An investigation of the effect of DNA degradation and inhibition on PCR amplification of single source and mixed forensic samples

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1. Abstract

The goal of this proposal was to examine the mechanisms for PCR inhibition and degradation and their effects on forensic DNA typing. The effects of these problems are well known; poor amplification and allele dropout. However, there are very few studies in the forensic literature that explore the issue of how inhibitors produce poor PCR results and even less is known about the mechanisms for degradation commonly present in typical forensic samples. A better understanding of these inhibition mechanisms could lead to the development of more sensitive, more robust analytical protocols.

In this proposal we performed controlled studies to clarify the mechanisms of environmental and chemical degradation and PCR inhibition on single source samples and mixtures. To do this we utilized real time PCR and HPLC/EC to evalutate the mechanisms of DNA degradation, oxidative damage and PCR inhibition on the recovery of STR profiles. Both degraded and pristine DNA were examined. In particular we performed the following experiments: 1) An analysis of the effects of various inhibitors on PCR amplification using real time PCR with high resolution DNA melt curves. 2) an analysis of the effect of natural and enzymatic degradation on PCR profiles. 3) An analysis of the effect of chemical oxidation on DNA profiles and 4) a correlation between PCR inhibition and DNA amplification.

Our overall conclusions are that 1) Environmental damage to DNA in tissue samples occurs rapidly to the point that DNA becomes nearly unrecoverable. The template in such samples breaks down to very small pieces in as little as 3 weeks.

2) The effects of oxidative damage on such samples was minimal. We utilized HPLC with electrochemical detection to monitor base damage to heavily degraded tissue samples. No oxidation of DNA bases was found for environmentally degraded DNA, although it was present in saliva samples.

3) . The combination of real time PCR and DNA melt curves is an effective tool for the detection of PCR inhibition and permits classification of various inhibitors based on their behavior. Our experiments on the effect of DNA template sequence, DNA template length and inhibitor concentration reveal that PCR inhibitors may affect STR results in several different fashions. Real time PCR results reveal that PCR inhibitors can affect Taq polymerase reactions reducing the total amount of DNA produced and/or can bind DNA, resulting in a loss of available template.

4) The effects of DNA binding also appear to be sequence and/or length specific. PCR inhibitors that mainly affect taq tend to inhibit DNA by affecting the largest alleles first, while inhibitors that bind DNA may affect smaller alleles as well as larger ones.

5) It has been widely reported that MiniSTRs improve resistance to PCR inihibition. Based on our results, a caveat should be that such improvements may depend on the type of inhibition. Sequence specific inhibition may still cause problems even with reduced sized amplicons.

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3. Executive Summary

The goal of this proposal was to develop methods to better understand the effect of inhibition, degradation and low copy number in the recovery of information in Forensic DNA casework. While STR multiplex analysis is now well established for the typing of samples in which high quality DNA can be recovered, the situation is quite different when poor quality DNA is present. This includes samples which contain highly degraded DNA, PCR inhibitors or both. These samples often exhibit problems such as allele loss, low intensity or inefficient amplification.

Because most developmental validation procedures do not explicitly deal with the interpretation of badly degraded or inhibited samples, the electropherograms produced from such samples can produce results which may fall outside general interpretational guidelines developed for standard forensic validation studies and can result in indeterminate results in court. This problem is especially significant for the interpretation of mixtures. Low level stochastic thresholds are usually determined using pristine single source samples. When degradation and/or inhibition is present problems with peak balance and allele dropout occur. Interpreting these effects can be difficult and may depend on the specific circumstances of the collection and recovery of the collected DNA sample.

. There are few studies in the forensic literature that explore the issue of inhibition mechanisms and the variation in PCR results with inhibitor concentration. Even less is known about the range of inhibiting substances present in typical forensic samples. A variety of techniques have been utilized to relieve inhibition. Sometimes diluting a DNA sample or increasing Taq concentration is all that is needed. Other times a more complex extraction and cleanup is needed. Knowing the mechanism of inhibition might also help in designing more

robust STR systems and better cleanup techniques. If certain primer sequences are more susceptible to PCR inhibition, better amplifications might be obtained by simply shifting the locations of the primer binding sites or utilizing a different form of Taq. The critical point is that nothing can be done until we know for certain what types of materials are co-extracted with the DNA and how these materials affect the results.

There is also a need to further study the effects of environmental conditions on DNA recovery. For example, how much of the problem of poor amplification is the result of PCR inhibition and how much is true degradation? PCR inhibitors may exist in many environmentally challenged DNA samples. Forensic analysts need to improve their ability to assess such samples. The goal of this proposal was to begin that process by developing methods to better define the mechanisms by which inhibition and DNA degradation affect PCR in order that low level indeterminate samples can be better defined and the analytical and stochastic thresholds can be clarified. A number of specific projects were performed:

1) **Examination of the effects of environmental degradation on DNA samples**

   In collaboration with the University of Tennessee's Forensic Anthropology Center in Knoxville Tennessee, we examined the rate of DNA decomposition in human tissue. In this study, tissue samples were removed from bodies placed at various locations – surface, brush covered, shallow graves and in water. Samples were collected from each body over a period of 8 weeks. Soil samples were also collected at this time to determine any specific changes to soil composition as a result of the placement of the bodies.

   Tissue samples were weighed and extracted using standard PCIA protocols and analysed using quantitative PCR. For the real time PCR work, two different Alu primer sequences were used to create a large and a small amplicon. The relative amplification quantity of the two amplicons was used to detect decomposition rates. This data was

compared with that produced by PCR amplification with the Powerplex 16 STR kit. The results confirmed the rapid loss of recoverable DNA that generally occurred within the first 2-3 weeks. Buried samples, figure 2, decomposed more quickly than those placed on the surface, figure 1. These samples were subsequently used in an analysis of the relative levels of oxidation and decomposition in environmentally degraded DNA, figure 3.

