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Assessment and *In Vitro* Repair of Damaged DNA Templates

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ABSTRACT

DNA extracted from biological stains is often intractable to analysis. This may due to a number of factors including a low copy number (LCN) of starting molecules, the presence of soluble inhibitors or damaged DNA templates. Remedies may be available to the forensic scientist to deal with LCN templates and soluble inhibitors but none presently exist for damaged DNA. In fact, only recently has the biochemical nature, the extent of DNA damage in physiological stains and the point at which the damage inflicted upon a particular sample precludes the ability to obtain a genetic profile for purposes of identification been examined. The primary aims of this work were first to ascertain the types of DNA damage encountered in forensically relevant stains, correlating the occurrence this damage with the partial or total loss of a genotype, and then to attempt the repair of the damage by means of *in vitro* DNA repair systems.

The initial focus of the work was the detection of damage caused by exogenous, environmental sources, primarily UV irradiation, but also factors such as heat, humidity and microorganism growth. Results showed that the primary causes of the damage that resulted in profile loss were strand breaks, both single and double stranded, as well as modifications to the DNA structure that inhibited its amplification.

Armed with this knowledge, the next focus was the repair of the damage by means of *in vitro* DNA systems. Efforts have been concentrated on single strand break/gap repair and translesion synthesis assays. By modifying the assays and employing various combinations of the systems, a genetic signature has been recovered from previously intractable samples.

Additionally, the effects that various storage conditions have on the DNA in physiological stains stored in a laboratory were examined. The optimal long term storage conditions for biological evidence has been a matter of debate in the forensic community for some time. But, no comprehensive study had previously been undertaken to describe the effects of dehydration and temperature on degradation and the ability to obtain a genetic profile on bloodstains kept in different types of storage media at a range of temperatures. To examine this, bloodstains were either allowed to dry overnight or placed in the storage medium while still wet and were stored at room temperature, 4^{o}C or 30^{o}C for up to four years. Results showed that specimens dehydrated prior to storage were very stable, and these bloodstains showed no degradation or loss of a genetic profile for up to four years.

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Executive Summary

- An experimental model for the assessment of DNA damage in physiological stains recovered from a crime scene was developed by subjecting DNA in the hydrated (naked DNA in solution) and dehydrated (naked, dehydrated and stain DNA) states to varying doses of UVC. The extent of the damage was first assessed by gel electrophoresis (native and alkaline). The point at which the damage inhibited DNA polymerase mediated primer extension, resulting in genotyping failure, was determined by attempting to amplify sample using a standard autosomal STR multiplex.

- UV photoproducts could not be detected in UVC exposed bloodstains. The primary lesions contributing to the loss of a genetic profile appeared to be strand breaks, both single and double. It was not possible to preclude the possibility, however, that a combination of damage types was responsible for the profile loss observed.

- The DNA in bloodstains is protected against damage by its state of dehydration and, to a lesser extent, by the presence of the cellular milieu. We hypothesize that this effect is due to 1) the change in the conformational state of the DNA (B → A) as it becomes dehydrated, and 2) the limited diffusibility in the dehydrated state.

- The study was extended to include an assessment of the effects of UVA and UVB irradiation. In general, the damage inflicted upon DNA can be described as follows:

UVC > UVB >UVA. The damage detected in each of the sample types can be described as follows: naked DNA > naked, dehydrated DNA > stain DNA.

- Bi-pyrimidine photoproducts could only be detected in naked DNA exposed to UVB. They were undetectable in any of the dehydrated sample types. Oxidative lesions were present in naked DNA exposed to both UVA and UVB light, but were not detected in the dehydrated samples. Strand breaks were observed in UVA/B irradiated naked, dehydrated and stain DNA. Exposure to UVA for up to 8.25 days was not sufficient to cause even the partial loss of a profile in naked, naked dehydrated or stain DNA. UVB – induced damage resulted in a partial profile loss in naked and naked dehydrated DNA after extended exposure, but in no instance caused a complete inhibition of amplification. UVA/B irradiation by itself did not cause sufficient damage to cause DNA genotyping failure.

- To evaluate the effects of humidity, bloodstains were incubated in a humidity chamber (~94% relative humidity) exposed to simulated natural sunlight. Double strand breaks were formed early (2 days' exposure), but UV photoproducts could not be detected. After 5 weeks of continuous exposure, amplification of a genetic profile was only partially inhibited. Sixteen weeks' exposure resulted in a complete profile loss. The ability to type true forensic samples can be impeded well before that time, so the contributions of heat, humidity and UV, while certainly contributory, are not the complete story. A major environmental factor was excluded from the initial experimentation – the contribution of microorganisms.

- A set of bloodstains was exposed, unprotected in any way, to the outside environment. A partial profile was obtained after 3 days' exposure, and was completely lost by 1 week. Microorganism induced DNA damage, in the form of double strand breaks, was found, and is expected to be a major contributor to the loss of DNA typing ability in forensic-type stains.

- A PCR based genome wide assay for the detection of specific types of damage was developed - lesion specific endonucleases nicked the DNA at damage sites, the single strand breaks were made into double strand breaks and the amount of damage estimated as a reduction in PCR product. However a number of problems were encountered using this method and these are delineated.

- The effects of temperature and substrate on the typeability of stored DNA were also studied in an effort to define the optimal storage conditions for DNA evidence held in a crime laboratory. Samples were stored 1) dried in a paper envelope, or 2) wet or dried in a plastic bag for up to 4 years at 4^{o}C, room temperature, or 30^{o}C. While there was no observable difference between the profiles of the control and exposed bloodstains up to 13 months, the four year samples stored at 30^{o}C showed signs of degradation in that the larger alleles became reduced in size relative to the others.

- Direct reversal of UV photoproducts, catalyzed by photolyase, resulted in the successful recovery of a genetic profile from UVC damaged naked DNA, but the success could not be extended to a wider variety of samples.

- Translesion polymerases were successfully incorporated into a PCR reaction with *Taq* polymerase. These polymerases allowed for the bypass of base modifications in UVC-damaged and environmentally exposed samples.

- A modified base excision repair (BER) strategy was developed for the repair of single strand breaks/gaps, bridging both UVC and environmentally induced lesions. Combining the modified BER strategy with translesion synthesis allowed for greater recovery of an *Alu* element genetic signature than did either method singly.

I - DNA DAMAGE ASSESSMENT

Phase I of this project involved the *in vitro* assessment of the damage done to DNA in physiological stains exposed to a variety of environmental insults including heat, light, humidity, UV radiation and microorganism growth. The effects of UVA/B/C light were studied separately, as were the damage caused by humidity and environmental exposure. Both gel- and PCR-based assessment assays were developed, revealing the state of the DNA in both the hydrated and dehydrated forms.

It has long been a matter of debate within the forensic community what constitutes the optimal, or acceptable, storage condition for a biological sample. Due to cost and space limitations in refrigerator/freezers in crime laboratories, biological evidence is often stored at room temperature or even in a warehouse setting. The dehydration state of the stain and the storage materials are also an important consideration. A study was conducted in which the effects of temperature and substrate on the DNA in stored bloodstains were examined and the results reported below.

UVC Damage

UVC Induced Damage to Naked DNA in Solution

Solar ultraviolet radiation consists mainly of UVB (290 – 320 nm) and UVA (320 – 400 nm) rays. The UVC portion of the spectrum (200 – 290 nm) is filtered by the ozone layer and does not reach the earth. For convenience, however, UVC was initially used to induce damage to DNA in the samples since it is likely that UVC induces the

same lesions as UVA and UVB, although the kinetics of their formation and their relative proportions may differ[1].

The strategy for the assessment of UVC damage was to focus initially on naked genomic DNA. Such DNA is not subject to the potential protective effects afforded by the constituents of the cellular environment *in vivo*. One to two micrograms of human genomic DNA (100 ng μl^{-1}) was irradiated with UVC light for various times at a flux of 104 mJ cm^{-2} min^{-1} and the effects of this treatment on both the structural integrity of the DNA and the ability to obtain a genetic profile were evaluated.

Gel Electrophoresis Analysis (naked DNA)

Native gel electrophoresis revealed that samples exposed for up to 25 minutes comprised high molecular weight DNA (~20 kb) with no apparent degradation in the form of double strand breaks. However, although the same quantity of DNA (~100 ng) was added to each lane in the gel, the putative high molecular weight DNA band began to exhibit retarded migration in comparison to the 20 kb size marker as the UVC exposure time increased. This observation is consistent with the presence of inter- or intra-strand DNA cross-links. Naked DNA samples exposed to UVC for 1 hour still showed a high molecular weight band, but double strand breaks became apparent. From 4 hours to 48 hours, the number of double stranded breaks steadily increased, until the sample was entirely degraded beyond 48 hours.

In order to detect the presence of single strand breaks, the treated DNA samples were run on denaturing alkaline gels. Single strand lesions were detected after 1 minute UVC exposure, the quantities of which increased noticeably thereafter. High molecular

weight DNA was still detectable under these denaturing conditions up to 4 minutes. After 16 minutes, the single strand breaks increased dramatically, with the concurrent loss of the high molecular weight (~20 kb) (HMW) band. Exposure times between 1 hour and 12 hours resulted in the steady increase of both single and double strand breaks and a concurrent steady reduction in the number average molecular weight (NAMW) of the DNA sample. Beyond 12 hours, the sample became completely degraded and could no longer be visualized. Interestingly, DNA that exhibited retarded migration compared to the ~20 kb marker was observed with increased UVC exposure from 1 minute to 1 hour, providing further evidence for the induction of DNA-DNA cross-links.

DNA Profiling (naked DNA)

To determine the effects of UVC treatment of naked DNA on the ability to obtain a genetic profile, the UVC treated naked DNA samples were amplified and typed using a nine locus autosomal short tandem repeat (STR) multiplex system plus the amelogenin gender marker (AmpFLSTR® Profiler™ PCR Amplification kit). A complete nine locus STR profile was obtained up to 1 minute UVC exposure (0.104 J cm^{-2}) but increased exposure to 2 minutes resulted in a partial loss of profile, in which the alleles at the D7S820 locus were lost with respect to the expected profile. The partial profiles obtained were characteristic of that expected from a degraded sample in that the larger loci signals were significantly reduced in intensity. As the UV dose was increased, the other loci progressively disappeared until the profile was completely lost at 16 minutes (1.664 J/cm^2). The loss of the genetic profile at this point corresponded to the loss of high molecular weight DNA observed by alkaline gel electrophoresis at the same time.

UVC Induced DNA Damage in Bloodstains

Next, the effects of UVC exposure on bloodstains were determined. Blood was spotted on filter paper in 50 µl aliquots (approximately one drop) and allowed to dry overnight. Dried bloodstains were exposed to UVC using the same flux rate as before ($0.104\ J\ cm^{-2}\ min^{-1}$).

Gel Electrophoresis Analysis (Bloodstains)

DNA was isolated from the bloodstains using a standard phenol:chloroform organic extraction procedure and visualized on a native agarose gel, or on an alkaline agarose gel. Samples visualized on the native gel consistently showed high molecular weight, non-degraded DNA through at least 102 hours exposure. However, when visualized on the alkaline gel, a significant number of single strand breaks appeared after 4 hours UVC exposure and increased thereafter, although the decline in number average molecular weight with increased exposure time was dramatically slower than that observed with naked DNA in solution. Single strand breaks were evident in all samples including the no exposure control, suggesting that the dehydration and rehydration of the DNA played a role in their formation.

DNA Profiling (Bloodstains)

With respect to the ability to obtain an STR profile, full nine locus profiles were obtained with UVC exposure up to 8 hours. At 12 hours, however, there was a significant loss of alleles at several loci and partial profiles continued to be obtained up to 79 hours. The profile was lost completely at 102 hours.

Collectively, the results indicate that, compared to naked DNA, DNA in bloodstains is protected somewhat against the damaging effects of UVC. For example, the genetic profile was lost in naked DNA samples exposed to 1.664 J cm^{-2} (16 minutes) of UVC, while it required 636 J cm^{-2} (102 hours) to produce the same effect in bloodstains, an approximate 360-fold increase in UVC dose. This protection from the harmful effects of UVC in bloodstains could be due to the dehydrated state of the nucleic acid in the stain, the local cellular milieu of the DNA or a combination of both. To further explore this issue, the effects of UVC irradiation on naked, but dehydrated, genomic DNA were investigated.

Effects of Dehydration on UVC Induced DNA Damage

Gel Electrophoresis Analysis (Dehydrated, Naked DNA)

Naked human genomic DNA was dried and exposed to UVC at the same flux as before. After exposure, the DNA was re-solubilized to a concentration of 100 ng μl^{-1}. Again, samples were visualized on native and alkaline agarose gels. Dehydrated naked DNA exposed to UVC up to 25 minutes showed high molecular weight DNA on the native gel, but began to degrade after 1 hour as evinced by the appearance of double strand breaks and the concomitant reduction of the intensity of the high molecular weight (HMW) band over time. The HMW band was lost at 6 hours and the DNA was degraded completely at 48 hours. Alkaline gel electrophoresis revealed a gradual decrease in the number average molecular weight of the DNA in samples irradiated from 6 minutes to 4 hours, indicative of increasing numbers of single strand breaks. The 8 hour sample showed an increase in number average molecular weight, consistent with the presence of

significant levels of DNA-DNA cross-links. As seen in the dehydrated stain DNA, single strand breaks were evident in all samples. They increased gradually over time until 12 hours after which significant numbers of single strand breaks were present and, like the native gel results, the 48 hour sample was completely degraded.

DNA Profiling (Naked, Dehydrated DNA)

Dehydrated naked DNA was capable of producing a complete nine locus STR profile up to 12 hours exposure (74.9 J cm^{-2}) but was totally lost at 24 hours and beyond. Thus the UVC exposure time needed to produce a total profile loss in dehydrated, naked DNA was longer than the 16 minutes (1.6 J cm^{-2}) required to produce the same effect in naked DNA in solution but, significantly, less than the 102 hours (636 J cm^{-2}) necessary to do so when DNA was present in bloodstain form. DNA in the latter is both dehydrated and present in a nucleoprotein (i.e. chromatin) complex within the cellular infrastructure. Therefore, dehydration *per se* affords DNA a measure of protection against the harmful effects of UVC irradiation.

