td>
<td>60–180</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>20</td>
<td>3</td>
</tr>
</tbody>
</table>

Note: Protocols vary from laboratory to laboratory. These are general conditions used.

<sup>1</sup> One spin is needed when using the Blood kit.

[9–11]. However, microfluidic SPE (μSPE) can be burdened by uneven packing of the solid phase and high backpressure [9,11–13]. In addition, μSPE increases the complexity of the extraction method with numerous syringe changes required, making automation more difficult. In contrast, liquid-based extractions eliminate the solid phase and are more amenable to automation.

Recently, a liquid-based DNA preparation technique was developed that uses a neutral proteinase from an Antarctic Bacillus sp. EA1. The enzyme is stabilized by divalent cations, and can be used to lyse cells and degrade proteins and nucleases at 75 °C, while leaving the nucleic acids intact, in a PCR-compatible buffer [14]. This method provides a closed environment with no sample transfer steps, reducing the opportunity for cross-contamination and/or sample loss. Additionally, centrifugation steps and the use of a solid phase are eliminated, making it more easily adaptable to a microdevice and amenable to automation as noted in Table 1. While this method has been demonstrated for numerous forensic samples [14], no efficiencies were reported with little to no optimization to improve DNA yield.

Using previously described glass PCR microdevices [15], the enzyme-based method can be adapted to a microfluidic platform. The DNA preparation reagents can be incubated in glass devices, which have a chamber volume of 400 nL, using the same infrared (IR)-mediated heating system used to perform PCR thermal cycling with software control of the heating/cooling (halogen lamp and fan) [16]. Concurrently, there has been a shift in the field of microfluidics toward polymer microdevices, which offer advantages over glass devices including simple fabrication methods, low-cost and disposability. PCR microdevices can be fabricated from polymeric substrates, such as poly(methyl methacrylate) (PMMA), using laser ablation and thermally bonding. This process removes the need for etching reagents or bonding solvents.

The work presented here characterizes the enzyme-based DNA preparation method for use with forensic biological samples, specifically, buccal swabs and bloodstains. The DNA yield obtained using this procedure was determined for both blood and saliva samples using a fluorescent intercalating dye assay and/or quantitative PCR. In addition, STR PCR was performed using a commercial 16-plex STR amplification kit and the results compared with a conventional DNA extraction and purification method. Finally, the new method was adapted to both glass and PMMA microdevices to facilitate future integration with downstream processes such as PCR and microchip electrophoresis.

2. Materials and methods

2.1. Enzyme-based DNA preparation of non-degraded samples

Two buccal swabs were collected from ten anonymous, healthy volunteers by using an Institutional Review Board (IRB) approved collection method. Swabs were obtained by vigorously rubbing inside both cheeks with a sterile cotton swab for a total of 30 s and swabs were allowed to dry overnight or until needed. Once an average buccal swab donor was determined (Person J), this volunteer donated numerous swabs (>20) for use in the remainder of the study. Fresh buccal swab eluates were used for each of three replicates. Venous blood samples were collected through an IRB approved method from three anonymous, healthy volunteers using EDTA vacutainers and stored in 1.7 mL microcentrifuge tubes at 4 °C until needed.

Epithelial cells were eluted from the cotton swab by agitating the swab in 500 μL of deionized water (DI H₂O) in a 1.7 mL microcentrifuge tube for 60 s. The elute was vortexed to ensure a homogenous suspension of cells and 20 μL added to the DNA extraction solution, which contained 10 μL 1 × forensicGEM<sup>®</sup> BLUE buffer (saliva kit), 1 μL forensicGEM<sup>®</sup> EA1 enzyme (ZyGEM Corp., Hamilton, New Zealand) and 69 μL 11 H₂O in a 0.2 mL PCR tube. After a brief vortex, the sample was incubated in a MyCycler<sup>™</sup> thermal cycler (Bio-Rad Life Science, Hercules, CA, USA) at 75 °C for 15 min, then 95 °C for 5 min.