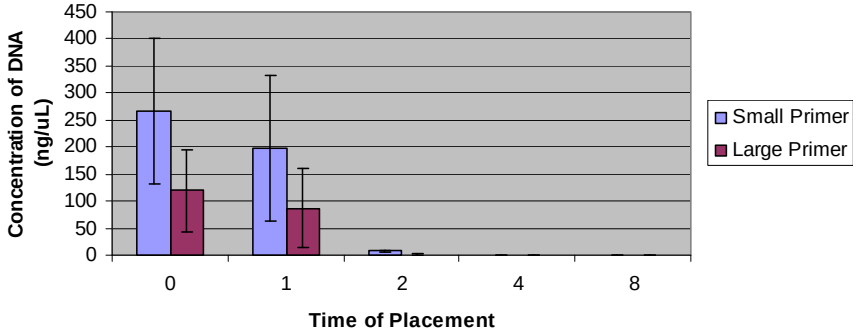

Figure 1: The relative concentration of DNA in a 60uL extract recovered from a tissue sample collected from a body placed on the surface at the Forensic Anthropology Center in Knoxville, Tennessee. Samples were collected over an 8 week period. Samples were analyzed using real time PCR and targeted 2 different lengths of Alu insert amplicons. As expected the greater amplification of the short 82 bp amplicon indicated DNA degradation in the sample.

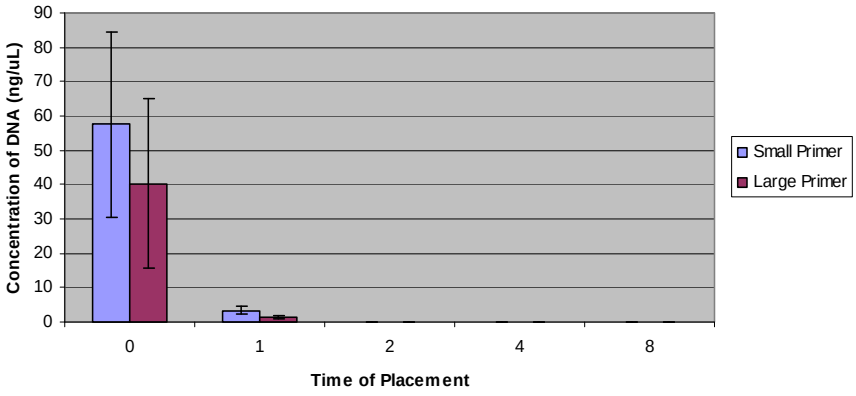

Figure 2: The relative concentration of DNA in a 60uL extract recovered from a tissue sample collected from a body placed in a shallow grave at the Forensic Anthropology Center in Knoxville, Tennessee. Samples were collected over an 8 week period. Samples were analyzed using real time PCR and targeted 2 different lengths of Alu insert amplicons. As in Figure 1, the greater amplification of the short 82 bp amplicon indicated DNA degradation in the sample. The figure also shows a more rapid decomposition of the buried tissue.

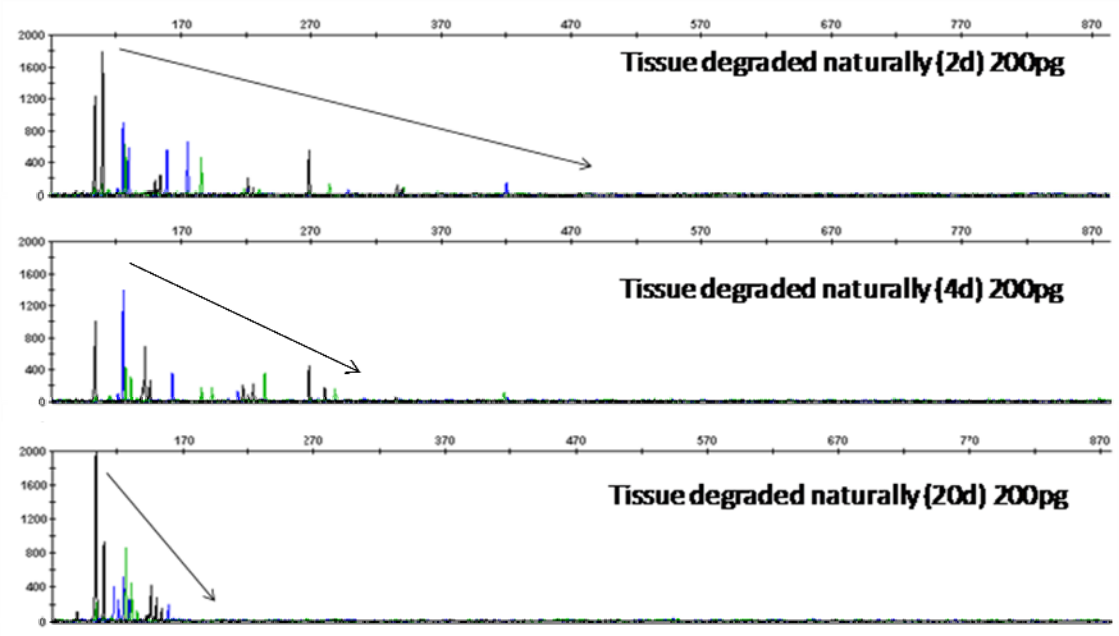

Figure 3: The amplification results from DNA recovered from tissue samples removed from a body placed on the surface at the Forensic Anthropology Center of the University of Tennessee. Samples were collected over an 8 week period. Samples were analyzed using the Powerplex 16 STR amplification kit. As expected the greater amplification of the short 82 bp amplicon indicated DNA degradation in the sample.

2) Examination of the mechanisms for PCR inhibition

Using a variety of DNA sequences and amplicon lengths, we examined various PCR inhibitors and measured their effect on DNA amplification. Depending on concentration, the effects of these inhibitors on the PCR reaction can vary from different levels of attenuation to complete inhibition of the signal. The inhibitors examined included heme, indigo, tannic acid, melanin, collegin, and humic acid. These inhibitors commingle with the DNA sample upon exposure to different environmental conditions and/or co-extract with the DNA sample.