Bi-Pyrimidine Photoproducts

Next, the formation of the two major types of bi-pyrimidine photoproducts (BPPP) in UVC treated DNA (CPDs and (6-4)PPs) were evaluated using, as before, naked DNA in solution, naked dehydrated DNA and DNA isolated from UVC exposed bloodstains. To accomplish this, three different lesion specific endonucleases, *Chlorella* virus pyrimidine dimer glycosylase (CV-PDG), T4 Endonuclease V (T4 EndoV), and *S.*

pombe ultraviolet damage endonuclease (UVDE), were used. CV-PDG is a DNA glycosylase with associated AP lyase activity that recognizes both *cis-syn* and *trans-syn* CPDs, leaving a single strand gap at the site of this damage [2]. T4 Endo V is also a pyrimidine dimer glycosylase with associated AP lyase activity, but recognizes only *cis-syn* CPDs, generating a single strand gap [3]. UVDE is a DNA glycosylase lacking AP lyase activity. It recognizes both CPDs and 6-4(PPs), generating a strand nick at the site of damage. The recognition specificity of this enzyme is not as limited as that of CV-PDG and T4 Endo V. UVDE has been shown to cleave at AP sites, but may also recognize other types of damage, such as adducts or modified bases, due to a relaxed structural constraint at its recognition site[4].

BPPPs in UVC Treated Naked DNA

The formation of CPDs in naked DNA in solution was investigated first. For each of the time intervals examined, both an enzyme treated sample and a 'no enzyme' treated control were run side by side. The latter control was used to take into account any heat, pH or oxidative induced damage inflicted during the DNA extraction and digestion procedures themselves. Samples were first digested using CV-PDG. Samples that contained CPDs were often indistinguishable from untreated controls when run on a native gel, but produced characteristic smears of DNA on an alkaline gel as the result of the formation of endonuclease induced single strand breaks. CPDs formed rapidly after only 5 seconds irradiation (0.009 J cm^{-2}), and steadily increased until 30 seconds (0.052 J cm^{-2}), at which point their formation appeared to level off. These results were confirmed by T4 Endo V digestion and alkaline gel electrophoresis of the same samples. These

observations are consistent with previous reports indicating that CPD formation reaches saturation at doses around 0.05 J cm^{-2}[1,5].

Next, naked DNA in solution was treated with UVDE to detect the formation of both types of BPPPs, including (6-4)PPs. Enzyme induced single strand breaks were apparent after 30 seconds, and increased linearly with dose until 16 minutes exposure. Thereafter the damage to naked DNA remained constant, until the sample could no longer be visualized at 12 hours. The linear increase in single strand breaks beyond the 30 second UVC exposure observed to produce CPD saturation is consistent with the continuous formation of (6-4) PPs, and is in accord with published reports. Interestingly, identical results were observed when the same samples were visualized on a native gel indicating the possible presence of double strand breaks. However the formation of DNA double strand breaks requires the input of an enormous quantity of energy, and has only been documented at the UVC doses described here when administered in the vacuum UV range (< 254 nm). Therefore, the degraded DNA observed here on native gels is most likely the result of a sufficient number of single strand breaks being generated in close proximity to one another on opposite DNA strands, such that the resulting fragmentation appears akin to that produced by *bona fide* double strand breaks.

Our previous data indicated that a genetic profile was partially lost after 2 minutes UVC exposure to naked DNA (0.21 J cm^{-2}), a full minute and a half after CPD formation had leveled off and subsequent to the appearance of single strand breaks and DNA-DNA crosslinks, which were observed after only 1 minute . The formation of (6-4)PPs (and other lesions recognized by UVDE) steadily increased with UVC exposure until 16 minutes (1.66 J cm^{-2}), which coincidentally was the point at which the genetic profile was

lost. UVDE detected damage did not increase appreciably beyond this. Collectively, these results are inconsistent with CPDs being the principal or only cause of genetic profile loss in the UVC treated naked DNA samples. However, the kinetics of profile loss are consistent with a role for single strand breaks and, possibly, DNA cross links in this process.

BPPPs in UVC Treated Bloodstains

Previous data indicated that naked DNA in solution experienced the effects of UVC induced damage more severely than DNA in other states, since it is unprotected by the cellular milieu and/or dehydration. Using the assays based upon the lesion specific enzymes developed for naked DNA, dried bloodstains exposed to UVC light were examined for the presence for BPPPs. However, a complicating factor with the analysis is the necessity of employing a DNA extraction procedure subsequent to UV exposure and prior to enzyme digestion, during which DNA could be subjected to further damage caused by the additional experimental manipulations required. To account for this a 'no enzyme' control was included for each time interval. Because each 'no enzyme' sample was subject to the same manipulations and incubations as the digested samples, any damage incurred by the physical processes of the experiment was controlled for.

To examine the formation of CPDs, DNA isolated from bloodstains exposed to UVC for times ranging from 25 minutes to 102 hours was restricted using CV-PDG, T4 EndoV, and UVDE, and visualized on an alkaline gel. To detect the presence of (6-4)PPs the same bloodstain extracted samples were restricted with UVDE, and run on an alkaline

gel. A comparison of the CV-PDG, T4 EndoV and UVDE restricted samples with their no enzyme digestion controls revealed little difference if any, indicating that BPPPs are not formed in dehydrated, biological stains in significant quantities as detected by this assay.

It has been shown above that a genetic profile is partially lost from bloodstain DNA exposed to UVC after 12 hours (75 J cm^{-2}), and completely lost after 102 hours (636.5 J cm^{-2}). Thus, the loss of the profile does not appear to be only, or even principally, due to the presence of UVC induced-BPPPs, or any other lesion recognized by UVDE. Although it is not entirely clear what type of damage is responsible for the profile loss, single strand breaks or gaps are formed, as evidenced by a decrease in number average molecular weight of the DNA on denaturing gels after UVC exposure. Although it was not possible to quantify the number of single strand breaks with our assay, the number of single strand breaks on opposite DNA strands was insufficient to produce products with the appearance of double strand breaks on native gels as was found, for example, with UVDE digested, UVC treated naked DNA .

BPPPs in UVC Treated Naked, Dehydrated DNA

The physical state of the DNA in bloodstains appeared to protect it against the formation of BPPPs. In order to determine the protective effects of dehydration the naked dehydrated DNA samples described previously were digested using the same lesion specific endonucleases. A comparison of the CV-PDG , T4 EndoV and UVDE restricted samples with their respective ‘no enzyme’ controls revealed the formation of a limited number of BPPPs, until the samples became so fragmented beyond 4 hours that

they could no longer be visualized on the gel. Significantly, a full genetic profile was still obtained with fragmented DNA after 12 hours exposure, but was completely lost by 24 hours. This situation is quite different than that observed using naked DNA in solution, indicating that the dehydrated state plays a significant role in the resistance of the DNA to damage, and the maintenance of the ability to obtain a genetic profile.

Conclusions: UVC Damage

As a model for DNA damage assessment in physiological stains recovered from crime scenes, human bloodstains and naked DNA in the hydrated and dehydrated states have been subjected to varying doses of UVC radiation. UVC irradiation of DNA in other model systems is known to produce bulky bipyrimidine photoproducts (BPPPs) that prevent the primer extension activity of DNA polymerase, and thus such treatment would be inhibitory toward the PCR process used in forensic genetic analysis. Indeed, as the work presented here shows, it was possible to damage the DNA sufficiently in a forensic-like bloodstain to cause a standard autosomal STR profile to be lost. However, a detailed analysis of the process, based upon assays developed to detect BPPPs, single and double strand breaks and DNA-DNA cross links, produced some unexpected findings.

Contrary to the situation with living tissues or cells in culture, the predominant UVC induced damage to DNA in bloodstains appears not to be pyrimidine dimers. Although some evidence for the presence of BPPPs and DNA crosslinks was obtained, the major forms of UVC damage causing genetic profile loss appear to be single strand breaks. It is not possible, however, to preclude the possibility that a combination of damage types was responsible for the profile loss observed.

A significant measure of protection against UVC-mediated genetic profile loss is afforded by the dehydrated state of the DNA and, to a lesser extent, the DNA cellular milieu. This is exemplified by the kinetics of profile loss in bloodstains versus naked DNA in solution and in the dehydrated states. It took an average of 102 hours of UVC at a flux of 104 mJ cm^{-2} min^{-1} to effect a profile loss in human bloodstains. In contrast, it took 16 minutes and 24 hours to produce the same effect with naked human DNA in solution and naked, dehydrated human DNA respectively. Thus, dehydration of the DNA alone protected the DNA such that a 90 fold increase in dose was required to produce enough damage to cause profile loss, whereas the cellular context afforded additional protection to the DNA (in addition to dehydration in the bloodstain) in that an additional 4 fold increase in dose was required to produce the same effect.

To explain these observations, it is hypothesized that is it the conformational state of the DNA, as well as cellular constituents, that protect the nucleic acid from UV induced damage. DNA in solution normally exists in a B conformation, an arrangement that facilitates the direct absorption of a photon of UV light by adjacent bases and the formation of bipyrimidine dimers. Dehydrated DNA, however, tends to assume an A conformation, in which adjacent pyrimidines may be positioned in a manner that is unfavorable for cyclobutane or pyrimidine-pyrimidone formation. Additionally, normal solution chemistry reactions in general are impeded in the dehydrated state. Protection by the cellular milieu may be due to other UVC absorbing species present including proteins and RNA, which may reduce the effective dose experienced by the genomic DNA.

UVA/UVB Damage

To explore the effects of UVA (365 nm) or UVB (315 nm) light on DNA, naked DNA in solution was initially used. One to two micrograms of human genomic DNA (100 ng μl^{-1}) were exposed to UV light for periods of time ranging from 0 to 198 hours (8.25 days) at a flux of 104 mJ $cm^{-2}min^{-1}$. Flux was measured by an internal sensor in the Stratalinker 1800 that was used to deliver the UV rays, and exposure times were normalized such that total exposure was accurately expressed in terms of hours (i.e. a 1 hour exposure = 6.24 J cm^{-2}). Because neither UVA nor UVB rays could penetrate the polypropylene of the microcentrifuge tube, it was necessary to place the tubes upright with the lids open to allow irradiation. When a sample was exposed in this manner, some of the liquid evaporated off, effectively increasing the concentration of the sample. To eliminate any possible concentration effects on the damage, samples were exposed for 2 hours, removed and water added to replace any volume lost due to evaporation before continuing the irradiation.

Although naked DNA in solution was the most convenient to work with, it did not mirror the situation in true forensic samples where a body fluid is deposited on a substrate and subsequently dehydrated. Therefore, the DNA extracted from dried biological stains was considered next. To prepare the bloodstains, 50 µl aliquots of human blood were spotted on cotton cloth and allowed to dry at room temperature overnight. Stains were then exposed to UV and the DNA extracted using a standard organic protocol. In this case, the stain DNA could potentially be guarded from harm by both its dehydrated state and the cellular infrastructure, but it would not be possible to ascertain which condition afforded a greater measure of protection.

To assess the contributions of each of these protective factors individually, a third type of sample was prepared in which naked DNA was dehydrated. Naked DNA was vacuum dried in a microcentrifuge tube, exposed to UV and then resolubilized overnight in sterile water. This facilitated a determination of the effects of dehydration in the absence of the cellular milieu.

The damaged DNA was analyzed using both native and alkaline agarose gel electrophoresis. A native gel allowed for the visualization of double strand breaks with a decrease in the number average molecular weight (NAMW) indicative of a greater number of breaks. An alkaline gel was used to provide information concerning the presence of single strand breaks, again with the NAMW inversely proportional to the number of breaks.

To correlate the damage observed with the ability to obtain a genetic profile, the damaged DNA was amplified using a standard autosomal short tandem repeat (STR) multiplex (Power Plex 1.2). PCR products were visualized by capillary electrophoresis.

Finally, the relative abundance of various types of damage including UV photoproducts and oxidative base modifications was determined through the use of lesion specific endonucleases. Each of these glycosylases recognized a particular type of damage, removed the offending base(s) and all but one subsequently functioned as a lyase, cleaving the phosphodiester backbone and resulting in a single strand gap. Post-restriction samples as well as 'no enzyme digest' controls were electrophoresed on an alkaline gel. A reduction in the NAMW of the restricted sample compared to its control was indicative of a greater number of breaks and, hence, lesions.

DNA exposed to UVA

UVA light ranges in wavelength from 320 to 400 nm. The longest of the UV rays, it is transmitted through the atmospheric ozone layer at a significantly greater rate than the more energetic wavelengths and so comprises 95% of the rays that reach the earth[1]. However, the direct absorption of UVA photons by DNA is insignificant, and studies have shown that few photoproducts can be detected after UVA irradiation of living cells. The ones that do form show a strong sequence dependence, with primarily T-T CPDs and, to a lesser extent, T-C and C-C CPDs being formed. (6-4) PPs are not formed by UVA rays[8]. UVA primarily causes damage indirectly through the action of photosensitizers, molecules that absorb the UV energy and transfer it to DNA. When the molecule is oxygen, this is known as the photodynamic effect[1,9]. The generation of reactive oxygen species (ROS) leads to the formation of strand breaks and oxidative products, the chief UVA-mediated lesions detected in living cells and cell-free systems[8]. The damage done by UVA irradiation of dried DNA is less well defined.

The effects of UVA on naked DNA in solution were considered first. Native gel electrophoresis revealed a high molecular weight (HMW) band (~ 20 kb) through 174 hours UVA exposure (1086 J cm^{-2}). Significant quantities of double strand breaks were absent until 126 hours UVA (786 J cm^{-2}), after which their formation slightly increased as evinced by the loss of the HMW band. The contribution of single strand breaks was assessed by alkaline gel electrophoresis , revealing the loss of a HMW band by 79 hours exposure (493 J cm^{-2}). A limited quantity of strand breaks were evident from 4 hours (25 J cm^{-2}), increasing linearly with dose. However, a UV dose as high as 1235 J cm^{-2} (198 hours) was not sufficient to cause even a partial profile loss.