For whole blood samples, 2.5 μL was added to the DNA preparation reagents, containing 10 μL 1 × forensicGEM<sup>®</sup> RED buffer (whole blood kit), 1 μL forensicGEM<sup>®</sup> EA1 enzyme, 2 μL 10 mM CaCl₂ from the ZyGEM RED kit and 86.5 μL 11 H₂O (total volume = 102 μL) in a 0.2 mL PCR tube. After a brief vortex, the sample was incubated using the same conditions as those described above for epithelial cells. Following incubation, the sample was centrifuged for 3 min at 12,100 × g.

2.2. Enzyme-based preparation of degraded samples

Whole bloodstains were prepared on 2" × 2" (5.08 cm × 5.08 cm) clean swatches of either cotton or blue denim by adding 1, 2.5, or 10 μL of blood directly onto the fabric and allowing to dry overnight at room temperature. Heat degradation was achieved by placing the stains in a 56 °C oven for one month, while UV degradation was completed by exposing the stains to 254 nm UV light (flux = 0.72 μJ/cm²) at a distance of 1 cm, provided by a 4W Mineralight<sup>®</sup> UVGL-25 multiband lamp (UVP, Inc., San Gabriel, CA, USA) for 158 min, corresponding to approximately 12 months exposure to noon-time sunlight in Los Angeles [17]. A 0.5 cm² portion of the substrate, containing the entire stain, was cut into eight smaller pieces and all the pieces were placed in a 0.2 mL polypropylene tube. The DNA preparation reagents, were added to the tube and the sample incubated (the same 100 μL (saliva) or 102 μL (blood) volumes were sufficient). The cotton or blue denim fragments were then separated from the supernatant using the piggy back method [18]. Briefly, a small hole was created in the bottom of the polypropylene tube and the tube was placed in a 1.7 mL microcentrifuge tube. The sample was centrifuged for 4 min.

at 12,100 × g and the polypropylene tube containing the substrate fragments discarded. DNA recovered from degraded samples was not quantitated and, therefore, a 10 μL aliquot was used for PCR analysis as described below.

2.3. Conventional DNA extraction

Another 0.5 cm² portion of the substrate was cut into smaller pieces and placed into a 1.7 mL microcentrifuge tube for conventional DNA extraction and purification following exactly the Qiagen® QIAamp® DNA Investigator Handbook (Qiagen, Valencia, CA, USA) protocol specific for body fluid stains except for the following three modifications. First, QIAamp® Mini Spin columns were used, as opposed to MiniElute columns, for the Mini Spin columns were readily available. Second, the residual lysate was collected from the substrate using a QIAshredder spin column, centrifuged at 12,100 × g for 2 min and the filtrate was added to the Mini Spin column to maximize DNA recovery from the cotton or blue denim. Finally, the DNA was eluted in 50 μL of Buffer AE rather than the recommended Buffer ATE as Buffer AE was readily available for use. Since the DNA was eluted in 50 μL instead of 100 μL (as with the ZyGEM method), only 5 μL of the eluted DNA was added to PCR as it was thought to be twice as concentrated as the enzyme-prepared DNA.

2.4. Fabrication and use of microdevices for enzyme-based DNA preparation

Glass microdevices were fabricated using borofloat glass following previously described photolithographic techniques [19]. The channels and chambers in the PCR devices were etched 175 μm deep, resulting in a chamber volume of ~400 nL. Reservoir holes were drilled using a 1.1 mm diameter diamond-tip drill bit (Crystalline Corp., Lewis Center, OH, USA) and a borofloat cover plate was thermally bonded to the etched plate. Prior to use, the internal surfaces of the glass devices were passivated with Sigmacote® (Sigma–Aldrich, St. Louis, MO, USA).

PMMA microdevices were fabricated in 1.5 mm thick PMMA (McMaster-Carr Supply Co., Atlanta, GA, USA) by laser ablation using a VersaLaser® 3.50 CO₂ laser system (λ = 10.6 μm, max. power = 25 W; Universal Laser Systems, Inc., Scottsdale, AZ, USA). PCR chambers were etched using a raster cut (60% power, 100% speed), channels were etched using a low power vector cut (3% power, 4% speed) and reservoirs were cut using a high power vector cut (60% power, 3% speed). This resulted in PCR chambers that were 433 ± 18.5 μm deep and 689 ± 72 nL in volume. Etched microchips were bonded using a previously described high temperature, low pressure bonding method [20]. After bonding, chips were filled with blue dye (30 μM eriochrysin) and visualized under a stereomicroscope to ensure that the chambers and channels were fluidically sealed.