To test for the effects of these inhibitors, we prepared a series of HUMTHO1 primers targeting different sequences and lengths surrounding the STR region. The goal was

to probe length and sequence effects on inhibition using real time PCR. In our results we found that DNA melt curves combined with real time PCR provided an exceptional method for the detection of the inhibitory effect. Based on the effect of a variety of inhibitors on product concentration, amplification rate, and DNA melt curve, we could develop a classification scheme for each inhibitor as well as produce initial recommendations on mitigation of their effects.

For example, figure 4 demonstrates the effect of increasing concentration of calcium on the real time PCR amplificaton of a HUMTHO1 9.3 STR . The figure shows the real time PCR curve, its first derivative, and the DNA melt curve. As can be seen in the figure, with an increase in calcium, there is a gradual loss of product and a reduction in the slope of the amplication curve, indicating the efficiency of the Taq polymerase is being affected, presumably by calcium displacing the enzyme's magnesium cofactor. The melt curve is not affected. Figure 5 shows the resulting amplification of the Powerplex STR kit with increasing levels of calcium. Larger amplicons are affected first by this type of inhibition.

When this result is compared with that for humic acid, a quite different plot is obtained. Figure 6 shows that with increasing concentration of humic acid, the Ct value for the amplification shifts to the right, indicating a loss of DNA template. The slope of the amplification plot however, does not change, indicating no effect on the polymerase. The melt curve also shows a strong effect with increased humic acid, indicating that this material binds to the DNA template and explaining the reduction in Ct. Thus humic acid inhibits DNA by binding it, effectively reducing the concentration of the DNA. Figure 7 demonstrates that this effect is sequence specific, and unlike calcium, humic acid affects both small and large amplicons.

Figure 4: The effect of increasing calcium on the amplification of a HUMTHO1 9.3 amplicon. Figure 4 A shows a loss of product and reduction in slope with increasing calcium concentration. Figure 4B is the first derivative of Figure 4A. Figure 4C is the melt curve. No change in melt temperature is seen with increasing calcium concentration.

Figure 5: The effect of increasing concentration of calcium on the amplification of 500 pg of a male DNA control by the Powerplex 16 STR kit. In the figure, the largest amplicons are affected first, indicating a general affect on amplification efficiency.

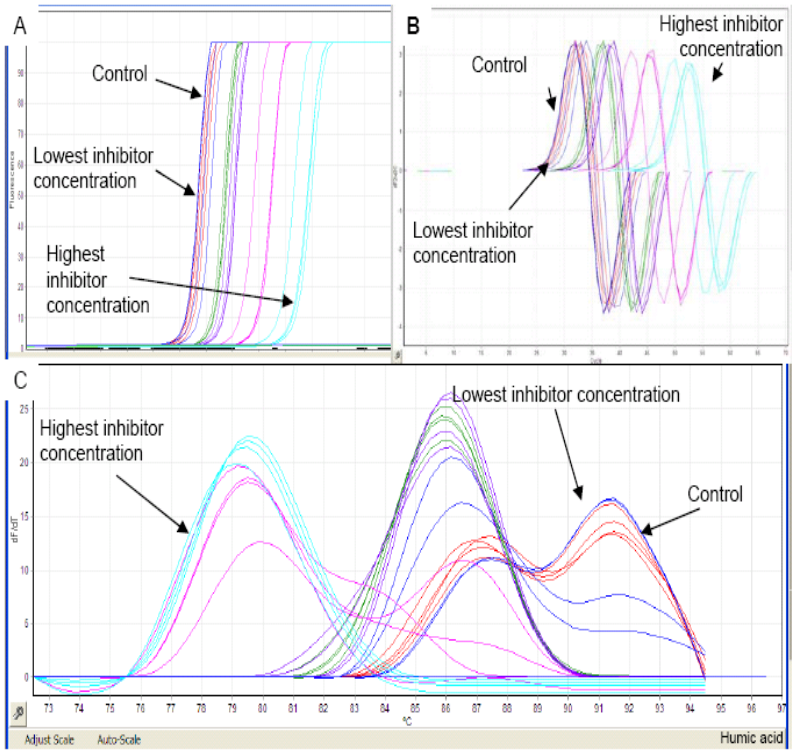

Figure 6: The effect of increasing humic acid on the amplification of a HUMTHO1 9.3 amplicon. Figure 6A shows a change in Ct with increasing humic acid concentration. Figure 6B is the first derivative of Figure 6A. It also shows this shift. Figure 6C is the melt curve. A shift to a lower melting temperature is seen indicating that humic acid is binding DNA, reducing the amount of available template.

Figure 7: The effect of increasing concentration of humic acid on the amplification of 500 pg of a male DNA control by the Powerplex 16 STR kit. In the figure, specific small amplicons are affected as well as larger ones, indicating some sequence specificity in the inhibition process.

These results demonstrate that inhibitors can function in two major ways- by affecting or binding Taq or by binding DNA template. As shown in the STR multiplex amplifications, the inhibition process is also sequence and length dependent. This is further demonstrated in Figure 8 which illustrates the effect of changing HUMTHO1 amplicon size on Ct and melt curve with increasing concentration of melanin. Like humic acid, melanin binds DNA affecting Ct and melt curve. Interestingly, the figure shows that minimal effects occur when the amplicon size is small, but when the size increases to 300bp, the Ct values shift and the melt curve shifts to lower temperatures.

Figure 8: The effect of amplicon size on real time PCR amplification curves and subsequent DNA melt curves with increasing melainin concentration. The results were obtained using HUMTHO1 amplicons with Sybr Green detection.

Overall our results strongly suggest that PCR inhibition affects samples in more than one way. There is no generic inhibitor. Inhibitors can affect Taq (calcium), can bind DNA(humic acid, melanin) or may do both (collegin). The results also show that inhibition generally affects larger amplicons first, however inhibitors that bind DNA may have additional sequence specific effects in addition to these generic length affects. The consequence of these processes are as follows

1. Persons interested in validating new extraction techniques should be careful to include a variety of inhibitors, for example calcium, humic acid and collegin all appear to inhibit DNA in different manners.
2. The inhibition processes are concentration dependent. Therefore, reaction volume and sample dilution should be carefully monitored
3. The inhibition process is size dependent, particularly for Taq inhibitors, such samples will benefit through the use of miniSTRs, however, template binding inhibitors such as melanin or humic acid, may still inhibit certain smaller amplicons in a sequence specific fashion.
4. Current control sequences used in real time kits should probably be longer. They would then be more sensitive to inhibition. More application of melt curve analysis would also be helpful.