Next, the effects of UVA exposure on the DNA in dried physiological stains were assessed. As visualized on a native gel, there was no sign of double strand break formation up to 198 hours. An alkaline gel did reveal some damage. A HMW band could be seen through 102 hours (636 J cm^{-2}), but single strand breaks were evident immediately, increasing linearly with UV dose. Noticeable after 4 hours exposure, the migration of a portion of the DNA through the gel matrix was retarded, running higher than the 20 kb molecular weight marker, an indicator of inter- or intrastrand crosslinks. After irradiation for up to 198 hours, there was no loss of a genetic profile in the DNA isolated from dried bloodstains.

Finally, the effects of UVA on naked, dehydrated DNA were studied. As seen on a native agarose gel, double strand breaks were present even in the 'no exposure' control sample, indicating that the processes of dehydration and subsequent rehydration of the unprotected nucleic acid were themselves a source of damage. The fragmentation remained relatively constant until 102 hours, at which point higher molecular weight DNA was lost. An alkaline gel told a similar story. Considering the two gels together, it appeared that, while single strand breaks certainly contributed to the fragmentation of the DNA, double strand breaks were ubiquitous. However, this damage was not severe enough to prohibit the amplification of the PowerPlex alleles in any of the samples tested.

A physiologically relevant UVA dose ranges from 18 – 36 J cm^{-2}, equivalent to the dose delivered during a typical tanning bed session or to a ten minute exposure to the noontime sun at 45^{o} latitude [8]. Equating this to exposure in the experimental system, the relevant range becomes 2.9 – 5.8 hours. The farthest time point tested, 198 hours (1236 J cm^{-2}), represented a 213 - 426 fold increase in UV dose over this range, but was still

insufficient to impair PCR amplification of the autosomal loci tested, indicating that, taken singly, UVA rays do not cause the DNA damage that results in the loss of a profile in samples taken from dried biological stains.

DNA exposed to UVB

The UVB portion of the spectrum ranges from 290 to 320 nm. Because it is mostly absorbed by atmospheric ozone, it comprises only about 5% of the rays that reach the earth[1]. The major effects of UVB on DNA are a result of the direct absorption of the energy residing in photons of light. The primary lesions observed in living cells and cell-free systems are bipyrimdine photoproducts (BPPPs)[10]. Their formation is sequence dependent and the three main types observed are (in order): cis-syn T-T CPDs, T-C (6-4) PPs, and T-C CPDs [8].

Other types of lesions are generated to a lesser extent. 8-oxo-guanine, the hallmark of oxidative damage, can be detected after UVB irradiation[11,12]. The mechanism of its formation is still unclear, but the oxidation of guanine by an -OH radical is implicated. The -OH radical can also cause single strand breaks through the abstraction a hydrogen from the C3, C4, or C5 of the deoxyribose sugar[11,12]. Cytosine photohydrates (6-hydroxy-5,6-dihydrocytosine), formed by the hydration of a singlet excited state cytosine, can also be UVB-induced[8].

The effects of UVB on naked DNA in solution were examined first. Human genomic DNA was diluted to a concentration of 100 ng μl^{-1} in a microcentrifuge tube and exposed to UVB light (315 nm) in the Stratalinker 1800, as described above. Double strand breaks began to form around 48 hours (299.5 J cm^{-2}) after which they increased

linearly with dose, and a HMW band could be seen through 79 hours. On the alkaline gel, the formation of a few single strand breaks could be seen as early as 4 hours (24.96 J cm^{-2}), gradually increasing with additional irradiation, with a HMW band only detectable up to 24 hours (150 J cm^{-2}) .

Considering the results from both gel types, it appeared that single strand breaks were formed early, their quantities increasing gradually until double strand breaks were formed. It was not possible to tell, however, whether these were true double strand breaks (dsbr), in which the phosphodiester backbones of the DNA are cleaved directly across from each other, or if the fragmentation was the result of single strand breaks formed on opposing strands in close enough proximity to mimic a dsbr. The quantities formed, however, were not sufficient to cause even a partial profile loss until 174 hours exposure (1086 J cm^{-2}).

Next, bloodstains were prepared by aliquotting 50 µl spots of blood on cotton cloth and allowing them to dry overnight at room temperature. The bloodstains were placed flat on the floor of a Stratalinker 1800 for exposure. Native gel electrophoresis of the DNA isolated from these bloodstains revealed few incidents of double strand breaks. Although a limited number could be detected at 24 hours and thereafter, a HMW band was present through 198 hours (1236 J cm^{-2}).

An alkaline gel revealed the presence of single strand breaks immediately, as was seen with UVA exposed samples, indicating again that the processes of dehydration and rehydration contributed to the nicking of the DNA . A HMW band was no longer present after 48 hours (300 J cm^{-2}), as the quantity of strand breaks increased, but a genotype was still obtainable through 198 hours. Evidence of DNA-DNA cross-links, in

the form of nucleic acid migrating more slowly than the 20 kb molecular weight marker, could also be seen on the alkaline gel.

The final damage substrate was naked, dehydrated genomic DNA. Naked DNA in solution was dehydrated using vacuum centrifugation. The tubes were held upright in a rack with the lids open for UV exposure, then rehydrated and analyzed. Double strand breaks appeared immediately and increased linearly with UV dose. A HMW band remained through 79 hours. Single strand breaks were also detected at all time points, gradually increasing over time.

Because the overall contribution of the UVB component to the collected rays that reach the earth's surface is relatively small (5%), the relationship between exposure to simulated UVB rays and natural sunlight is not as well defined as that for UVA irradiation.

UV Photoproducts

The formation of UV photoproducts in naked DNA in solution, DNA extracted from physiological stains, and naked, dehydrated DNA was investigated using lesion specific endonucleases. These enzymes, players in the first step of the base excision repair pathway, specifically recognize CPDs and/or (6-4) photoproducts. For the experiments described here, the enzymes T4 Endonuclease V (T4 Endo V), *Chlorella* virus pyrimidine dimer glycosylase (CV-PDG), and *S.* Pombe ultraviolet damage endonuclease (UVDE) were employed.

Each of the sample types tested was restricted with all three enzymes. For each time point, a no enzyme control was also run. All of the samples were processed under

identical conditions, to account for any heat, pH or oxidative damage caused by the DNA extraction and incubation procedures themselves. Therefore, a comparison of each restricted sample with its control allowed a fair assessment of the damage. A positive control was also run with each experiment, confirming that the nuclease was functional.

Naked DNA in solution was exposed to UVA rays and subsequently digested with T4 Endo V. As confirmed by experiments with CV-PDG and UVDE, UV PPs formed slowly, beginning between 4 (25 J cm^{-2}) and 8 (50 J cm^{-2}) hours as indicated by the reduction in the NAMW caused by enzyme induced single strand gaps. These increased linearly with dose through 198 hours (1235 J cm^{-2}). Strand breaks were obvious in the control samples, but there was a marked difference in their relative quantities when compared with their enzyme treated counterparts. Although UV PPs were formed in naked DNA samples, they were not present in sufficient quantities to hamper autosomal STR profiling through 198 hours (8.25 days), as described above.

Next, the DNA extracted from bloodstains exposed to UVA was probed for UV PPs. No BPPPs could be detected, but strand breaks were present and a HMW band was absent at all time points, including the no exposure control, confirming earlier observations that the processes of dehydration and rehydration contributed to strand breakage. From the 4 hour sample, DNA migrating more slowly than the 20 kb molecular weight marker was evident, indicating the presence of inter-or intra-strand cross-links. Still, these lesions did not result in even a partial profile loss.

Endonuclease restriction of UVA irradiated, naked, dehydrated DNA produced similarl results. The alkaline gel subsequent to a UVDE digest did not reveal the presence of any UV PPs, but did provide evidence of inter-of intrastrand cross-links with

the slowly migrating nucleic acid visualized above the 20 kb molecular weight marker. Also present were strand breaks in each sample, likely a result of the hydration processes, as was the absence of a HMW band. However, full genetic profile was still obtainable from samples exposed up to 198 hours.

A repeat of the experiments on UVB irradiated DNA yielded similar results. After a digestion of naked DNA in solution with CV-PDG, enzyme induced strand breaks and a reduction of the HMW band were seen as early as 4 hours, a trend which increased proportionally with dose, and resulted in the complete loss of the HMW band by 48 hours (300 J cm^{-2}), indicative of the formation of BPPPs. Although strand breaks were present in the 'no enzyme' controls after this point, UV dimers could be detected by a comparison of the NAMW of the enzyme treated sample with that of its respective control. In this case 174 hours (1086 J cm^{-2}) exposure caused a partial profile loss.

Next, the DNA extracted from UVB exposed dried bloodstains was examined. While no photoproducts were evident, the formation of strand breaks and the absence of a HMW band from the no exposure control through 198 hours could be observed. The slowly migrating DNA, seen above the 20kb molecular weight marker, again indicated the formation of inter- or intrastrand cross-links, but even their presence did not inhibit the amplification of STR DNA, as discussed above .

An analysis of naked, dehydrated DNA exposed to UVB with CV-PDG revealed the formation of photo-dimers after 8 hours, their numbers increasing slightly with dose, as evinced by reduction of the NAMW in the later time points. This increase, however, was insignificant when compared with that observed in naked DNA irradiated with UVA or UVB. While there was no evidence of cross-links, strand breaks were present in all

samples, consistent with the observed effects of dehydration/rehydration. Despite the detection of some DNA damage to UVB it was insufficient to cause even a partial profile loss in any sample.

Oxidative Damage

Oxidative damage to DNA is mediated by reactive oxygen species (ROS), which can be endogenous or exogenous in origin. The major intracellular source of these species is the leakage associated with cellular respiration in which oxygen is reduced to H_2O in the mitochondria[13,14]. Common extracellular sources include heat, drugs, certain redox cycling compounds, and radiation, especially ionizing radiation and near UV light (320 – 380 nm)[1,15].

ROS attack of DNA can produce a plethora of lesions. There is little doubt that the hydroxyl radical is the chief culprit. Guanine is the base most susceptible to attack, followed by thymine. 8-oxo-guanine, the hallmark of oxidative stress, is the most abundant base modification, comprising 50% of modified guanine residues. It is a miscoding lesion, pairing with adenine and leading to a G → T transversion[1]. Another 20% of the damaged guanine moieties take the form of formamidopyrimidine (FaPy), a polymerase stalling lesion[16,17]. Around half of all oxidized thymine bases can be classified as hydroperoxides, formed by the substitution of the C5-C6 bonds. They have the ability to halt enzyme-mediated polymerization[16,18]. Hydroxyl radicals mediate sugar damage by the abstraction of electrons from the deoxyribose sugar carbons, giving rise to single strand breaks[19]. They can also initiate chain reactions in which the DNA at a site far removed from that of the initial contact is damaged[1,14].

Previous experiments, as described above, involved the use of UVC light to induce DNA damage. Under these conditions, oxidative lesions could not be detected in any of the sample types tested. It should be noted that some of these modifications were likely present, but at levels beneath the detection limits of the system, or in forms not recognized by the enzymes used. Their relatively low abundance can be explained by a consideration of the radiation involved. The UVC range encompasses 260 nm, which is the nucleic acid absorption maximum. Therefore, one would expect that the predominant types of lesions would be the result of the direct absorption of energy by the DNA, rather than through the action of radical intermediates, which is precisely what was found in naked DNA in the form of UV photoproducts. Interestingly, however, these dimers could not be detected in dehydrated DNA.

Because UVA and UVB have both been shown to generate ROS, the observation of these lesions was expected. To assess the oxidative damage done to naked DNA in solution, the DNA in bloodstains, and to naked, dehydrated DNA by both UVA and UVB irradiation, the lesion specific endonucleases human 8-oxo-guanine glycosylase 1 (hOGG1) and formamidopyrimidine dimer glycosylase (FPG) were employed. Both enzymes display a dual glycosylase/lyase action *in vitro,* and result in a single strand gap at the sit of damage. Damaged samples and their 'no enzyme' controls were restricted with each of the endonucleases and analyzed on alkaline gels as previously described.

UVA Oxidative Damage

Naked DNA in solution was exposed to UVA rays (365 nm) and then incubated with both hOGG1 and FPG. A FPG digest showed the absence of oxidative lesions until

79 hours, after which gradual increase in their formation was detectable. As shown by the 'no enzyme' controls, strand breaks began to form around the same time, increasing until the HMW band was lost at 126 hours (786 J cm^{-2}). Even after this fragmentation of the DNA, a reduction in the NAMW of the treated vs. non-treated DNA was obvious, indicating the continued formation of oxidative products. However, in no case was the genetic profile of a sample affected.

No oxidation products were detected in bloodstain DNA as evidenced by hOGG1 digestion. There was, however, evidence of cross-links with the retardation of the migration of a portion of the nucleic acid though to gel matrix, confirming the results seen on the alkaline gel of stain DNA exposed to UVA with no further treatment. As observed with all dehydrated samples analyzed, strand breaks were present at all time points, including the no exposure control. It is possible that oxidative lesions were present, but undetectable due to sensitivity limitations. In fact, the absence of detectable oxidative damage was somewhat surprising given that the Fe^{2+} catalyst of the Fenton reaction that mediates the formation of ROS, is present in blodstains. A careful consideration of the situation in a dehydrated stain, however, may provide an explanation. The iron component of hemoglobin is sequestered and therefore unavailable to participate in Fenton-like reactions. In a living cell, there exist pools of free iron that feed into the hemoglobin biosynthetic pathway. These would be available to participate in ROS generating reactions prior to their incorporation. Once the cell becomes dehydrated, however, their diffusibility would be severely limited and access to them limited. An inhibition of the diffusibility of any radical generated would similarly inhibit its ability to contact the nucleic acid and cause damage.

Likewise, oxidative damage could not be detected in naked, dehydrated DNA using FPG digestion; a comparison of the number average molecular weight of the restricted DNA with that of the 'no enzyme' control revealed no difference. There was no indication of the formation of cross-links, but strand breaks were evident in all samples as was typical with the dehydrated types. Of course, it remains possible that some oxidative products were formed, but at levels below the detection threshold of our assay.