Epithelial cells were eluted from buccal swabs and prepared in the enzyme-based DNA preparation solution as described above for a final volume of 100 μL (one swab for each replicate). A 2 or 6 μL portion of the prepared solution was loaded into a glass or PMMA microdevice, respectively, using a pipette and incubated using an IR-mediated PCR system [16–21] for 0.5, 1.5, 5, 10 or 15 min at 75 °C and 0.5, 1, 5 or 5 min at 95 °C. After incubation, the sample was removed from the microdevice and added to the conventional PCR master mix as described below.

2.5. Quantitation protocols

The DNA in enzyme-treated non-degraded samples was quantitated using the Quant-iT™ RiboGreen® ssDNA assay kit (Invitrogen by Life Technologies, Carlsbad, CA, USA), as 90% of the DNA in solution is single-stranded due to the denaturing capability of the DNA preparation reagents [22]. However, since RiboGreen® also quantitates RNA, 5 μL of each sample was treated with 0.25 μL RNase Cocktail™ (Ambion, Inc., Austin, TX, USA) and incubated at 37 °C for 15 min, followed by 5 min at 95 °C. Samples were then snap-cooled (placed into an ice bath) to ensure the DNA remained single-stranded. RiboGreen® was added to the samples and the fluorescence measured using a NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific, Wilmington, DE, USA).

For the enzyme versus no-enzyme studies, after quantitation with RiboGreen®, quantitative PCR (qPCR) was performed on the samples using a previously described method [23]. Briefly, a standard curve (from 50 ng to 10 pg DNA), along with samples, was run in duplicate using an iCycler™ with the iQ™ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories). The reaction mixture consisted of 2× JumpStart™ Taq ReadyMix™ (Sigma–Aldrich Corp.), 300 nM forward and reverse primers for a 63-bp region of the human TPOX locus (InVitrogen Corp., Carlsbad, CA, USA), and 250 nM TaqMan® TPOX probe labeled with VIC (fluorophore) and TAMRA (quencher) (Applied Biosystems). The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.6. PCR amplification

Enzyme-treated samples were amplified in a BioRad MyCycler™ using the AmpliFSTR® Identifier® PCR amplification kit according to the manufacturer’s instructions. The amount of sample added to the PCR solution depended on the source of the DNA. Since there were no deviations from the manufacturer’s protocol for the non-degraded samples, a 1 μL aliquot was used for PCR, which contains 1–1.5 ng DNA. For degraded samples, 10 μL was added to the PCR to maximize the probability of obtaining high quality STR profiles. For the conventionally extracted degraded samples, 5 μL was added to PCR since the elution volume was 50 μL (half of the enzyme-based method) so that comparisons could be made between the two methods. Finally, for samples incubated on glass microchips, the entire sample (2 μL) was added to the PCR solution and for those samples incubated on PMMA, a 2 μL portion was added to PCR in order to be comparable to those samples from the glass microdevices. The amplified product was separated and detected using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The raw data were analyzed and deconvoluted using GeneScan® analysis software with the allele detection threshold set to 100 relative fluorescent units (RFUs).

3. Results and discussion

3.1. Optimization of the enzyme-based DNA preparation method

Efficient recovery of DNA during purification is essential for subsequent successful amplification of STR loci; low recoveries (<500 pg DNA) reduce the likelihood of obtaining a full STR profile. Therefore, to gain widespread use in the forensic community, the enzyme-based preparation method must produce consistent DNA yields in a timely manner – even with compromised samples. For the characterization and optimization studies, the blood used had a white blood cell (WBC) count of 7.1 × 10⁸/μL and the blood used for the degraded stains study had a WBC count of 2.4 × 10⁷/μL. Since the amount of cheek cells collected with buccal swabs is known to vary, two buccal swab samples were collected from 10 individuals and analyzed with the enzyme-based method. The DNA yield ranged from 0.72 ± 0.09 to 3.78 ± 0.07 ng/μL. Four of the ten samples gave similar yields and so it was decided to select one individual from these, Person J, as a provider of an ‘average’ buccal...
swab (1.42 (±0.21) ng/µL). These swabs were used for all further studies.