3) Evaluation of chemical and environmental DNA degradation

When DNA degrades, it tends to fragment into smaller and smaller segments. A number of mechanisms have been postulated to account this effect including hydrolytic cleavage, chemical oxidation and enzymatic degradation. The goal of this portion of the proposal was to determine the relative amount of oxidative damage present in degraded DNA. Therefore, a study was performed on the relative effects of hydrolytic damage and base

damage through chemical oxidation. We utilized environmentally exposed DNA from the University of Tennesse's forensic anthropology center as well as enzymatically and chemically degraded DNA from controlled laboratory studies.

The major site of oxidative attack on the DNA bases is the C=C double bond of pyrimidines, and purines, leading to ring fragmentation and base modifications. Many of these oxidized base products will block replication, negatively impacting amplification with the standard Taq-DNA polymerases used in PCR (3). While there have been a number of papers and reports suggesting potential mechanisms to repair damaged forensic DNA, there has been very little research on methods to detect the actual damage to degraded forensic DNA. In particular, there has been little work done examining oxidative damage in forensic samples, in spite of the fact that such damage is well documented in a number of disease processes such as cancer.

Guanine nucleobases are frequently targeted by oxidants due to the fact that their oxidation potential is the lowest among the DNA bases. 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-OH-dG), is an adduct for which specific cellular repair enzymes exist and it has been shown to cause GC→TA transversions. Its presence in DNA causes mutations resulting in mispairing and multiple amino acid substitutions. As such, the detection of this oxidative product provides a bellwether for the presence of oxidative DNA damage. Thus it is likely that this compound may provide insight into the relative amount of oxidative damage to target tissues used in forensic STR and mitochondrial analysis. The aim of this study was to evaluate the relative contribution of oxidative damage and hydrolytic damage to DNA by determining the 8OHdG concentration in DNA from both degraded and non-degraded biological samples, and comparing these data with amplification success using multiplexed STR typing.

To perform this study we performed a complex set of enzymatic reactions to break down the genomic DNA in order that the presence of oxidation of individual bases could be detected and quantified. We utilized DNaseI, Nuclease P1, alkaline phosphatase, and phosphodiesterase to completely digest the DNA. We then measured the relative amount of guanosine dG to 8OHdG using HPLC with UV and electrochemical detection. We utilized the environmentally degraded tissue samples discussed above. We also prepared control samples consisting of genomic DNA as well as genomic DNA treated with oxidizing solutions of bleach and hydrogen peroxide. The effect of treatment of genomic DNA with hydrogen peroxide and bleach when compared to the untreated DNA is demonstrated in Figure 9. The figure shows a sample of genomic DNA amplified using the Powerplex 16 STR kit. The treated samples show the characteristic degradation curve seen with the loss of larger amplicons due to the fragmentation of the genomic DNA

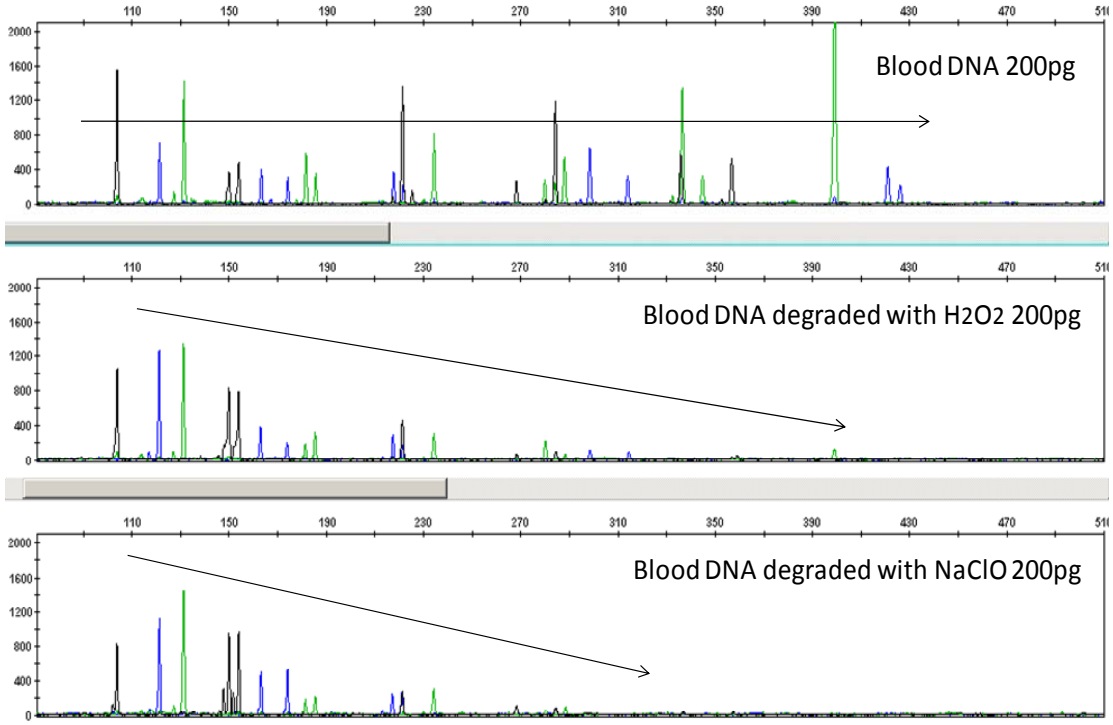

Figure 9: A comparison of the amplification of a DNA sample extracted from blood with the Powerplex ® 16 STR multiplex kit with that same sample treated with bleach and peroxide. The concentration of the DNA template was 200pg. PCR amplification

and genotyping were performed using manufacturer's suggested protocols using an ABI 310.