UVB Oxidative Damage

Next, UVB (315 nm) irradiated DNA was screened for oxidative lesions. By reacting naked DNA in solution with hOGG1, the formation of these products was detected as early as 24 hours, at which time the HMW band was also lost. The fragmentation of the DNA due to enzyme induced single strand gaps increased proportionally with time, remaining detectable even with the introduction of UVB induced breaks ('no enzyme' controls) from 48 hours. UVB induced damage was severe enough to cause the partial loss of a profile in samples exposed for 174 hours (1086 J cm^{-2}) or longer.

UVB irradiation of stain DNA was not sufficient to bring about the formation of detectable oxidative modifications, as determined by FPG digestion. However, as was seen in stain DNA exposed to UVA , cross-links began to form as early as 4 hours. Also present in all samples were strand breaks, damage common to dehydrated sample types. Through 198 hours, there was no inhibition of the amplification of STR DNA.

FPG interrogation of naked, dehydrated DNA was similarly fruitless, but there was some evidence of cross-link formation in the earlier time points with the migration of nucleic acid slower than the 20 kb molecular weight marker.

Conclusions: UVA and UVB Damage

The damage observed in naked DNA in solution, stain DNA, and naked dehydrated DNA is summarized in the following tables. The earliest time point at which each of the lesions could be detected is given, and the results of the previously described

	UVC (to 48 h)	**UVB (to 198 h)**	**UVA (to 198 h)**
UV PPs	**5 sec**	**25 min**	**4 h**
oxidative lesions	**no**	**24 h**	**24 h**
cross-links	**1 min**	**no**	**no**
ssbr	**1 min**	**25 min**	**24 h**
dsbr	**1 h**	**24 h**	**126 h**
partial profile	**2 min**	**174 h**	**no**

Table 1. Summary of damage detected in naked DNA in solution

UVC experiments are included for comparison. Samples were exposed to UVC for up to 48 hours (2 days), and to UVA/B for up to 198 hours (8.25 days).

Table 1. Summary of the damage detected in stain DNA.

	UVC (to 48 h)	**UVB (to 198 h)**	**UVA (to 198 h)**
UV PPs	**no**	**no**	**no**
oxidative lesions	**no**	**no**	**no**
cross-links	**4 h**	**4 h**	**4 h**
ssbr	**4 h**	**0 h**	**0 h**
dsbr	**102 h**	**no**	**no**
partial profile	**12 h**	**no**	**no**

Table 2. Summary of the damaged detected in naked, dehydrated DNA.

	UVC	**UVB**	**UVA**
UV PPs	**no**	**no**	**no**
oxidative lesions	**no**	**no**	**no**
cross-links	**4 h**	**4 h**	**24 h**
ssbr	**0 h**	**0 h**	**0 h**
dsbr	**no**	**0 h**	**0 h**
partial profile	**12 h**	**126 h**	**no**

As described previously for UVC, a model for DNA damage assessment in physiological stains recovered from crime scenes was developed by subjecting human bloodstains and naked DNA in the hydrated and dehydrated states to varying doses of UVA and UVB radiation.

The direct absorption of UVA photons by DNA is insignificant, and studies have shown that few photoproducts can be detected after UVA irradiation of living cells. UVA primarily causes damage indirectly through the action of photosensitizers, molecules that absorb the UV energy and transfer it to DNA or use it to generate ROS[1,8]. Although moderate quantities of CPDs, formed as the result of *photosensitization*, oxidative lesions, and strand breaks in naked DNA were detected subsequent to UVA irradiation, the combined effects of the insults was not sufficient to cause even the partial loss of a genetic profile up to 8.25 days exposure.

The only lesions detected in dehydrated DNA, both naked and in stain form, were cross-links and strand breaks, but again these did not inhibit the amplification of STR DNA. These results were somewhat surprising since UVA comprises 95% of the UV rays that reach the earth and it is a known damaging agent in living cells. In fact UVA irradiation has been associated with the formation of melanomas[20]. One possibility for the absence of DNA profile loss in the experimental systems is that the UVA generated base modifications are primarily mutagenic rather than polymerase stalling and thus would not affect the ability to genotype. With the cell-free samples, the lack of available photosensitizers certainly protected the DNA from damage. Additionally, the absence of fully hydrated cells which facilitate the free diffusion of photosensitizers is likely contributory.

According to published reports, the major effects of UVB on DNA are the result of the direct absorption of the energy residing in photons of light. The primary lesions observed in living cells and cell-free systems are bipyrimdine photoproducts (BPPP) [8]. However, BPPPs in naked DNA were not observed until 4 hours exposure (25 J cm^{-2}), and they were absent from the dehydrated sample types altogether. Oxidative modifications, such as 8-oxo-guanine, can also arise from UVB irradiation, but these were not formed in significant quantities until 24 hours exposure (150 J cm^{-2}), and were undetectable in dehydrated naked and stain DNA.

Confounding the analysis of the data is the observation that the STR profile was partially lost by 126 hours (786 J cm^{-2}) in naked, dehydrated DNA, but was not affected in naked DNA until 174 hours (1086 J cm^{-2}). The amplifications were repeated a number of times with the same results. It should be noted, however, that the profiles obtained for the higher time points from both types of naked DNA samples were similar with the larger alleles present at very low relative fluorescence units (rfu) at 102 hours and thereafter. Because more damage was detected in naked DNA in the hydrated state than in the dehydrated state, there must be present additional as-yet-uncharacterized damage in the latter that was not detectable using the systems employed, but resulted in amplification inhibition.

It was previously theorized that a dehydrated state afforded a significant measure of protection to DNA, a result of the B → A conformation change, in which adjacent bases assumed positions relative to each other that were unfavorable to dimerization by direct absorption of energy. This state also limited the diffusibility of reaction

components, impeding the normal solution chemistry. Finally, the presence of the cellular milieu afforded an additional measure of protection.

It is apparent, however, that any damage incurred by crime scene samples that is severe enough to prevent the primer extension activity of DNA polymerase, thus inhibiting the PCR process used in forensic genetic analysis is not caused solely by UVA/B irradiation.

Effects of Humidity

The previously described experiments provided convincing evidence that UV light, especially the physiologically relevant longer wavelengths, alone was not the primary cause of the DNA damage resulting in the loss of the genetic profile from a forensic specimen. To further investigate the cause of DNA profile loss, additional variables were studied. It was previously demonstrated that the dehydrated state of the DNA in a stain afforded it a significant measure of protection from damaging agents. However, it was reasoned that since a stain exposed to the outside environment rather than in a climate controlled laboratory would experience higher levels of humidity, the resulting partial rehydration of the nucleic acid may render it more susceptible to damage.

To investigate this, a humidity chamber was constructed in a normal glass aquarium with airtight seals. A plexiglass lid with an opening to which a UV light fixture was affixed was fashioned. These seams were also sealed airtight. A humidity/temperature meter was attached inside the tank to measure conditions and samples were placed flat on 2” high microcentrifuge racks to avoid wetting them with any moisture that gathered at the bottom of the tank. To simulate natural sunlight, samples were irradiated using a 302 nm bulb for time periods ranging from 1 day to 14

weeks at a flux of 0.024 J cm^{-2} min^{-1}. Ninety-four percent humidity was maintained chemically by the inclusion in the sealed tank of a saturated solution of copper II sulfate pentahydrate. These conditions created an internal temperature of approximately 27^{o}C (81^{o}F).

After exposure in the humidity chamber, DNA was isolated from the stains using a standard organic protocol and visualized on a native agarose gel. Double strand breaks were formed as early as 2 days, albeit in low quantity, and their numbers increased with time. The HMW band was lost by 5 weeks, the point at which genotyping was partially inhibited.

Although UV PPs were not detected in any of the stain DNA tested in previous experiments, it was possible that the increase in the humidity with the concomitant change in hydration state would prove conducive to their formation. The samples were digested with T4EndoV and electrophoresed on an alkaline gel together with the 'no enzyme' controls. Comparing the treated and untreated samples, the NAMW of the DNA looked the same and it did not appear that any dimers had formed. However, considering the state of the nucleic acid, an alternative explanation was possible. The DNA was so degraded prior to enzymatic restriction that the additional breaks indicative of dimer removal simply could not be visualized.

The alkaline gel also provided additional information concerning the state of the phosphodiester backbone. The native gel showed the presence of a limited number of double strand breaks through 3 weeks, so the breaks seen in the 1, 2, and 3 week samples must have been single stranded.

The results indicated that breaks, both single and double stranded, were the primary lesions formed under conditions of high humidity and simulated sunlight exposure. However, it is still probable that UV PPs and oxidative base modifications were formed but could not be detected due to the analytical insensitivity of our procedures. Still, it took 5 weeks (12,096 J cm^{-2}) of direct exposure to cause even a partial inhibition of STR amplification. But, the ability to type true forensic samples can be impeded well before that time, so the contributions of heat, humidity and UV, while certainly contributory, must be exacerbated by other factors.

A major environmental factor was excluded from the initial experiments – the contribution of microorganisms. All of the stains were made from freshly drawn blood and were dried overnight prior to their use. Experiments were carried out in a controlled laboratory environment. Additionally, the continuous UV irradiation of samples at a short distance from the source would likely have inhibited microorganism growth. In fact, an attempt to culture any microorganisms present on the exposed stains on both PDA (potato dextrose agar) and TSA (tryptic soy agar) gave negative results.

Environmentally Impacted Samples

The next logical step in the quest to describe the DNA damage in dehydrated stains was their unprotected exposure to the environment, subjecting them to insults such as heat, light, humidity, precipitation, and UV and rendering them susceptible to microorganism growth. Fifty microliter drops of blood were dried on cotton cloth and placed in direct sunlight on an unenclosed patio in Orlando, Florida. Two sets of samples were exposed for 3 days, 1 week, 3 weeks, 6 weeks and 9 weeks. The average

temperature over this time was 78°C (high – 85°C; low – 37°C) and the average humidity was 83% (high – 89%; low – 77%).

After only 3 days outdoors, the stains became faded, losing their dark red color, and there appeared to be microorganism growth. A full genotype was obtained through 3 days, a partial through 1 week. Subsequently ($\geq$ 3weeks), the profile was completely lost.

Gel analysis of the DNA produced interesting findings. The 3 day sample was heavily degraded, as visualized on both native and alkaline gels. But, high molecular weight was once again present in the 1 and 3 week samples. Highly fragmented DNA was again detected at the 6 week time point, in contrast to the HMW band seen in the 9 week sample. Due to the exposure conditions and the physical appearance of the stains, we reasoned that the HMW DNA probably belonged to microorganisms such as molds or yeasts. To test for the presence of human DNA the QuantiBlot® Human DNA Detection Kit, a generally accepted and widely used method for the quantification of human DNA, was employed. Human DNA was observed in the 0 day, 3 day and 1 week samples, but the remaining samples were negative, supporting the contention that the DNA detected with higher time points was contributed by microorganisms.

For additional confirmation of microbial growth, the species found on the bloodstains were cultured. A sterile, cotton-tipped swab was wetted with sterile water. This was rubbed across the bloodstain and transferred to both PDA and TSA plates. These types were chosen because they are general purpose media and allow the growth of a wide range of organisms. The plates were incubated at room temperature for 3 – 6 days. For each time point an unexposed stain was also swabbed and plated as a control. By 1 day, microorganism growth was obvious, the number of colonies increasing with

prolonged environmental exposure. Significantly, the predominant colonies appear to be the eukaryotic forms (mold and/or yeast) .

A T4Endo V digestion provided no evidence of UV photoproducts. It is expected that these dimers were formed, but could not be detected using the gel-based method due to the extensive degradation of the samples. Therefore, the endonuclease mediated detection of additional lesions was not attempted.

These results indicate that microorganism growth is a significant cause of DNA damage leading to the non-typeability of forensic samples. To utilize the cellular constituents for sustenance, these organisms secrete digestive enzymes that introduce DNA double strand breaks. The heat and humidity that promote such growth are certainly contributory factors as well. The availability of water in the form of humidity for the hydration of the DNA would lead to a greater diffusibility of free radical species, allowing them access to the nucleic acid for the formation of oxidative lesions.

Damage Assessment Conclusions

Taken as a whole, the results of the UVA/B/C, humidity, and environmental experiments indicate that no single factor is responsible for damaging forensic-type samples sufficiently to cause a profile loss. A significant measure of protection is afforded to DNA in the dehydrated state, a result of the B→A conformation change. The cellular milieu acts as an additional shield from damage, although to a lesser extent.

Surprisingly, UV photoproducts were not detected in dehydrated DNA in significant quantities. Instead, strand breaks, base modifications and crosslinks were the primary lesions. Microorganism induced DNA damage, in the form of double strand

breaks, was certainly a major concern, and is expected to be a major contributor to the loss of DNA typing ability in forensic-type stains. There is currently no method available for the in vitro repair of double strand breaks in human genomic DNA, but systems to reapair single strand breaks/gaps and to accommodate certain types of base modifications have been developed and are reported below.

Damage Detection: Genome-Wide Scan

While the above described gel-based methods have proven successful in the detection of different types of DNA damage, they are somewhat insensitive. PCR based assays involving the *Alu* insert are under development to eliminate these problems. *Alu* repeats are a class of short interspersed elements (SINEs). They are ubiquitous in the human genome, comprising ~5%-10% of the total DNA, and found at a frequency of approximately one per 3000 bp[21,22]. Therefore, an assessment of damage indicated within the repeats is expected to be representative of the complete genome. These *Alu* elements can be divided into a number of families and sub-families based on particular mutations that have accumulated in certain families over evolutionary time. Primers were designed specifically to complement as many of these groups as possible, avoiding primer placement at sites containing sequence differences[21,23].

The basis of the assay is shown in Figure 1. The ability to detect DNA damage using the PCR based method depends upon polymerase stalling at the site of a strand break, prematurely terminating strand elongation, with a concomitant reduction of the specific PCR product. To detect DNA lesions, DNA is restricted with a lesion-specific

nuclease, generating a single strand gap and halting polymerization. Therefore, the reduction of amplicons can be seen as a lowering of the *Alu* peak in the restricted sample relative to the unrestricted control. Initially, both linear and exponential amplification techniques were investigated, but abandoned the former due to limitations in its sensitivity.