When following the manufacturer’s protocol, the enzyme-based method produced DNA yields of 1.42 (±0.21) and 1.40 (±0.14) ng/µL for buccal swabs and whole blood samples, respectively, in 20 min (Table 2). To decrease the processing time, shorter incubation times were evaluated to determine whether there was an impact on the DNA yield. Buccal swab eluate and whole blood samples were incubated for 10 or 5 min at 75 °C, followed by 5 min at 95 °C, and the quantity of DNA recovered from the preparation was determined. The results of these studies (Table 2) show that incubation for 10 and 5 min, respectively, provided 1.26 (±0.22) and 1.00 (±0.06) ng/µL for buccal swab eluate and 1.06 (±0.06) and 1.09 (±0.22) ng/µL for whole blood, respectively, with the recommended amount of enzyme. All incubation times tested, for both buccal swab eluate and whole blood, resulted in similar DNA recoveries for each sample type. This supports that a 3-fold reduction in incubation time can be used without a significant decrease in the amount of DNA recovered.

To further optimize the enzyme-mediated DNA preparation method, modifications were made to improve the DNA yield. First, the amount of enzyme needed in the reaction was tested. DNA preparations were made using enzyme amounts of 1 µL (manufacturer’s protocol), 2 µL, and 3 µL with buccal swab eluate and whole blood samples at the incubation times that were discussed above (5, 10 or 15 min at 75 °C, followed by 5 min at 95 °C). With a 2- and 3-fold increase in amount of enzyme, an increasing quantity of DNA can be recovered from both whole blood and buccal swab lysate (Table 2). Specifically, with a 3-fold reduction in incubation (from 15 to 5 min) and 3-fold increase in enzyme amount (from 1 µL to 3 µL), a 5-fold increase in DNA yield is observed for both buccal swab lysate (from 1.00 (±0.06) ng/µL to 5.25 (±1.42) ng/µL) and whole blood (from 1.09 (±0.22) ng/µL to 5.79 (±0.50) ng/µL). These results suggest a linear relationship between the DNA yield and the amount of enzyme added to the reaction, between 1 and 3 µL, given the same amount of starting cellular material, i.e. 20 µL for buccal cell eluate and 2.5 µL for whole blood. When 4 or 5 µL of enzyme is added, however, a plateau is reached which is most likely the result of the number of cells in the preparation reaction becoming the limiting factor (Fig. 1). Furthermore, these results support the results obtained by Moss et al. for increased amounts of enzyme, where they showed increased peak heights with higher enzyme amounts.

To yet further optimize the DNA yield, the effect of varying the quantity of starting cellular material was evaluated. The volume of buccal swab eluate added to the reaction mixture was increased (from 2.5 µL to 80 µL), while the volume of water added was reduced to keep the total reaction volume at 100 µL. The amount of DNA recovered from buccal swab eluate using the enzyme-based method improved with increasing eluate added to a maximum of 40 µL, at which point a plateau was reached (Fig. 2A). The DNA yield could be augmented from the 40 µL data point onward when using increasing enzyme concentrations (data not shown). Additional studies between 0 and 10 µL buccal swab eluate a linear relationship exists (Fig. 2A, inset). This provides a way to estimate the DNA yield by extrapolating from the known volume of buccal swab eluate added, for a particular sample, as long as a constant volume of enzyme is used in the reaction. In addition, the unprocessed sample (i.e. the eluted cells) can be placed in a storage buffer and saved for future testing, if necessary.