Portions of these samples as well as the environmentally degraded samples were put aside prior to amplification, enzymatically digested and checked for the presence of 8OHdG using HPLC with electrochemical detection. We utilized a dual electrochemical/UV detection scheme that permitted determination of unoxidized bases by HPLC/UV while simultaneously measuring the oxidized bases via electrochemical detection. The results of the HPLC analysis are shown in Figure 10. The main figure shows the separation of the individual bases dG, dA, dT, and dC while the inset reveals the results from electrochemical detection. The electrochemical determination is 2-3 orders of magnitude more sensitive than the UV method and is very specific. Only oxidized bases are detected. The figure also shows no oxidation for an untreated blood sample while the blood sample treated with peroxide shows loss of signal for the individual bases and an easily detectable quantity of 8OHdG.

Figure 10: A chromatogram showing the results of the hydrolysis of two blood samples. The fist sample is untreated blood. The second sample was oxidized with peroxide in the presence of iron. The insert shows the detection of the oxidation product 8OHdG. Samples were analyzed using HPLC with UV and EC detection using an eluent consisting of 92.5% 50mM KH_2PO_4, (pH=5.5) with 7.5 % Methanol. C18 column, flow: 1.0 mL/min, injection 50 µL, 260 nm. Insert shows amperometric detection at 600mV.

Once the procedure was optimized, a series of samples were examined including blood, saliva, human tissue and beef tissue. Certain samples were treated with oxidants to determine their effect. The results indicated 8OHdG was present in saliva as well as oxidized samples of blood and animal tissue. However, no oxidation of dG was seen in tissue samples recovered from badly degraded DNA left in the environment for 20 days. We interpreted these results to indicate that oxidative damage in not a significant source of degradation of tissue samples in forensic investigations. The finding of oxidized DNA in saliva may be a result of constant exposure to air and the presence of amylase.

SAMPLE / TREATMENT	Exposure time	8OHdG/10^6dG (mean ± SD)
Saliva DNA control	-	18.6 ± 3.2
Human blood control *	-	-
Environmentally degraded Human tissue	20 days	-
Blood + 0.3% H_2O_2 *	1 hour	94 ± 13
Blood + 0.3% H_2O_2 + Fe^{+2} *	3 hours	117 ± 22
Blood + 0.6% NaClO *	1 hour	4.2 ± 1.3
Blood + 0.6% NaClO + Fe^{+2}*	3 hours	15.8 ± 2.7
Bovine Tissue control*	-	-
Bovine Tissue in 1% H_2O_2 *†	18 hours	59.2 ± 5.6
Bovine Tissue in 2% NaClO *†	18 hours	2.7 ± 0.6

* 100 μg DNA samples were digested with 40 U DNAseI for 0.5 h, followed by 1 U NP1 for 1 h, and 0.01 U PDE I and 0.02 U PDE II for 1 h, all digestions were performed at 37 °C in triplicate.
† After oxidation treatments, DNA samples were extracted from bovine tissue treated with 1% H_2O_2 and 2% NaClO, all digestions were perfomed at 37 °C with the optimized protocol.

Conclusions

The goal of this proposal was to investigate the mechanisms responsible for allele dropout and loss of signal in forensic DNA typing. In this proposal we examined environmentally damaged DNA as well as DNA treated with a variety of oxidants and inhibitors. In general we verified the well described ski slope pattern for degraded DNA. We found that this pattern was also present in certain types of inhibited DNA and oxidatively

damaged DNA. For other types of inhibited DNA this was not true. Certain inhibitors are length and sequence specific.

In general the forensic community can benefit from this work through an improved understanding of the underlying causes of allele dropout due to degradative and inhibitive effects. Interestingly we found that realtime PCR can be an effective tool for inhibition determination, particularly when longer amplicons are used in combination with melt curve effects. In fact, utilizing real time PCR in combination with STR typing we determined that at least three different mechanisms for inhibition could be ascertained, inhibition affecting taq and altering amplification rates, inhibition through DNA binding, altering Ct and melt curves, and a hybrid type of mechanism affecting both. Real time assays for inhibition are also more effective when BSA is not present in the reaction mixture.

Our work on oxidative damage demonstrates that oxidation of DNA can be an effective way to produce degraded DNA and may be responsible for effects seen with saliva, however the rapid degradation of DNA in tissue samples is not the result of oxidative effects. It is reasonable to assume this degradation is from bacterial attack and studies on bacteria recovered from the soil seem to indicate that this degradation occurs mainly through internal bacteria rather than from the soil.

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4. Introduction and overview

The goal of this proposal was to develop methods to better understand the effect of inhibition, degradation and low copy number in the recovery of information in Forensic DNA casework. While STR multiplex analysis is now well established for the typing of samples in which high quality DNA can be recovered, the situation is quite different when poor quality DNA is present. This includes samples which contain highly degraded DNA, PCR inhibitors or both. These samples often exhibit problems such as allele loss, low intensity or inefficient amplification. To deal with such samples most laboratories establish general interpretational guidelines which are based on published developmental validations. Specific thresholds are next determined during the laboratories own internal validation process. These threshold values are usually based on single source samples and then further used to define the interpretation of mixtures.

Figure 1 shows the comparison of an amplified DNA control sample with that of a recovered bone sample. The results clearly demonstrate the effects of degradation and/or inhibition on a STR profile. Loss of larger sized alleles and locus specific inhibition effects are evident among the smaller sized alleles.