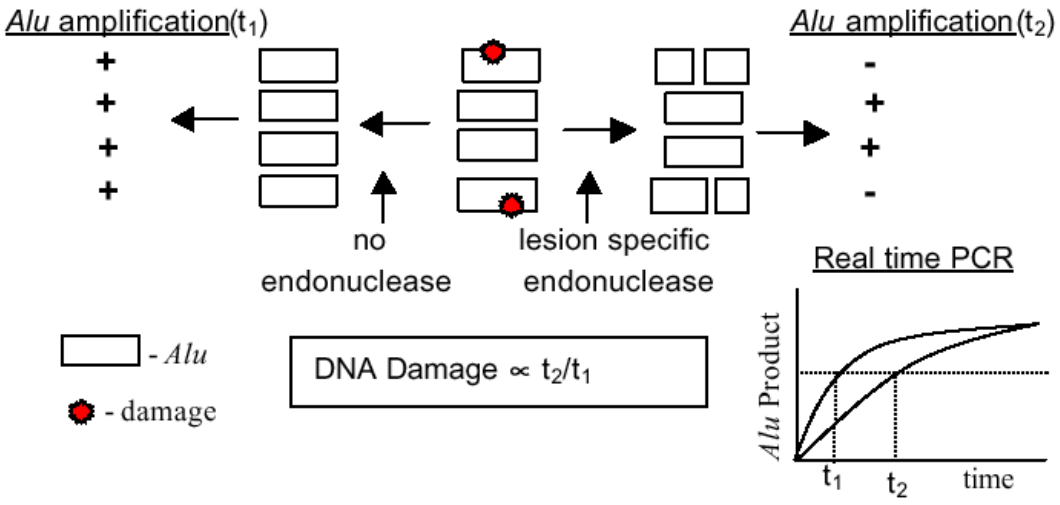

Figure1. *Alu* assay for the *in vitro* detection of damaged DNA.

The technique has been somewhat successful. A semi-quantitative assay has been developed by halting amplification in its exponential phase, and ensuring that template is the limiting factor. A titration of UVC damaged template (0 to 6 minutes exposure) amplified using the optimized protocol showed that the 265 bp product was reduced with increased irradiation until it was absent.

Although the technique is successful when used in this context, problems arise when trying to detect different types of damage using lesion specific endonucleases. DNA lesions stall the polymerase, making difficult the comparison of *Alu* peak heights before and after treatment. Naked DNA in solution was exposed to UVC. The DNA was restricted with CV-PDG, replacing CPDs with single strand gaps. Subsequent digestion with S1 nuclease, an enzyme capable of cleaving the DNA strand opposite a gap, resulted in a double strand break at the site of the BPPPs. PCR following the incubation of undamaged DNA with the enzyme pair or in buffer alone ('no enzyme' control) resulted in the amplification of nearly equivalent quantities of the *Alu* inserts, as determined by peak height. However, incubation of UVC exposed naked DNA (1 minute) with no enzyme, CV-PDG/S1, CV-PDG only or S1 only gave similar results with no significant difference in the peak heights of the respective amplimers.

To solve this problem, we attempted to alter bypass properties of polymerase, allowing translesion synthesis. We experimented unsuccessfully with different polymerases, including proofreading deficient types, and alternate divalent metal ion co-factors. It is evident that the DNA damage must be repaired, either by pre-amplification enzymatic processing or by the inclusion of a translesion polymerase in the PCR reaction, to allow *Alu*-mediated lesion specific damage detection.

After the development of the semi-quantitative PCR/CE based system, the *Alu* assay was transferred to a quantitative real-time format. The primers and probe were designed to complement sequences found in the oldest *Alu* families, reasoning that these inserts would be present in higher copy number and would more accurately represent the

genome as a whole. Specifically, primers and probes were placed at sites that were conserved among the majority of families to maximize genome coverage.

Initially an absolute quantification protocol in which a forward and reverse primer were added and in a SYBR green buffer was used. The dye fluoresces when bound to double stranded DNA, allowing the instrument to quantify total DNA. Two nanograms template DNA was added to the reaction, the expected result a linear decrease in PCR quantifiable DNA. Looking at a limited number of points over time, this is what was observed. However, when additional time points were added, a different effect was observed. There was a decreasing trend over the course of time, but sample to sample there was a see-saw effect in which a sample appeared to have more DNA than the previous time point. Interestingly, a higher quantity of DNA was detected in the 30 second sample with respect to the no exposure control. Because the DNA in the various samples was aliquotted from the same stock prior to UVC exposure, it was not likely that the observed effect was due to quantification errors prior to processing. The assay was repeated using naked DNA exposed to UVB in both the hydrated and dehydrated state, and our humidity chamber samples, with similar results.

Reasoning that this may be an artifact of the SYBR Green system, the samples were next analyzed using a real-time TaqMan assay in which a primer set as well as a probe having the reporter molecule were included. The results were comparable to those obtained from the SYBR green assay.

Next, to rule out the possibility that this was an artifact of the *Alu* protocol, the samples were analyzed using the Quantifiler® Human DNA Quantitation Kit, a

commercially available real time PCR quantification system. Again, the same trend was observed.

It was possible that this effect was a result of the real-time PCR process, so the amplification was next transferred to a traditional thermocycler followed by CE detection, but with similar results.

Finally, we reasoned that the observed phenomenon was a PCR artifact. To investigate this, the samples were quantified using the QuantiBlot®, in which the DNA is immobilized on a nitrocellulose membrane and detected by incubation with a probe complementary to satellite DNA, as described above. The results were as expected, with a gradual decline in detectable DNA as the satellite sequences were damaged, but no see-saw effect as in the PCR amplified samples.

Once it had been established that this was probably a PCR artifact, the nature of it needed to be determined. One possibility is jumping PCR which has been described in the ancient DNA literature. This is a phenomenon seen in degraded samples in which the primer binds and is elongated to the site of the damage where the polymerase stalls. In the next round of PCR, this truncated amplimer can act as a primer, effectively increasing the primer concentration with respect to that available to the undamaged control DNA, resulting in a greater PCR yield[24,25]. The effect is pronounced with smaller amplimers; it was not observed with the amplification of the 265 bp *Alu* product.

If this technique is to be used to detect specific lesions in DNA that has been exposed to a variety of insults, it will be necessary to first repair the other types of damage. The assay would be useful, in its present form, for the detection of intra-

laboratory created lesions in which a single damaging agent is used and so the type of damage is known, but information on damage quantity is lacking.

Effects of Temperature and Substrate on Stored DNA

It has long been a matter of debate within the forensic community what constitutes the optimal, or acceptable, storage condition for a biological sample. Due to cost and space limitations in refrigerator/freezers in crime laboratories, biological evidence is often stored at room temperature or even in a warehouse setting. The dehydration state of the stain, as has been demonstrated, and storage materials are also an important consideration.

To examine the effects these variables have on the degradation of the DNA in forensic-type samples, and ultimately on the ability to obtain a STR profile, 50 µl bloodstains were spotted on cotton material. Two sets of the stains were allowed to dry overnight. One of these was then transferred to a plastic bag and the other to a paper envelope. A third set was not allowed to dry, but was placed immediately in a plastic bag. A control group of stains was stored at -35^{o}C, and the remaining sets were stored at 4^{o}C, room temperature (~25^{o}C), or 30^{o}C (to simulate conditions, for example, in a non-temperature controlled evidence warehouse). Samples were collected and analyzed at 1 day, 1 week, 5 weeks, 3 months, 6 months, 13 months, and 4 years. The DNA was extracted using a standard organic procedure and analysis was completed using the autosomal STR systems Profiler (Applied Biosystems) or PowerPlex 1.2 (Promega).

Remarkably, all samples provided a DNA profile no matter the storage conditions although there was evidence of DNA degradation in some of the samples (see below).

After 4 years, a strong high molecular weight (HMW) band could be seen in all of the samples stored at 4^{o}C or less. The samples kept at room temperature (~ 25^{o}C) also showed HMW bands, although they were significantly fainter, especially in the sample that had been placed in a plastic bag while still wet. In contrast, all of the samples stored at 30^{o}C showed evidence of partial degradation. Additionally, the 30^{o}C samples placed in a plastic bag while still wet were more degraded than those that were dried before being placed in the bag. Similarly, the sample dried then stored in an envelope showed a stronger, less degraded HMW band.

While there was no observable difference between the profiles of the control and exposed bloodstains up to 13 months, the four year samples stored at 30^{o}C showed signs of degradation in that the larger alleles became reduced in size relative to the others . Although the profile was not lost in these cases it began to deteriorate and it is envisioned that samples stored in a non environmentally controlled warehouse type setting (i.e. with high heat and humidity) could eventually become so damaged that it becomes intractable to DNA analysis. It appears that there is a clear advantage to storing biological samples at 4^{o}C or less for extended periods of times.

PHASE II - DNA REPAIR

The Phase I assessment of the DNA damage in dried physiological stains immediately suggested remedies for the non-typeability of damaged templates. Three methods for the in vitro repair of such samples were developed – direct reversal by

photolyase, translesion synthesis, and single strand break/gap repair. The latter have proven successful in the recovery of a genetic signature from both laboratory-damaged and environmentally exposed bloodstains.

Photoreactivation

Prokaryotic and lower eukaryotic cells have evolved a direct reversal mechanism, photoreactivation, to cope with UV induced damage. Photolyases are the enzymatic effectors of this type of repair. In the first steps of the reaction pathway, the light independent phase, the electrostatic lip of the photolyase cleft associates with the DNA, scanning the genome for helical distortions. When an ultraviolet induced lesion is detected, the enzyme interacts specifically with the base and phosphate immediately 5' to the damage and with the bases and phosphates three to four nucleotides 3' to the dimer, acting upon a region totaling six to eight nucleotides. After the formation of this enzyme-substrate complex, the dimer is flipped out of the DNA helix into the photolyase pocket[26,27]. In the second light dependent step, the enzyme absorbs a photon of blue light (377 nm). This energy cascades down the inside of the photolyase cleft ultimately being transferred to the BPPP to form an anion which spontaneously rearranges, breaking the C-C bond of the dimer and returning the bases to their original forms. Photolyases exist that are specific for each of the two major types of UV lesions, namely CPDs and (6-4) PPs[26].

The experiments described below incorporated the CPD photolyase, gauging its success by the recovery of a genetic profile subsequent to treatment. It has been shown

previously that, with respect to intra-laboratory damaged samples, only the exposure of naked DNA to UVC resulted in the formation of BPPPs in quantities sufficient to inhibit autosomal STR amplification. Therefore, only these samples were included in repair attempts.

Naked DNA (100 ng ul^{-1}) was exposed to UVC for times ranging from 20 seconds to 4 minutes. Five hundred nanograms of this DNA was reacted with photolyase by exposure to UVA light (365 nm) in a closed cabinet at room temperature for 2 hours. A 'no enzyme' control consisting of 500 ng of the same UVC sample was incubated under the same conditions. The amplification of the damaged DNA was then attempted using the autosomal STR multiplex Power Plex 1.2. DNA subjected to 20 seconds of UVC was so damaged that the genotype was almost completely lost. Treatment of the same DNA with photolyase prior to amplification, however, facilitated the recovery of a full profile.

While the initial results were promising, the repair could not be repeated using DNA exposed to greater quantities of UVC. CPD formation reaches saturation at doses of approximately 0.05 J cm^{-1} (30 seconds, in this system)[7], thereafter (6-4) PPs are the primary BPPP formed and so the CPD photolyase would cease to be effective. Because damage detection experiments had shown that UV PPs, while certainly contributory, were not the chief cause of amplification inhibition, no further work was done with photolyase.

Translesion Synthesis

A novel group of DNA polymerases termed the Y family has been described. Its members are capable of translesion synthesis (TLS), a process by which a polymerase is able to incorporate nucleotide(s) opposite a damaged DNA template, bypassing lesions

that normally block synthesis. The ability of any polymerase to incorporate the correct nucleotide into a growing strand depends upon the structure of its active site[28]. The template nucleotide and the incoming dNTP are held and allowed to react in this site, which normally only allows the Watson-Crick (WC) pairing of intact molecules. Standard replicative polymerases have "palm," "finger," and "thumb" domains which can only accommodate WC base pairing. Should one of these enzymes encounter a damaged base, it idles, locked into futile dNTP incorporation/incision cycles since none is a good fit[29].

Y-family polymerases have smaller thumb and finger domains than their counterparts, but also have a "little finger" domain. This is the least conserved structure of the translesion polymerases and is thought to contribute significantly to the different biochemical properties of family members such as bypass ability and processivity[30]. There is substantial movement of this little finger to accommodate the various types of lesions, thus it is a relaxed constraint at the active site that allows for lesion bypass. Under physiological conditions, however, these polymerases are distributive rather than processive, adding only about six to ten bases before dissociating from the DNA strand[30].

Translesion synthesis is an attractive option for the *in vitro* repair of damaged DNA. The incorporation of a Y-family polymerase into a PCR reaction with a thermostable replicative polymerase, such as Taq (from *Thermus aquaticus*), would allow for a direct bypass of the damage without the need for repair processing prior to amplification.

The PCR reaction incorporating *Taq* and a TLS is envisioned to proceed as follows: *Taq* polymerase (present in higher quantity) extends the primer until it

encounters a lesion, stalls and then dissociates from the DNA. The TLS is then loaded and bypasses the lesion. Since the translesion polymerase is distributive, it dissociates shortly after translesion synthesis, allowing Taq polymerase to reassociate and resume template DNA synthesis.

A number of thermostable translesion polymerases have recently been isolated and characterized[30,31]. The following experiments involve the use of four naturally occurring DNA polymerase 4-like proteins from the archaeal bacteria *Sulfolobus solfataricus* (Dpo4), *Sulfolobus shibatae* (Ssh), *Sulfolobus tengchongensis* (Ste), and *Acidianus infernus* (Ai), as well as with two laboratory-created chimeras – Ai/Sso (S*ulfolobus acidocaldarius*) and Ai/Ste.