Similarly with whole blood, the volume added to the reaction mixture was incrementally increased from 0.5 µL to 90 µL while reducing the volume of water (total volume remained 102 µL). Fig. 2B (inset) demonstrates that the yield increases until 2.5 µL of whole blood is added, following which the yield begins to decrease. Beyond 10 µL, and up to 90 µL, a steady increase in DNA yield is observed (Fig. 2B). The results are anomalous in that the relationship does not hold true between 5 and 25 µL but may be explained by recognizing that measured yields are changed by both actual yield and inhibition. It is possible that higher quantities of blood have a carrier effect assisting in the precipitation of inhibitors. In addition, the theoretical DNA yield was calculated using the WBC count provided and plotted along with the experimental results. The theoretical and experimental values track well below 5 µL, but above 5 µL, the theoretical yield remains linear, while the experimental results first decrease then rebound and significantly increase. The reason for deviation from the theoretical values is still under investigation and may be related to behavior of the EA1 enzyme in the presence of large quantities of blood.

Again the results show that the analyst has some control over the amount of DNA prepared from a given sample by modulating the amount of sample used. As 500 pg–1.5 ng of DNA is required for STR PCR, it would not be necessary to purify DNA from the entire sample. Hence, some unprocessed sample can be set aside for other analyses such as blood drug or alcohol content.

Although the enzyme is responsible for some of the cell lysis that occurs, the method requires an enzyme deactivation step at 95 °C which alone would result in some cell lysis [24,25]. In addition, vortexing of the sample can also disrupt cell membranes and cause a small amount of cell lysis. However, without the enzyme present to remove the inhibitors released from the cells (e.g., proteins and nucleases [26,27]), any PCR that is performed will likely be inhibited and produce little to no product. To determine the extent of cell lysis due to temperature and vortexing, buccal swab eluate was incubated for 0–15 min at
75 °C with or without enzyme present, followed by the requisite 5 min at 95 °C. In addition to quantitation with RiboGreen®, a qPCR method using a TaqMan® probe was used to determine DNA yield and/or inhibition from cellular or extracellular material present in the sample [23]. Fig. 3 shows that when no enzyme is present in the reaction, the fluorescent assay reveals a DNA yield of approximately 1 ng/µL (blue trace). However, when the same samples are quantified using qPCR, the yield is approximately 200 pg/µL (red trace), indicating that even though there is ~1 ng/µL DNA present in the solution, only one-fifth of it is available for PCR. The different yield obtained from qPCR may be caused by inhibition from cellular debris and other components still present in the solution.

When enzyme is added to the reaction, not only does DNA yield increase significantly (nearly 4-fold), but the quantitation values from both the fluorescent and qPCR assays are statistically similar (Fig. 3). Also worthy of note, DNA yield is not significantly altered from 0 to 15 min, corroborating previous studies, and may allow a reduction of sample preparation time. This would offer a 15-fold reduction in time needed (from 15 to 1 min) to generate PCR-ready DNA over that suggested by the manufacturer, and a 30-fold reduction in time (from 30 to 1 min) compared to conventional silica-based purification columns (i.e., Qiagen).

The optimization and modification of this DNA preparation method has shown that two parameters can be altered to effect the DNA yield. DNA yield can be enhanced from the same amount of starting cellular material by simply increasing the amount of the EA1 enzyme. Alternatively, holding the enzyme concentration constant, the DNA yield from brush swab eluate can be increased by supplying more starting cellular material. Concurrently, it was demonstrated that there is little to no effect on DNA yield with a decrease in the incubation time at 75 °C. Furthermore, the enzyme was shown to play a critical role in not only the lysis of cells, but in the removal of PCR inhibitors released from the cells, such as nucleases [28].