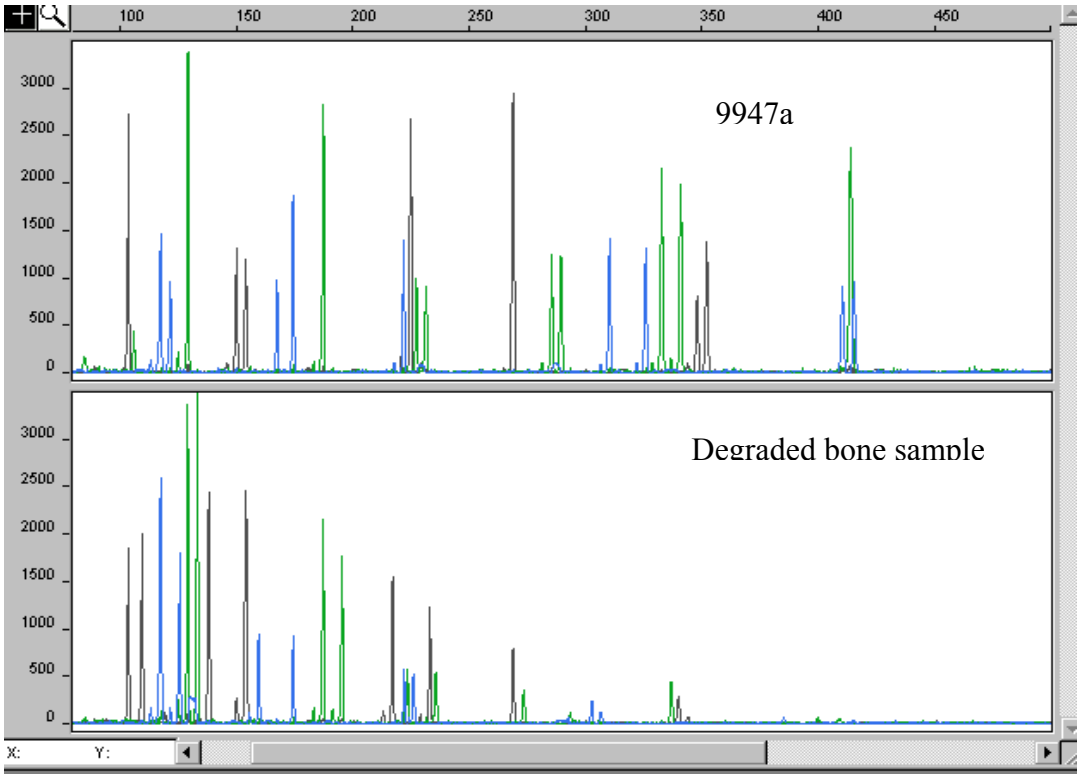

Figure 1: The comparison of a standard DNA sample with a DNA extract from a bone sample. Samples were extracted using and organic extraction and amplified via the Powerplex STR kit. The bone sample shows allele dropout at larger amplicon sizes.

Because most developmental validation procedures do not explicitly deal with the interpretation of badly degraded or inhibited samples, such results can fall outside the guidelines developed for standard forensic validation studies and can result in indeterminate results in court. This problem is especially significant for the interpretation of mixtures. Low level stochastic thresholds are usually determined using single source samples. How does the presence of the major contributor affect these results, especially when degradation or inhibition is present?

. There are few studies in the forensic literature that explore the issue of inhibition mechanisms and the variation in PCR results with inhibitor concentration and even less is known about the range of inhibiting substances present in typical forensic samples. A variety of techniques have been utilized to relieve inhibition. Sometimes diluting a DNA sample or

increasing Taq concentration is all that is needed. Other times a more complex extraction and cleanup is needed. Knowing the mechanism of inhibition might also help in designing more robust STR systems and better cleanup techniques. If certain primer sequences are more susceptible to PCR inhibition, better amplifications might be obtained by simply shifting the locations of the primer binding sites or utilizing a different form of Taq. The critical point is that nothing can be done until we know for certain what types of materials are co-extracted with the DNA and how these materials affect the results. There is also a need to further study the effects of environmental conditions on DNA recovery. For example, how much of the problem of poor amplification is the result of PCR inhibition and how much is true degradation? PCR inhibitors may exist in many environmentally challenged DNA samples. Forensic analysts need to improve their ability to assess such samples. The goal of this proposal is to begin that process.

Research goals and objectives

The overall goal of this proposal was to better define the mechanisms by which inhibition and DNA degradation affect PCR in order that low level indeterminate samples can be better defined and the analytical and stochastic thresholds can be clarified. A number of specific goals were defined.

1) Examination of the mechanisms for PCR inhibition

Using a variety of DNA sequences and amplicon lengths, we examined various PCR inhibitors and measured their effect on DNA amplification. Depending on concentration, the effects of these inhibitors on the PCR reaction can vary from different levels of attenuation to complete inhibition of the signal. The inhibitors examined included heme, indigo, tannic acid, melanin, collegin, and humic acid. These inhibitors commingle with the

DNA sample upon exposure to different environmental conditions and/or co-extract with the DNA sample.

To test for the effects of these inhibitors, we found that high resolution melt curves combined with real time PCR provided an exceptional method for the detection of the inhibitory effect. Based on the inhibitors effect on product concentration, amplification rate, and DNA melt curve, we could develop a classification scheme for each inhibitor as well as produce initial recommendations on mitigation of their effects.

2) Evaluation of chemical and environmental DNA degradation

A study of the effect of environmental factors on sample degradation was performed. In particular we examined the relative effects of hydrolytic damage and base damage through chemical oxidation. We utilized environmentally exposed DNA from the university of Tennesse's forensic anthropology center as well as enzymatically and chemically degraded DNA from controlled laboratory studies.

5. The determination of the effect of inhibitors on DNA amplification by real time PCR

Introduction

Degraded and environmentally challenged samples can produce numerous problems in forensic DNA typing including loss of signal, peak imbalance and allele dropout. However, DNA degradation is not the only issue encountered when analyzing challenging samples. Many such samples contain substances which are co-extracted with the DNA and inhibit the PCR reaction. While the effect of the presence of inhibitors is well known, the mechanism for PCR inhibition often is unclear. A better understanding of these processes should help the analyst recognize and troubleshoot problematic samples. This paper describes the utilization of real time PCR to study the mechanism of various PCR inhibitors and examines the effect of amplicon length, sequence and melting temperature on the process.

While a number of methods have been developed to improve PCR amplification in the presence of inhibition (1-3), little is known of the underlying causes of inhibition in PCR. Three potential mechanisms include: 1) binding of the inhibitor to the polymerase (4-5); 2) interaction of the inhibitor with the DNA; and 3) interaction with the polymerase during primer extension.