Dpo4 was the earliest characterized thermostable member of the Y-family. Published data indicates that it can bypass a number of lesions including abasic sites, thymine-thymine CPDs, (6-4)PPs, cisplatin adducts, and N-acetyl-2-aminofluorne adducts[31]. More recent data indicates that it can traverse oxidative lesions as well (personal communication, Dr. Roger Woodgate). The wide range of moieties that can be accommodated by this polymerase is reminiscent of the situation with the relaxed structural constraint of the active site of the ultraviolet damage endonuclease (UVDE), which allowed it to recognize a plethora of lesions. This hints that Dpo4 may have the ability to bypass more types of damage than have been shown experimentally.

Ssh, Ste, and Ai are closely related to Dpo4 and possess similar enzymatic properties, but are approximately two to three times less active *in vitro*[30](personal communication, Dr. John McDonald). Ai in particular is much less processive than the others. To enhance its activity, the two chimeras were constructed. Briefly, restriction

sites were introduced into the Ai and Ste genes immediately prior to the region encoding the LF domain. Then, the Ai LF domain was replaced by subcloning the Ste LF to create the Ai/Ste polymerase. The Sso LF domain was likewise used to replace the endogenous Ai LF, generating the Ai/Sso protein[30].

To incorporate a TLS polymerase into an amplification reaction with *Taq*, a buffer had to be developed in which both were active. Reasoning that the TLS was a less robust enzyme and so would be harder to accomodate, the first attempted PCR employed a buffer recipe developed for use with Dpo4 and Pol ι[31,32], but under these conditions *Taq* failed to amplify the target. By considering the buffer requirements for each, a number of alternative mixtures were subsequently developed, altering the concentrations and constituents until finding the optimal blend.

Once there was a functional buffer, the PCR components were optimized. An important consideration was the polymerase concentration. Because the reaction is envisioned proceeding as described above, the *Taq*/TLS ratio was critical. The inclusion of too much enzyme restricted TLS access to the DNA template, but too little resulted in amplification failure. A low quantity of the TLS proved insufficient to effect lesion bypass because the enzyme was unable to out-compete *Taq* for template contact, but the inclusion of higher quantities of the protein tended to inhibit PCR altogether.

It should be noted that the use of alternative Taq-like DNA polymerases was considered. Because of the cycling conditions necessary to accommodate a TLS (below), any hot start polymerase was eliminated as a possibility. As described above, an excess of Taq restricted TLS access to the DNA. Additionally, *Taq* is a relatively processive polymerase *in vitro*, meaning that is does not tend to dissociate from the template as often

as would a less processive enzyme, such as *Pfu* (from *Pyrococcus abyssi*). However, attempting the incorporation of *Pfu* required the addition of a large quantity of the protein (3 units compared to 0.5 units *Taq*), defeating the purpose.

The remaining reaction components – dNTPs, $MgCl_2$, BSA, and template concentration – were next optimized. A *Taq* titration ranging from 0.25 to 5 units was performed to determine the lowest concentration at which amplification was successful, with 0.5 units the choice. The optimal $MgCl_2$ was likewise determined to be 3 mM (*Alu*) or 1.6 mM ('YAP', see below), and the template requirement 1 ng (*Alu*) or 2 ng (YAP).

The cycling conditions were an especially important consideration. Although the translesion polymerases were thermostable, their activity was reduced by continued incubation at 95^{o}C, the denaturation phase of the cycles[30]. This meant, first, that a hot start could not be used. Next, an 85^{o}C incubation was not sufficient to denature the whole of the genomic DNA and only alleles of approximately 200 base pairs or fewer were successfully amplified under those conditions.

Typically, the extension phase of a PCR cycle ranges from 30 seconds to 1 minute. However, this amount of time may be insufficient to allow the polymerase switching at a damage site, and so the incubation time was increased to 5 minutes.

To evaluate the success of TLS incorporation into a PCR reaction, two systems were used– YAP and *Alu*. The YAP primers amplify two alleles – 81 bp and 85 bp - present in a single copy in the genome, and the *Alu* product is a single peak at 128 bp. Due to the inclusion of the 85^{o}C denaturation it was necessary to re-design the *Alu* primers described earlier since they amplified a 265 bp allele. The new primers were

again designed to complement sequences conserved among *Alu* families to maximize genome representation.

After titrating each of the translesion polymerases over a range of 1 to 200 nM with an invariable quantity of *Taq*, it was decided that 100 nM was the optimal concentration for inclusion in both the YAP and *Alu* assays. Because the addition of the TLS to the reaction effectively increased the total polymerase concentration, it was necessary to be sure that any increase in the signal of a 'repaired' allele with respect to that amplified by *Taq* alone was truly the result of repair activity and not simply due to the increased availability of enzyme. Therefore, as a control, a reaction was included in which the concentration of *Taq* was doubled.

Naked DNA damaged with UVC for 0, 6, 11, or 28 minutes was amplified in a YAP reaction containing '*Taq* only' (0.5 u), 'double *Taq*'(1 u), or *Taq* (0.5 u) + 100 nM TLS. Adding twice the *Taq* to the PCR reaction of unexposed DNA resulted in a significant increase in peak height with respect to the '*Taq* only' condition. However, when compared to the peaks amplified in the presence of a TLS there was, in general, no significant difference. Dpo4 did not seem to perform as well as the other enzymes, but this may have been due to the isolate used, that can vary in quality between batches. Ai also shows a reduced activity, consistent with earlier reports[30]. From these results, it appears, first, that the TLS enzymes have been successfully incorporated into the PCR reaction, and next that the addition of two times the *Taq* has compensated for any polymerase concentration effects, and can be used as a control in further experiments.

YAP amplification of the DNA exposed to UVC for 6 minutes resulted in no significant difference in peak height in any of the samples with respect to the standard

Taq reaction, indicating that either the TLS were not traversing the lesions, or that the UV-induced DNA damage was not the type that could be bypassed. Due to further experimentation, described below, the latter is likely the case. Again, Ai was not as robust as the other TLS enzymes.

Similarly, there was no significant difference in the quantities of DNA amplified from the 11 minute or 28 minute samples, with the exception of the Ai reactions which resulted in amplification failure. Of interest is the observation that neither doubling the concentration of *Taq* nor adding a Y-family polymerase affected the efficiency of amplification, again symptomatic of DNA too damaged to be bypassed.

The results of these experiments are summarized in Table 4. The observed peak heights, in relative fluorescence units are listed. It is obvious that the TLS were active in the amplification, as evinced by the peak heights equal to that of the 'double *Taq*' no exposure and 6 minute samples, but there was no damage present that they could bypass. This is feasible given the small size of the amplimers (81bp, 85 bp). These results were confirmed using environmental samples. Fifty microliter bloodstains were exposed outdoors for 0 or 8 days and amplified using the YAP protocol incorporating the bypass polymerases. The results are summarized in Table 5. As was seen with the UVC exposed samples, the enzymes were successfully incorporated into the PCR, but did not participate in lesion bypass.

This is the first report of an polymerase enzyme blend, comprising various Y-family polymerases and a standard replicative enzyme, being used to successfully amplify genomic DNA templates.

	0m (rfu)	6m (rfu)	11m (rfu)	28m (rfu)
Taq	782, 259	485, 354	156, 148	0
Double Taq	6347, 1588	540, 315	226, 202	88, 81
Dpo4	4869, 1781	625, 313	197, 207	254, 60
Ssh	7570, 1286	353, 195	55, 49	184, 120
Ste	7440, 3194	620, 441	135, 326	187, 146
Ai	4163, 1169	275, 145	0	0
Ai/Sso	7060, 1513	674, 645	261, 217	95, 89
Ai/Ste	7510, 1607	557, 449	280, 290	80

Table 3. Summary of the YAP-TLS experiments. Naked DNA exposed to UVC was amplified using the YAP primers and incorporating the translesion polymerases (100 nM each), as listed. The peak heights, as detected by capillary electrophoresis, are listed in relative fluorescent units.

	0 d (rfu)	8 d (rfu)
Taq	7133, 4218	308, 140
Double Taq	6895, 6077	883, 524
Dpo4	7473, 4779	349, 423
Ssh	7825, 5401	473, 486
Ste	7810, 4571	670, 406
Ai	7397, 4343	460, 115
Ai/Sso	7802, 5812	568, 357
Ai/Ste	7789, 5869	460, 235

Table 4. Summary of the YAP amplified environmental samples. The DNA extracted from bloodstains was exposed outdoors for 0 or 8 days and amplified using '*Taq*,' 'double *Taq*,' or *Taq* + 100 nM TLS. The numbers represent peak height in rfu.

The use of a modified *Alu* system, with a 128 bp amplimer, allowed further testing of the abilities of the translesion polymerases that had been incorporated into a PCR. Naked DNA from the same stock used in the YAP experiments was exposed to UVC for 0, 6, 30 or 40 minutes then amplified with '*Taq* alone' (0.5 u), 'double *Taq*' (1 u), or *Taq* (0.5 u) + 100 nM TLS.

Surprisingly, there were significant differences in some of the amplified peaks in the 'no exposure' samples. The '*Taq* alone' peak was approximately half the height of the 'double *Taq*' peak, as expected, but had nearly the same RFUs as those resulting from the addition of Dpo4 and Ai/Ste, seemingly indicating that those enzymes had been inactive. The Ai/Sso and Ssh amplimers were around the same height as that seen with 'double Taq' suggesting that the enzymes were active, but not engaged in lesion bypass. The Ste and Ai peaks, however, were significantly larger than even the 'double Taq.' Taking into consideration the YAP results described above, this is indicative of translesion bypass activity. Of course, confounding this interpretation was the fact that it was observed in a no exposure control. Detailed observation of the quality of the DNA used on a native agarose gel, however, offers a possible explanation. The sample is fragmented and there is no discrete high molecular weight band – the sample is obviously somewhat damaged, an observation consistent with the activity seen from Y-family polymerases.

After 6 minutes' exposure, the peak height difference between 'Taq' and 'double Taq' was not as pronounced and the Dpo4, Ssh, and Ai/Ste PCR products were nearly equal in size to the latter. In contrast to the situation with the no exposure control, the Ai/Sso peak was almost twice the size of that of the 'double *Taq*,' while the Ai and Ste peaks were even greater. These results are consistent with increased translesion synthesis activity with the polymerases most able to bypass the types of damage encountered in a particular sample yielding an increased PCR product.

The general trend continued after 30 minutes' exposure. The Dpo4 and Ai/Ste products were approximately the same size as the 'double Taq,' while Sh and Ai/Sso

amplimers were around twice as numerous. Again, the inclusion of Ai and Ste in the reaction resulted in a significant increase in PCR product with respect to the use of twice the concentration of the typical polymerase.

DNA exposed to UVC for 40 minutes was not amplifiable using the standard *Alu* reaction, but a peak could be obtained by doubling the *Taq* concentration. Dpo4, Ssh, Ai/Sso and Ai/Ste containing PCRs produced products approximately equivalent to the latter, but Ai and Ste again amplified more than twice the DNA than did their counterparts . These results, taken together, provide evidence that not only have Y-family polymerases been incorporated into a standard PCR reaction with *Taq* DNA polymerase, as seen with the YAP assay, but they are engaged in damage bypass. Although the majority of the TLS enzymes displayed translesion synthesis Ai and Ste provided superior performance.

As described above, when the same set of experiments was attempted with the YAP locus, there was no evidence of lesion bypass, only an indication that the enzymes were active. It was theorized that this was due to the abbreviated amplimer size and the absence of damage that could be traversed. By designing a system representing approximately 10% of the human genome, this obstacle was successfully overcome. The enzymes were provided with a repairable template and have demonstrated for the first time the in vitro repair of damaged DNA by the inclusion of a Y-family polymerase with a replicative polymerase in a standard PCR reaction.

An obvious concern when gauging repair success by peak height is that the observed differences are due to pipetting differences when adding amplified product, rather than a true recovery of damaged DNA. Therefore, ten replicates of a number of

both the unreacted and repaired samples were prepared for injection on the 310. The maximum, minimum, and mean were determined. In every instance, the minimum repaired peak was significantly larger than the maximum untreated peak, indicating that the observed difference was not an artifact of CE analysis.

	0m (rfu)	6m (rfu)	30m (rfu)	40m (rfu)
Taq	581	725	190	0
Double Taq	1677	1141	435	230
Dpo4	667	1125	421	205
Ssh	1894	1300	821	237
Ste	4613	6488	2611	781
Ai	4219	3463	1806	509
Ai/Sso	1433	2984	976	229
Ai/Ste	843	1084	555	228

Table 5. Summary of the *Alu*-TLS experiments. Naked DNA exposed to UVC was amplified using the *Alu* primers and incorporating the translesion polymerases (100 nM each), as listed. The peak heights, as detected by capillary electrophoresis, are listed in relative fluorescent units.

Single Strand Break/Gap Repair

We have shown that single strand breaks and/or gaps are significant contributors to the inability to type compromised DNA samples. Therefore, the repair of these lesions has been attempted, first with a simple gap filling/ligation reaction, then by modifying the base excision repair (BER) pathway *in vitro*.

In theory, the repair of a single strand gap should be easy – simply add the missing bases using a DNA polymerase and re-join the backbone with a ligase. A single strand break should be even simpler, only requiring a ligase to seal the nick. Unfortunately, in practice, it may not be so straightforward. Polymerase mediated extension of a DNA end requires the presence of a 3' –OH, which may not be found at the broken end of the strand. Additionally, the ligase requires a 5' phosphate group, which again may not be readily available. There have been reports from the ancient DNA field, however, of a successful repair accomplished by reacting damaged DNA with DNA polymerase I and DNA ligase [33,34]. Repair using this method was attempted using damaged DNA, experimenting with various enzyme concentrations, incubation conditions, and substrates. Subsequent to a repair reaction, samples were analyzed on an alkaline agarose gel to determine the success. In no case was degraded DNA recovered. The probable explanation is that the broken ends were subtended by alternative chemical moieties that were neither extensible nor ligatable. *In vivo*, these ends can be dealt with by enzymes of the base excision repair pathway, Specifically, an apurinic (AP) nuclease, such as endonuclease IV, can recognize abasic sites as well as gaps and nicks, processing the ends and leaving an extensible 3' –OH. Next, a repair competent polymerase that can fill in the gap, but also possesses a deoxyribophosphatase activity capable of 'polishing'

the altered 5' end and restoring to it a ligatable phosphate group, such as polymerase β, is allowed to react. Finally, a DNA ligase seals the nick.