3.2. PCR amplification of DNA from enzyme-prepared whole blood and brush swab eluate

Following optimization of the enzyme-based DNA preparation method, it was essential to demonstrate that the purified DNA was of sufficient quality for forensic analysis, specifically PCR amplification of STR loci. A common commercial STR amplification kit, Identifiler®, involves the amplification of 15 autosomal STR loci and the sex-typing locus, Amelogenin. Processing non-degraded brush swab eluate and whole blood as described in Section 2, 1 µL of the prepared DNA was amplified using the Identifiler® PCR amplification kit in the conventional manner. Samples without the enzyme in the reaction solution resulted in partial profiles for all samples (8 of 16 loci [12 of 26 alleles]), demonstrating the importance of EA1 in removing PCR inhibitors (data not shown). Full STR profiles (16 of 16 loci) were obtained from both brush swab eluates (Fig. 4A) and whole blood (Fig. 4B). These results demonstrate that not only is the DNA PCR amplifiable from the enzyme-based method but it can also be used for complex PCR reactions, such as STR amplifications. The average peak heights were 419 (±230) and 353 (±185) RFU for brush swabs and whole blood respectively, with peak height ratios above 0.7 for all heterozygote loci for both.

Fig. 2. Effect of additional starting material in the conventional ZyGEM reaction. (A) Water in the reaction mix was increasingly replaced by brush swab eluate, until no water was used. Inset: close-up view of the first five data points. (B) Increasing amounts of whole blood (stored in EDTA) were added to the reaction mix until no water was used. Inset: close-up view of the first seven data points. All points represent n = 3, with errors bars for the standard deviations (one brush swab used for each replicate). Whole blood samples are not from Person J, but rather collected as described in Section 2. Using the WBC count provided, the theoretical yield (of efficiency of enzyme-based method were 100%) of DNA was calculated and is represented as the red dashed line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3. Comparison of samples in the presence or absence of the enzyme EA1. qPCR (red) and quantitation with RiboGreen® (blue) show that without the enzyme, cells are still lysed during the 95 °C incubation (blue), but PCR is significantly inhibited by cellular materials left in the solution (red). However, qPCR (green) and quantitation with RiboGreen® (pink) show that in the presence of the enzyme, DNA is released from the cells and PCR is not inhibited or hindered by the enzyme solution. Error bars represent the standard deviation for n = 3 (one brush swab used for each replicate). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
sample types (data not shown). In addition, the inter-color peak balance for both sample types was above a 20% threshold and though the intra-color balance for each sample type was below the 50% threshold (data not shown), all alleles were still above the allele detection threshold of 100 RFU. Additionally, the method is shown to be sufficiently robust to varying quantities of cellular material (protein content in buccal swab eluate (~0.30 μg protein/ng DNA) [29] versus whole blood (~4.29 μg protein/ng DNA) [30]).

Although the results shown in Fig. 4 demonstrate the applicability of the enzyme-based method, most samples presented in forensic laboratories are dried biological stains on a variety of substrates. Therefore, the method was tested on dried samples. A portion of a dried buccal swab (5.0 ± 0.1 mg; ~1/3 of a swab) or a 0.5 cm² portion of blue denim, encompassing a 2.5 μL dried bloodstain, was cut into pieces, added to 100 μL of the enzyme DNA preparation cocktail and processed using the manufacturer’s protocol as stated in Section 2. Increased amount of enzyme was not used so that the results from the stain could be compared to the results from the liquid blood sample (both used 2.5 μL of whole blood). Results show that the enzyme-based method generates full STR profiles (16 of 16 loci) from dried buccal swabs (Fig. 4C) or dried bloodstains (Fig. 4D) without a loss in STR peak height. With buccal swab samples, only a portion of the sample is processed when the cellular material is eluted first (20 μL). In comparison, the entire sample (~1/3 of the swab) was processed for the dried buccal swab, without eluting any cellular material. As a result, there is an overall increase in average peak height to 1177 (±669) RFU for buccal swabs. In addition, the results demonstrate that the method can produce DNA from stains on both cotton and blue denim substrates and produce comparable results to those obtained using SPE methods (Supplemental Figs. 1 and 2). Typically, SPE methods involve the incubation of the stain in lysis buffer for up to 1 h prior to the main extraction procedure. With the enzyme-based method, the DNA from the stain is ready for direct addition to PCR in 20 min, representing at least a 3-fold reduction in analysis time, with no additional wash or concentration steps required.