In previous work (6) we have determined that certain primers with a higher melting temperature are less affected by inhibition (Figure 1), and that not all inhibitors have the same effect on different STR loci. This suggests that the sequence of the amplicon or primer may have an affect on PCR inhibition. Primers with higher melting temperatures are more strongly bound to the DNA and may possibly prevent the

inhibitor from binding to the DNA. Alternatively, the inhibitor may bind to the DNA and block or interfere with primer extension. This could provide one explanation as to why shorter amplicons improve PCR sensitivity.

Inhibitors can also affect PCR efficiency through binding to the polymerase and/ or blocking necessary reagents. The purpose of this research is to examine inhibited PCR reactions in an attempt to better understand the general mechanisms of these interactions. If inhibitors bind to the polymerase and deactivate it, template size, melting temperature, and sequence should not affect results and all amplicons should be inhibited at roughly the same rate. If the inhibitors bind to the DNA and are influenced by primer or sequence, sequences with different melting temperatures should be inhibited at different rates and the total amount of template available to the polymerase at that locus may be reduced. If the inhibitor interacts with the polymerase or template during primer extension, longer amplicons should be inhibited at lower inhibitor concentrations than shorter amplicons for the same locus.

Real time PCR (qPCR) was selected as a means of testing inhibition for several reasons. First, since it is a PCR process, inhibition can be detected due to changes in either the efficiency of the reaction (7) or by changes in the threshold cycle (Ct), which indicates that lower concentrations of DNA are being amplified (8). Second, analysis of the PCR product is possible through a measurement of the melt characteristics of the amplicon (9). A change in the melt curve demonstrates modification of the PCR product presumably due to inhibitor binding. Third, a variety of inhibitor treatments may be directly compared by examining the relative amounts of PCR product produced by different levels of inhibition. Examination of these criteria should provide important information on how various types of inhibitors affect the amplification of DNA

template during PCR and aid the analyst in identifying the particular class of inhibitor that is interfering with sample analysis.

Materials and Methods

DNA standards

DNA standard K562 was used for primer optimization. For the inhibition tests, a standard solution of genomic DNA (TH01 9.3 homozygous genotype) was collected via multiple buccal swabs. The swabs were extracted by organic separation (phenol/ chloroform/ isoamyl alcohol (Sigma Aldrich, St. Louis, MO)) using a previously published protocol (10). The extracts were combined into one stock solution, quantified using the Alu qPCR protocol published by Nicklas et al (11), and diluted to approximately 2 ng/µL concentration.

Primer Design

Primers for the HUMTH01 locus were designed using the GenBank sequence accession number D00269 and the online primer design program Primer3 (12). The default settings available were used for all parameters except product size, primer length, and primer melting temperature. A primer length of 20 bp was used as a default unless it was necessary to increase the length to improve specificity. Target amplicon size ranges were: 100-150 bp, 200-300 bp, and 300-400 bp; and target melting temperatures were: 58°, 60°, and 62° C. Nine sets of primers were designed to produce three amplicons (100, 200, and 300 bp) at each of the three melting temperatures. The oligonucleotide primers were manufactured by Integrated DNA Technologies (Coralville, IA) and were purified by standard de-salting by the manufacturer. In order to confirm the specificity of the amplification, amplification of the K562 standard DNA was performed for each of the nine primer sets using the Miniplex PCR protocol described previously (13) with 5 ng of template DNA. The products were separated and

analyzed on the Agilent 2100 Bioanalyzer (Waldbronn, Germany) using the DNA 1000 Assay kit according to manufacturer's protocols (14).

Real time PCR analysis

Real time PCR was performed on the Corbett Rotorgene 6000 (Corbett Robotics, Sydney, Australia), with SYBRGreenI (Invitrogen, Carlsbad, CA) intercalating dye. The reaction components were based on a previously published protocol (11), with three modifications. To enhance the effect of the various inhibitors, BSA was not added, the amount of Taq polymerase was reduced by half to 0.02 U/µL, and the primer concentrations were reduced by an order of magnitude to 0.21 µM. Additionally, Ramp Taq® polymerase (Denville Scientific, Metuchen, NJ) was used instead of AmpliTaq® Gold. A genomic DNA standard (homozygous 9.3 HUMTHO1 STR allele) was added to the reaction mixture for a final concentration of 2 ng/µL. The inhibitor was added last to reach a final reaction volume of 20 µL. Control (non-inhibitor) samples were performed using the same protocol, with an equivalent volume of ddH_2O used in place of the inhibitor.

Cycling conditions for the reaction were as follows: an initial hold for 10 minutes at 95° C; then cycling for 20 seconds at 95 °C to denature, 20 second at an annealing temperature of 53° C, 55°C, or 58° C, depending on the melting temperature of the primer, and a 20 second extension at 72 °C. The melt cycle involved a 90 second pre-melt at a temperature of 72 °C followed by a temperature ramp from 72°C to 95°C, with a 5 second hold at each 1 degree step of the ramp.

Inhibitor Preparation

The inhibitor stock solutions were prepared as follows: hematin (ICN Biomedicals, Aurora, OH), 100 mM in 0.1 N sodium hydroxide (Fisher Scientific, Waltham, MA); calcium hydrogen phosphate (Aldrich, Milwaukee, WI), 100 mM in 0.5

N hydrochloric acid (Fisher Scientific); indigo (Tokyo Kasei Kogo Co, LTD, Tokyo, Japan), 100mM in 2 % Triton X(Sigma, St. Louis, MO); indigo carmine (MP Biomedicals, Aurora, OH), 100 mM in water; melanin (ICN Biomedicals), 1mg/mL in 0.5 N ammonium hydroxide (Fisher Scientific); collagen (from calf skin) (Sigma), 1 mg/mL in 0.1 N acetic acid (Fisher); humic acid (Alfa Aesar, Ward Hill, MA), 1 mg/mL in water; and tannic acid (Sigma), 1 mg/mL in water. All subsequent dilutions were prepared in water.