An *in vitro* reconstitution of this pathway was attempted. Because the ultimate goal was the repair of the DNA from forensic stains, i.e. dehydrated body fluids subjected to a variety of insults and many times present in low copy number, it was necessary to optimize the conditions such that the three enzymes functioned synergistically in a single reaction, eliminating the need for buffer switching between multiple incubations.

First, a common 'BER Buffer' was developed, in which all enzymes were active. To do so, the buffer components such as metal ions and salt concentrations were tested. pH was another concern - the three enzymes had optimal pH requirements ranging from 7.4 to 8.1, but it was found that all were still active at 7.8. Likewise, all three were functional at 37^{o}C.

To test the system, naked DNA was exposed to UVC for 0, 6 and 10 minutes and repair attempted with the following enzyme combinations: Endo IV / Pol β / Ligase; No Endo IV / Pol β / Ligase; Endo IV / No Pol β / Ligase; Endo IV / Pol β / No Ligase; No Endo IV / Pol β / No Ligase; Endo IV / No Pol β / No Ligase. The inclusion of Pol β in the reaction results in the recovery of a significant amount of DNA, as evidenced by an increase in the NAMW. While there didn't appear to be any difference between the samples incubated with or without ligase, it is likely that the gel-based method is simply not sensitive enough to detect the differences.

Although these results were promising, it is unlikely that a single repair mechanism will suffice when dealing with true forensic-type samples in which the DNA is subject to a myriad of insults and incurs a diverse array of lesions. Therefore, an

attempt was made to repair environmentally exposed bloodstain DNA by combining BER and TLS.

Samples were exposed outdoors for 0, 6, 7, or 8 days. The DNA was extracted and BER attempted with the 8 day sample. A third of each sample was incubated with EndoIV, Pol β and ligase, another third with only EndoIV and ligase, and last fraction was not treated. The DNA was amplified using the YAP primers and incorporating '*Taq* only,' 'double *Taq*' or 100 nM Dpo4, Ai/Sso, or Ste. The results are summarized in Table 7. Peak height is described in relative fluorescence units.

A difference in peak heights was seen in the 0 day sample amplified with Taq only, but doubling the enzyme concentration or including a TLS in the reaction rendered the remaining peaks approximately equal.

The 8 day sample, however, showed evidence of repair. From the untreated DNA, YAP peaks of 470 and 667 were obtained. When the sample was incubated with EndoIV and ligase the heights increased to 917 and 1073. Surprisingly, reaction with all three BER enzymes resulted in only a slight increase – to 1332 and 1125 rfu. A similar trend was seen with the remaining samples, indicating that the damage was primarily single strand breaks rather than gaps which would require the polymerase mediated addition of dNTPs.

The incorporation of translesion polymerases into the post-BER amplification to facilitate the recovery of even more of the lost profile was also attempted. To ensure that any signal increase seen was the result of lesion bypass rather than of the increased polymerase concentration, the 'double *Taq*' condition was taken as the baseline. Compared to this, the *Taq*/Dpo4 amplified peaks were actually lower, and no repair

polymerization had taken place. However, both the *Taq*/Ai-Sso and *Taq*/Ste reactions showed evidence of repair - the 'no repair' control peak was not increased significantly in any case, but both of the enzyme blends yielded an increased PCR product in the BER treated samples. Prior to BER, the same environmental samples were amplified using the YAP system (summarized in Table 5), but also failed to display any repair polymerization.

Taken together, these results indicate that damage done to the YAP locus comprised both single strand breaks/gaps and base modifications. The breaks were the primary cause of polymerase stalling, as evinced by the necessity of their repair prior to any observable bypass activity by the Y-family polymerases. But, once the template was restored, the TLS polymerases were able to effect translesion synthesis.

The present report presents evidence of the first system to effect the successful *in vitro* repair of single strand breaks/gaps in human genomic DNA damaged via natural processes. The addition of a translesion polymerase to a PCR reaction with a standard replicative enzyme is likewise a novel mechanism, and the use of the two methods together has proven successful in initial experiments. In the future, these systems will be used to repair damage found in various types of forensic-type samples, attempting the recovery of an autosomal STR profile.

	0 days (rfu)	8 days (rfu)
TAQ ONLY		
EndoIV / Pol β / Ligase	2659, 1186	1332, 1125
EndoIV / No Pol β / Ligase	8604, 8515	917, 1073
No Repair	5662, 2198	470, 667
Double TAQ		
EndoIV / Pol β / Ligase	9242, 6278	1573, 1340
EndoIV / No Pol β / Ligase	8205, 8632	1741, 1539
No Repair	8696, 3916	584, 238
TAQ + DPO4		
EndoIV / Pol β / Ligase	9202, 5258	1298, 838
EndoIV / No Pol β / Ligase	8715, 8428	1341, 1771
No Repair	8776, 3657	536, 342
TAQ + AI/SSO		
EndoIV / Pol β / Ligase	8653, 8995	2314, 1609
EndoIV / No Pol β / Ligase	7510, 8288	2544, 1712
No Repair	8022, 5634	338, 510
TAQ + STE		
EndoIV / Pol β / Ligase	8880, 7439	2273, 1534
EndoIV / No Pol β / Ligase	8606, 8505	2417, 2829
No Repair	8744, 4538	583, 205

Table 6. Summary of BER results. Environmentally exposed bloodstains were repaired using the modified BER strategy and amplified with the inclusion of TLS.

DNA Repair Conclusions

Forensically-relevant stains, i.e. dehydrated physiological samples deposited at the scene of a crime, are subject to a myriad of insults including heat, light, humidity, UV and microorganism growth, which can cause various types of DNA damage. The principal concern from the forensic science standpoint is that many of these environmentally induced lesions are expected to be inhibitory towards DNA polymerase-mediated primer extension and may result in amplification, and hence DNA typing, failure. The results presented here first showed that the types of damage that are most frequently encountered in dehydrated samples are not UV photoproducts, but rather strand breaks, base modifications and, to a lesser extent, DNA-DNA crosslinks.

Attempting the repair of such damage, with the ultimate goal of recovering a genetic profile from a previously intractable sample, three systems were successfully developed. The first was a direct reversal of the damage by photolyase. This enzyme, capable of breaking the CPD bonds and restoring the DNA to its undamaged state, was limited in its usefulness since, as described above, UV photoproducts are not the major lesions that result in non-typeability.

Next, a set of thermostable translesion polymerases were incorporated into a PCR reaction with a standard replicative polymerase, facilitating the recovery of *Alu* genetic signatures from both laboratory-damaged samples and true forensic specimens exposed to the environment, and representing the first time such a combination of enzymes had successfully performed *in vitro* repair.

A modified base excision repair system was optimized for the repair of single strand breaks and/or gaps. By optimizing a buffer in which the three enzymes of the

pathway were functional, repair was effected in a single tube, without the need for buffer switches. Breaks/gaps were successfully repaired in both UV exposed samples generated in the lab, and in environmentally exposed samples.

Finally, DNA repaired using the BER system was subsequently amplified using the *Taq*/TLS blend described above, resulting in the successful recovery of a PCR amplified peak. While these two systems are not likely to be the only methods for the repair of damaged DNA in forensic stains, they are certainly promising, and the work presented here suggests the direction for future research in the *in vitro* repair of damaged DNA templates. As was shown with a number of sample types including UVC exposed specimens and environmentally exposed blood stains, double strand breaks are major contributors to the loss of DNA typeability and it should be possible to develop *in vitro* assays for their correction.

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APPENDIX 1: MATERIALS AND METHODS

Bloodstain Preparation

Blood was drawn by venepuncture from human subjects in accordance with the University's Institutional Review Board and spotted within 24 hours of collection to minimize naturally occurring damage. Blood was aliquotted in 50 µl spots onto Whatman paper and allowed to dry at room temperature. Dried stains were stored at -20^{o}C until use.

Naked DNA

To prepare the naked DNA samples in solution, human genomic DNA (Promega Corporation, Madison, WI) was diluted in sterile water to a concentration of 100 ng µl^{-1} in a 1.5 mL microcentrifuge tube (Fisher Scientific, Norcross, GA). To generate the dehydrated naked DNA samples, the human genomic DNA in solution was pipetted into a microcentrifuge tube and spun in a Speed-Vac (Albertville, MN) until dehydrated. After the indicated UVC exposure, the samples were resolubilized in sterile water to a concentration of 100 ng µl^{-1}, making use of an overnight incubation in a 56^{o}C water bath.

Naked Dehydrated DNA

To prepare the samples, naked human genomic DNA in solution was pipetted into a polypropylene tube. The liquid was evaporated in a Speed-Vac (Savant, Albertville, MN) and exposed to UVC in this state. Subsequent to exposure, the samples were resolubilized in sterile water to a concentration of 100 ng µl^{-1} by incubation overnight in a 56^{o}C water bath.

UVC Exposure

DNA samples were exposed to UVC light (254 nm) in a Stratalinker 1800 (Stratagene, LaJolla, CA). Microcentrifuge tubes, containing DNA either in solution or in the dried state were placed, closed, on their sides on the floor of the crosslinker. Bloodstains were likewise placed flat on the floor so each stain received equal exposure. An energy delivery rate of 0.104 J/cm^2/min was used to convert all exposure to time in minutes. Exposure times and doses were: 4 hr – 25.0 J cm^{-2}, 8 hr – 50.0 J cm^{-2}, 12 hr – 74.9 J cm^{-2}, 24 hr – 150 J cm^{-2}, 48 hr – 300 J cm^{-2}, 79 hr – 493.0 J cm^{-2}, 102 hr -636.5 J cm^{-2}J cm^{-2}, 126 hr – 786.2 J cm^{-2}, 150 hr – 936.0 J cm^{-2}, 174 hr – 1085.8 J cm^{-2}, 198 hr – 1235.5 J cm^{-2}. Subsequent to UV exposure, samples were stored at -20^{o}C until their use.

UVA/B exposure

DNA samples were exposed to UVA (365 nm) and UVB (315 nm) light in a Stratalinker 1800 (Stratagene, LaJolla, CA). Because UVA/B rays cannot penetrate the polypropylene tubes used for storage, the microcentrifuge tubes, containing DNA either in solution or in the dried state were placed open in a rack in the crosslinker. Because DNA concentration may affect the types of damage done the samples were exposed for 2 hours, removed and water added to replace any volume lost due to evaporation before continuing the irradiation. Bloodstains were placed flat on the floor so each stain received equal exposure. An energy delivery rate of 0.104 J cm^{-2} min^{-1} was used to convert all exposure to time in minutes. Exposure times and doses were: 4 hours – 25 J cm^{-2}, 8 hours – 50 J cm^{-2}, 12 hours – 75 J cm^{-2}, 24 hours – 150 J cm^{-2}, 48 hours – 300 J

cm^{-2}, 79 hours – 493 J cm^{-2}, 102 hours – 636 J cm^{-2}, 126 hours – 786 J cm^{-2}, 150 hours – 936 J cm^{-2}, 174 hours – 1086 J cm^{-2}, 198 hours – 1236 J cm^{-2}.

Isolation and Purification of DNA

DNA was extracted from blood stains using a standard phenol:chloroform method[35]. Briefly, stains were extracted in DNA IQ™ spin baskets (Promega Corporation), incubated overnight at 56°C in 400 µl DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL Proteinase K). The crude extract was purified by 25:24:1 phenol/chloroform/isoamyl alcohol (Fisher, Norcross GA), and spun in a Phase Lock Gel (PLG) Tube (2 mL, heavy, Eppendorf, Boulder, CO) according to the manufacturer's protocol. DNA was further purified using a Microcon (Millipore, Bedford, MA) according to the manufacturer's protocol, and stored in sterile water.

DNA Quantification

Yield Gel

Extracted DNA was quantified using a yield gel. An aliquot of each extract was electrophoresed on a 1% agarose gel along with DNA quantification standards, and stained using a 1% ethidium bromide solution. DNA was visualized using a short wave UV light transilluminator. A film of the gel was taken, and quantification completed by a visual comparison of the samples with the standards.

DNA standards (Applied Biosystems) were prepared – 10 ng, 2.5 ng, 1.25 ng, 0.625 ng, 0.3125 ng, and 0.15625 ng. To test the accuracy of these dilutions, two calibrators were prepared. Five microliters of each standard and calibrator were added to 150 µl spotting solution (0.4 N NaOH, 25 M EDTA, 0.00008% bromophenol blue). Samples to be quantitated were diluted if necessary and 5 µl of each added to spotting solution.

A piece of positively charged Biodyne B membrane was cut (~11 cm x 7.9 cm) to fit the Convertible® Filtration Manifold System (Gibco-BRL) slot blot apparatus and incubated in 50 mL pre-wetting solution (0.04 N NaOH, 25 mM EDTA) at room temperature for up to 30 minutes. The membrane was then placed on the gasket of the slot blot and covered with the top plate. To ensure the formation of a tight seal, the sample vacuum was turned off while the clamp vacuum was turned on and a vacuum source applied.

Each sample (~155 µl) was slowly added to the center of a different well in the slot blot. After all were loaded, the sample vacuum was slowly turned on until the liquid had been completely drawn through the membrane (~30 seconds). The vacuums were turned off, and the slot blot disassembled. The membrane, supporting the bound DNA, was immediately transferred to a HybriBoat (Gibco BRL) containing 5 mL of 30% H_2O_2 in 100 mL of Hybridization Solution (0.9 M NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA, 0.5% w/v SDS) pre-warmed to 50°C. The boat was rotated at 50 rpm in a 50°C water bath for 15 minutes, after which the solution was decanted.