3.3. PCR amplification of DNA from degraded biological samples

In addition to encountering biological samples in the form of stains, many samples analyzed by forensic laboratories have experienced harsh environmental conditions including heat, humidity and/or exposure to UV radiation from sunlight. To test whether the enzyme-based method could be used to purify DNA from degraded samples, simulation of outdoor conditions were created by placing samples in a 56 °C oven or exposing them to ultraviolet-C (UVC) radiation for a prolonged period of time. UV radiation exposure causes numerous photoproducts and DNA strand breaks, reducing the likelihood of obtaining a full STR profile [17]. Specifically, UVC (200–290 nm) radiation, which causes similar damage to UVB (290–320 nm) and UVA (320–400 nm) (the main components of sunlight), was used in this work to create the common photoproducts seen. 0.5 cm² cuttings of blue denim or cotton fabric spotted with 10 μL of whole blood were exposed to UVC radiation for 158 min (equivalent to ~12 months of outdoor exposure). The material was cut into smaller pieces and processed using enzyme-based or conventional SPE methods as described in Section 2.

When purifying DNA from a degraded bloodstain on blue denim, the enzyme-based method gave a partial profile with 14 of 16 detectable loci (15 of 26 alleles), whereas the STR profile generated from conventionally purified DNA (SPE method) gave only 11 of 16 loci (13 of 26 alleles) (Fig. 5A). However, if the allele detection threshold can be set to 75 RFU, the STR profile produced following the enzyme-based method has 26 of 27 alleles present, while the STR profile produced from the SPE method has 21 of 27 alleles present. A similar result was obtained when DNA was purified from a bloodstain on cotton, where the enzyme-based method outperformed conventional SPE giving 10 of 16 loci (18 of 27 alleles) compared to 4 of 16 loci (11 of 27 alleles) (Fig. 5B). Although full STR profiles were not obtained from these samples, Hall and Ballantyne [17] only demonstrated partial profiles from bloodstains that had been exposed to UV radiation for the equivalent of ~4 years outdoors using an organic extraction method and concentration step.
Another common environmental factor impacting the quality of forensic samples is elevated temperature. Exposure of blood samples to 56 °C has been demonstrated cause allelic and/or locus dropout [31]. To determine whether heat-degraded samples could be prepared using the enzyme-based method, bloodstains on cotton or blue denim substrates were placed in a 56 °C oven for a period of one month. DNA was extracted from the stains using either SPE or enzyme-based methods. No significant difference was found between the two methods when used for the heat-degraded bloodstains on blue denim (Fig. 6A), but the enzyme-based method yielded a full (16 of 16 loci) profile for bloodstains on white cotton outperforming SPE, which yielded 12 of 16 loci (21 of 27 alleles) (Fig. 6B). These results demonstrate the first use of an enzyme-based method for the purification of DNA from both UV- and heat-degraded biological samples.

3.4. Microchip enzyme-based DNA preparation

Translation of the method to microfluidics offers numerous advantages including a closed environment, further reducing entrance points for contaminants, reduced analysis time, and potential integration with downstream processes [8]. To test whether the enzyme-based method can be easily adapted for microfluidics, glass microdevices using a 400 nL incubation chamber were filled with the enzyme extraction cocktail containing epithelial cells from buccal swab eluate (whole blood was not incubated on the microdevice due to possible clogging of the microchannels that may occur from the precipitate that forms). Currently, no substrate can be directly added to the microdevice, so elution of the cells from the substrate first is a necessity. After a 20 min incubation (15 min at 75 °C and 5 min at 95 °C) using the non-contact IR-PCR system as previously described for PCR on a microfluidic device [15,16,21], the sample was removed from the device and conventionally amplified using Identifier®. Fig. 7A shows that a full STR profile can be obtained using this method. To determine whether the analysis time could be further reduced with the microdevice, buccal swab eluate with enzyme-based preparation reagents were again incubated in a glass microdevice using the IR-heating system for only 60 s (30 s at 75 °C and 30 s at 95 °C). Following conventional PCR amplification with the Identifier® kit and separation and detection, a full STR profile resulted (Fig. 7B). Successful reduction of the analysis time to 60 s provides approximately a 20-fold reduction in analysis time (from 20 min to 60 s) compared to the conventional enzyme-based method and at least a 30-fold reduction (from 30 to 60 min to 60 s) when compared to conventional SPE. These results demonstrate the first application of an enzyme-based DNA preparation method to a microdevice with an incubation time of only 60 s.