Thirty milliliters of hybridization solution was then poured into the boat, it was tipped to the side and 20 µl of the biotinylated D17Z1 probe added. The boat was rotated

at 50 rpm in the 50°C water bath for 20 minutes. The solution was decanted and the membrane rinsed briefly in 100 mL of pre-warmed Wash Solution (0.27 M NaCl, 15 mM $NaH_2PO_4 \cdot H_2O$, 0.5% w/v SDS). Another 30 mL of the pre-warmed Wash Solution was added, along with 180 µl of the Enzyme Conjugate (horseradish peroxidase/streptavidin). The membrane was incubated in a 50°C rotating water bath for 10 minutes. This solution was poured off and the membrane washed for 15 minutes on an orbital shaker (50 rpm) at room temperature in 100 mL of Wash Solution. The membrane was next washed briefly in Citrate Buffer (0.1 M sodium citrate, pH 5.0)

The bound probe was detected using a colorimetric reaction. The membrane was covered with Color Development Solution (30 mL Citrate Buffer, 1.5 mL chromogen:tetramethyl benzidine solution in 100% ethanol, 30 µl 3% H_2O_2) and incubated at room temperature on an orbital shaker (50 rpm). DNA was quantitated by a comparison of the intensity of the color reaction of the DNA standards with that of the questioned samples.

Real-Time PCR: Alu

SYBR Green

The 25 µl reaction contained 2.5 µl SYBR Green Buffer (Applied Biosystems, proprietary), 3 mM $MgCl_2$, 2 µM dNTPs, 1.25 units AmpliTaq Gold DNA Polymerase, and 22.5 pmol each primer. Cycling conditions were as follows: 1) 95°C 10 m; 2) 40 cycles: 95°C 15 s; 3) 60°C 1 m.

TaqMan® Assay

The 25 µl reaction contained 12.5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems, proprietary), 22.5 pmol each primer (F: TET- aac ccc gtc tct act aaa aat aca aaa a; R: atc tcg gct cac tgc aac ct; designed using Primer Express software, Applied Biosystems), and 6.25 pmol probe (agc tact cg gga ggc tga ggc agg a; designed using Primer Express software). Cycling conditions were as follows: 1) 95^{o}C 10 m; 2) 40 cycles: 95^{o}C 15 s; 3) 60^{o}C 1 m.

PCR Amplification

Autosomal Multiplex

Autosomal STR analysis was carried out with 2 ng of genomic DNA using a mutiplex comprised of Power Plex 1.2 primers (Promega Corporation) to determine a eight-locus (plus amelogenin) genotype or with nine-locus (plus amelogenin) AmpFLSTR® Profiler™ PCR Amplification kit (Applied Biosystems). The analysis was performed in accordance with the manufacturer's instructions.

Alu Amplification

Protocol 1

The *Alu* protocol, yielding a 265 bp amplimer, was adapted from published reports [21]. The 25 µl reaction was carried out with 2 ng genomic DNA, 2.5 uM dNTPs. 3.25 mM $MgCl_2$, 10 µg non-acetylated BSA, 2.5 units AmpliTaq DNA Polymerase (Applied Biosystems), and 20 pmol each of the forward and reverse primers (F: FAM -

gcg gtg gct cac gcc t; R: gga gtc tcg ctc tgt cg) in 1X Buffer D3 (40 mM Tris-HCl. pH 8.0, 10 mM DTT, 6 mM KCl, 2.5 % glycerol). Cycling conditions were as follows: (1) 95^{o}C 11 m; (2) 17 cycles – 95^{o}C for 30 s, 56^{o}C for 30 s, 72^{o}C for 30 s, (3) 72^{o}C for 5 m.

Protocol 2

The *Alu* amplification protocol yielded a 125 bp amplimer. Primers were designed in-house using Primer Express software (Applied Biosystems). The 25 µl reaction included, 1 ng template DNA, 2.5 µM dNTPs, 2.5 mM $MgCl_2$, 0.5 units Amplitaq DNA polymerase, and 5 pmol each of the forward and reverse primers (F: TET- aac ccc gtc tct act aaa aat aca aaa a; R: atc tcg gct cac tgc aac ct; designed using Primer Express software, Applied Biosystems), in 1X Buffer D3 (40 mM Tris-HCl, pH 8.0, 10 mM DTT, 6 mM KCl, 2.5% glycerol). If applicable, 100 nM of a translesion polymerase was added. Cycling conditions were as follows: (1) 85^{o}C 1 m; (2) 22 cycles – 85^{o}C for 30 s, 56^{o}C for 30 s, 60^{o}C for 5 m, (3) 60^{o}C for 5 m.

YAP Amplification

The YAP locus was amplified in a 25 ul reaction containing 2 ng template DNA, 2.5µdNTPs, 1.6 mM $MgCl_2$, 8 µg non-acetylated BSA, 0.5 units AmpliTaq DNA Polymerase, 10 pmol each of the forward and reverse primers (F: TET- agg act agc aat agc agg gga aga; R: cag ggc caa ctc caa cca ag)[36] in 1X Buffer D3. Cycling conditions were as follows: (1) 85^{o}C for 1 min, (2) 32 cycles: 85^{o}C for 30 s, 59^{o}C for 60 s, 60^{o}C for 5 minutes, and (3) final extension at 60^{o}C for 5 min.

Autosomal Multiplex

Autosomal STR analysis was carried out with 2 ng of genomic DNA using a mutiplex comprised of Power Plex 1.2 primers (Promega Corporation) to determine a eight-locus (plus amelogenin) genotype. The analysis was performed in accordance with the manufacturer's instructions.

PCR Product Detection

Amplified fragments were detected using the ABI Prism 310 capillary electrophoresis system. A 1.5 µl (Profiler, *Alu*) or 0.5 µl (Power Plex 1.2) aliquot of each amplified sample was added to 24 µl Hi-Di formamide (Applied Biosystems) and 1 µl of *GeneScan 500 ROX* (Profiler), *GeneScan 500 TAMRA* (*Alu*) internal lane standard or with 0.25 µl of the CXR internal lane standard (Power Plex 1.2). Tubes were heated at 95^{o}C for three minutes and snap cooled on ice for at least three minutes. Samples were injected through the capillary using the module *GS STR POP4(1 mL)C* (5s injection, 15 kV, 60^{o}C, run time 28 minutes, Filter Set F - Profiler), *GS STR POP4(1 mL)C* (5s injection, 15 kV, 60^{o}C, run time 28 minutes, Filter Set C - *Alu*) or *POP4(1 mL)A* (5s injection, 15 kV, 60^{o}C, run time 28 minutes, Filter Set A – Power Plex 1.2). Samples were subject to laser induced fluorescence, and analyzed with GeneScan 3.1.2 software (Applied Biosystems).

Lesion Specific Endonuclease Restriction

Chlorella virus pyrimidine dimer glycosylase (CV-PDG)

CV-PDG (Trevigen, Gaithersburg, MD) is a DNA glycosylase with associated AP lyase activity which recognizes both *cis-syn* and *trans-syn* CPDs, leaving a single strand gap at the site of this damage. Human genomic DNA samples were digested in a 20 µl reaction containing 0.008 units ng^{-1} CV-PDG in 1X REC Buffer 11 (Trevigen) (25 mM $NaPO_4$ pH 6.8, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.1 mg/mL BSA), with an overnight incubation in a 37°C water bath. The reaction was stopped with a 20 minute incubation in a 65°C heating block.

T4 Endonuclease V

T4 Endo V (Epicentre, Madison WI) is a pyrimidine dimer glycosylase with associated AP lyase activity that recognizes *cis-syn* CPDs, generating a single strand gap. The 20 µl reaction contained 5 x 10^{-4} units ng-1 T4 Endo V, 1X REC Buffer 11 (Trevigen) (25 mM $NaPO_4$ pH 6.8, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.1 mg mL^{-1} BSA). Samples were incubated in a 37°C water bath for 30 minutes, and the reaction stopped with a 20 minute incubation in a 65°C heating block.

Ultraviolet Damage Endonuclease (UVDE)

UVDE (Trevigen) is a DNA glycosylase that lacks AP lyase activity. It recognizes both CPDs and 6-4(PPs). The 20 µl reaction included 0.004 µl ng^{-1} UVDE, 1X REC Buffer V (Trevigen) (20 mM HEPES pH 6.5, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM NaCl). Digests were allowed to proceed overnight in a 30°C water bath, and halted with a 20 minute incubation in a 65°C heating block.

Formamidopyrimidine Glycosylase (FPG)

FPG (Trevigen) is a DNA glysolase with associated lyase activity. It recognizes a number of oxidatively modified bases including open ring forms of 7-methylguanine (2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine,4,6-diamino-5-formamidopyrimidine), 8-oxo-guanine, 5-hydroxycytosine, 5-hydroxyuracil, alfatoxin bound imidazole ring opened guanine, and imidazole ring opened N-2-aminofluorene-C8-guanine. The 20 µl reaction included 0.001 units ng^{-1} FPG, 1X REC Buffer 10 (Trevigen) (10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM EDTA, 0.1 mg mL^{-1} BSA). The reaction was allowed to proceed at 37°C overnight, and stopped by a 20 minute incubation in a 65°C heating block.

Human 8-Oxoguanine Glycosylase 1(hOGG1)

hOGG1 functions as both a DNA glycosylase and a lyase. It recognizes 8-oxo-guanine/cytosine base pairs, formamidopyrimidine/cyosine base pairs, and to a lesser extent, 8-oxo-guanine/thymine base pairs, removing the oxidized base and leaving a single strand gap. The 20 ul reaction contains 0.01 units ng^{-1} hOGG1, 1X REC Buffer 6 (Trevigen) (1 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1 mM EDTA). The reaction proceeded at 37°C overnight and was halted with a 20 minute incubation in a 65°C heating block.

Alkaline Gel Electrophoresis

One percent alkaline gels were made by dissolving the appropriate quantity of molecular biology grade agarose (Fisher Scientific) in alkaline gel buffer (50 mM NaCl,

1 mM EDTA). After the gel hardened, it was soaked for at least 30 minutes in alkaline gel running buffer (30 mM NaOH, 1 mM EDTA). To each DNA sample was added an equal volume of alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% glycerol, 0.025% bromocresol green). The entire sample was loaded onto the gel, as was a λ HindIII lane standard for sizing. Electrophoresis proceeded for 3.1 hours at 70 V (217 volt hours), after which the gel was soaked in neutralization solution (1 M Tris-HCl pH 7.6, 1.5 M NaCl) for one hour to allow SYBR Gold (Molecular Probes, Eugene, OR) staining. Finally, the gel was visualized using a short wave UV transilluminator.

Humidity Chamber Exposure

The humidity chamber was a normal glass aquarium with airtight seals. A hole was cut in the plexiglass lid to allow for the attachment of a 302 nm UV light fixture (flux = 0.204 J $cm^{-2}min^{-1}$), and the seams sealed. 94% humidity was maintained chemically by including four 250 mL beakers of a saturated copper (II) sulfate pentahydrate solution. 50 µl bloodstains were placed on low racks on the bottom of the tank to protect them from gathering water and exposed for times ranging from 1 day to 14 weeks. Subsequently, stains were collected and stored at -20^{o}C until use.

Photoreactivation

The CPD photolyase catalyzes the direct reversal of CPDs. The 20 ul reaction contained 500 ng DNA, 0.08 ng photolyase per ng DNA in 1X REC Buffer 14 (20 mM Tris-HCl, pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT). The microcentrifuge tubes

were placed open in a rack on the top shelf on an exposure stand approximately 12 inches from the light source. The reaction was initiated by exposure to UVA light (365 nm) and allowed to proceed for 2 hours.

Modified Base Excision Repair

The modified base excision repair strategy included a 30 µl reaction containing 0.015 units ng^{-1} human Polymerase B, 0.0025 units ng^{-1} *E. coli* Endonuclease IV, and 0.0025 units ng^{-1} T4 DNA ligase in 1X BER Buffer 4 (50 mM Tris-HCl, pH 7.8, 10 mM DTT, 100 mM $MgCl_2$, 10% glycerol). It was allowed to proceed overnight at 37°C and was halted with a 20 minute incubation in a 65°C heating block.

APPENDIX 2: PRESENTATIONS AND PUBLICATIONS

Publications

Characterization of UVC-Induced DNA Damage in Physiological Stains: Forensic Implications. Hall AM, Ballantyne J. *Analytical and Bioanalytical Chemistry;* Sep 2004; 380(1):72-83.

Novel thermostable Y-family Polymerases: Applications for the PCR Amplification of Damaged or Ancient DNAs. McDonald, J.P., Hall, A., Gasparutto, D., Cadet J., Ballantyne J and Woodgate, R. *Nucleic Acids Res* (2006) 34 (4) 1102-1111

Assessment of DNA Damage induced by terrestrial UV irradiation of dried bloodstains: forensic implications. Hall, AM, Ballantyne, J. Manuscript in preparation.

Development of *in vitro* systems for the repair of damaged DNA from dried biological stains. Hall, AM, Ballantyne, J. Manuscript in preparation.

Presentations

Ashley Hall, Jack Ballantyne. "Assessment and Repair of Damaged DNA." Future Directions in Forensic Biology Workshop. SWAFS Training Conference, Scottsdale, AZ, 2002.

Ashley Hall, Jack Ballantyne . "Assessment of Damaged DNA Templates. NIJ fourth Annual DNA Grantees Workshop, Washington DC, 2003.

Ashley Hall, Jack Ballantyne . "Assessment and *In Vitro* Repair of Damaged DNA Templates." Florida Academy of Sciences Annual Meeting, Orlando, FL, 2004.

Ashley Hall, Jack Ballantyne . "Assessment and Repair of Damaged DNA Templates." NIJ Fifth Annual DNA Grantees Workshop, Washington DC, 2004.

Ashley Hall, Jack Ballantyne . "Assessment and Repair of Damaged DNA Templates." Joint Meeting of the Canadian Society of Forensic Scientists, MAFS, MWAFS and SAFS, Orlando, FL, 2004

Ashley Hall, Jack Ballantyne. "Assessment and *In Vitro* Repair of Damaged DNA Templates-Forensic Implications." American Society for Microbiology Conference. DNA Repair and Mutagenesis: From Molecular Structure to Biological Consequences, Southampton, Bermuda, 2004

Ashley Hall, Jack Ballantyne. "Assessment and *In Vitro* Repair of Damaged DNA Templates." AAFS Annual Meeting, New Orleans, 2005.

Ashley Hall, Roger Woodgate, John McDonald and Jack Ballantyne. “Biochemical Repair and Lesion Bypass of Damaged DNA”, to be presented at AAFS Annual Meeting, Seattle, 2006