Due to the success seen with glass microdevices for enzyme-based DNA preparation, the next logical step was to determine whether plastic microdevices could be used. Plastic microdevices were fabricated in PMMA using a laser ablation system, as described in Section 2. A 6 nL portion of the enzyme-based preparation solution was loaded into these devices and incubated for 20 min using the IR-PCR system. Results show that DNA purified on PMMA using the enzyme-based method and IR-PCR temperature control provides a full STR profile (Fig. 7C). Similar to the studies in the glass microdevices, DNA preparation on the PMMA microdevices was completed in 60 s and gave a full STR profile (Fig. 7D) with little decrease in STR peak height. The differences between STR results for PMMA and glass are most likely due to the differences in volumes, both chamber and overall, between the two microchips. The glass microchips have a ~400 nL chamber volume with an overall volume of ~2 μL (a ~5-fold dilution). Alternatively, the PMMA microchips have a ~689 nL chamber with an overall volume of ~6 μL (a ~8.7-fold dilution).
Fig. 6. Identifiler<sup>®</sup> profiles (representatives of n = 3) obtained after ZyGEM treatment of bloodstains, which had been heated in an oven at 56 °C for a period of one month. (A) 1 μL bloodstain on blue denim jeans (13 of 16 loci). Inset: electropherogram obtained after SPE of the same sample type (13 of 16 loci). (B) 1 μL bloodstain on cotton (16 of 16 loci). Inset: electropherogram obtained after SPE of the same sample type (14 of 16 loci).

Fig. 7. Identifiler<sup>®</sup> profiles (representatives of n = 3) obtained after incubation on microdevices. (A) Full Identifiler<sup>®</sup> profile (16 of 16 loci) after incubation on a glass microdevice using conventional incubation times (The extra peaks in D19S433 and vWA are pull-up from D3S1358 and TH01, respectively). (B) Full Identifiler<sup>®</sup> profile (16 of 16 loci) after incubation on a glass microdevice using reduced incubation times of 30 s at 75 °C and 30 s at 95 °C. (C) Full Identifiler<sup>®</sup> profile (16 of 16 loci) after incubation on a PMMA microdevice using conventional incubation times. (D) Full Identifiler<sup>®</sup> profile (16 of 16 loci) after incubation on a PMMA microdevice using reduced incubation times of 30 s at 75 °C and 30 s at 95 °C.
The increase in this dilution lowers the concentration of DNA in the sample and therefore, in the PCR. These results demonstrate that the enzyme-based method is adaptable to both microdevices made from glass or plastic, which result in a 30-fold reduction in incubation time compared to conventional SPE methods (1 min versus 30 min).

4. Conclusion

This work demonstrates the utility of an enzyme-based DNA preparation method for forensic DNA analysis of buccal swabs, whole blood and bloodstains on blue denim or cotton. The method was shown to produce STR profiles that were comparable to those from conventional SPE, and in many cases outperformed the SPE method. In addition, the enzyme-based method produced PCR-ready DNA after a 20 min incubation and requires no centrifugation or sample transfer steps. Furthermore, the DNA yield may be controlled by increasing the amount of starting material or enzyme added to the reaction, demonstrating the adaptability of the method to a wide variety of sample types. The enzyme-based method was also shown to be adaptable to microdevices and allowed for the incubation time to be reduced to 60 s providing a 20-fold and 30-fold reduction in analysis time compared to conventional enzyme-based preparation and conventional SPE, respectively. Implementation of this method into the workflow for forensic cases could reduce sample and DNA preparation time, leading to a reduction in overall analysis time. The adaptability of this method, due to its simplicity, also allows for the integration of this method with downstream processes, such as PCR, on a single, disposable microdevice, providing the quintessential on-site analysis tool.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsiagen.2012.01.011.

References


[22] Quick start guide – DNA extraction using forensicGEM™ saliva, Available at http://www.eyegem.com/Products/Products-FG-Saliva.html.