Inhibitor Concentrations

A range of concentrations was tested to determine the concentration of inhibitor that would produce a change in the signal output. The starting concentrations were based on previous work with these inhibitors, where the concentration required for allele dropout with the miniSTR primer sets was determined (6). These qPCR tests were conducted using a primer set producing a 200 bp amplicon with a Tm of 60 °C (Primer set 2). The final range of concentrations for each inhibitor is presented in Table 1.

Polymerase and Magnesium Tests

The maximum concentration of each inhibitor was used to test the effects of increased Taq polymerase and Magnesium. Three concentrations of Taq were tested: 1X, 1.5X, and 2X of the standard concentration (0.02 U/µL); and three concentrations of Magnesium were tested: 1X, 2X, and 3X of the standard concentration (62.5mM). Additionally, a range of Taq concentrations from 1/4X to 2 X were tested on non-inhibited DNA to determine the effect of lower Taq concentrations on amplification with the TH01 primers.

Data Analysis

In examining the mechanism of PCR inhibition on amplification by real time PCR, four effects were examined, amplification efficiency, product quantity, takeoff

cycle and melt curve. The first effect, differences in relative amplification efficiency were evidenced by changes in the slope of the exponential amplification curve compared to the non-inhibited control sample. The second effect was determined by the relative quantity of product. When the intensity of the qPCR amplification curve levels off at a lower relative fluorescence than the control, there is evidence of a limiting effect produced by a reduction in the availability of one or more of the components of the PCR reaction mixture (primers, Taq, magnesium, dye, or dNTPs). The third effect, a change in Ct value or "takeoff cycle," indicates a relative decrease in the amount of DNA template available for amplification. The fourth effect is the melt curve for the PCR products produced following the qPCR. A lower melt temperature for the amplified products indicates that the strength of the hydrogen bonding of the product has decreased. Melt curve analysis is generally used to determine a change in the sequence of the PCR product. In these studies, the DNA sequence was held constant while the inhibitor concentration was varied. Thus a change in the melt curve indicates the presence of inhibitor binding to the DNA.

A comparison between amplicons of different lengths (with the same melting temperature) and primer sets with different melting temperatures (with the same amplicon length) was made to determine the effect of size and primer melting temperature on PCR inhibition. A ratio of the Ct cycle between the inhibited sample and the uninhibited sample (Io/I) was calculated for each inhibitor concentration to determine the effect of the range of concentrations on the various primer sets.

Results and Discussion

The experimental design for this study utilized a series of primer sets to compare the effect of amplicon length and primer melting temperature (Tm). Three primer sets with the same melting temperature of 60°C producing amplicon lengths of

100 bp, 200 bp, and 300 bp were used to determine the effect of length on PCR inhibition. In addition, a second set of primers producing an amplicon length of 300 bp but with melting temperatures of 58, 60, and 62°C were used to determine the effect of melting temperature. Other primers producing 100 and 200 bp amplicons were not used as they were less efficient or did not produce clean amplification products. Overall, five primer pairs were selected. (Table 2). Seven inhibitors were examined and their effects on PCR amplification were determined using the real time system.

Calcium

Calcium, a major inorganic component of bone (5) was the first inhibitor examined. Inhibition by calcium reduced the efficiency of the amplification, showed evidence of limiting reagents, and produced no change in the melt curve for all primer sets. (Figure 2) Addition of magnesium and Taq polymerase up to three times the normal concentration produced a minor increase in the amplification efficiency. There was no difference in Ct for the different size amplicons or the primer sets with different melting temperatures. These results were consistent with our expectation that calcium is a Taq inhibitor, competing with magnesium and reducing the reaction efficiency and total amount of product.

Humic Acid

Humic acid is a component in soils (15) and may be encountered in samples that have been buried, particularly in skeletal remains. Inhibition by humic acid did not reduce the efficiency of the amplification or show evidence of limiting reagents (Figure 3). However, a change in the melt curve was observed for the two larger amplicons and for all primer sets there was an increase in the Ct cycle as the concentration of inhibitor rose. The smallest amplicon dropped out at the lowest inhibitor concentration and additional Taq or magnesium did not relieve inhibition. These results indicate that

humic acid inhibits the PCR reaction through sequence specific binding to DNA, limiting the amount of available template.

Collagen

Collagen is a component in connective tissue and bone (16), and may be encountered in DNA extracts from skeletal samples. Inhibition by collagen reduced the amplification efficiency, and produced a change in the melt curve for all primer sets. There was slight reduction in Ct with inhibitor concentration for all amplicons, although the larger amplicons required higher inhibitor concentrations for the Ct to increase. Interestingly, for the larger amplicons, a loss of signal was observed during later cycles, presumably due to fluorescent quenching (Figure 4). Additional Taq and magnesium did not appear to improve amplification of inhibited samples. Collagen, different from humic acid, appears to bind DNA but does not alter the availability of DNA template. Instead the binding appears to affect Taq processivity.

Melanin

Melanin is a pigment found in hair and skin, and is a possible inhibitor present in telogen hair samples (17). No change in efficiency, melt curve, or Ct cycle was observed for the smallest amplicon with the addition of melanin to the reaction mix. For all other amplicons, a loss of signal occurred at the highest inhibitor concentrations, an increase in the Ct cycle with inhibitor concentration was observed, and melt curve effects were observed (Figure 5). The 100 bp amplicon was less affected by inhibition than the larger two amplicons, and the 60 Tm amplicon required a higher inhibitor concentration to produce a change in the Ct cycle. Additional Taq and magnesium did not improve amplification for inhibited samples. Thus melanin, like humic acid inhibits the PCR reaction through sequence specific binding to DNA, limiting the amount of

available template. Smaller amplicons appear to be less inhibited by this material presumably due to fewer binding sites.

Hematin

Hematin is a metal chelating molecule found in red blood cells (18), and may be encountered in dried blood stains. Inhibition by hematin produced a reduction in final product formation (limiting effect) for all amplicons. A shift in the Ct cycle at high inhibitor concentrations was observed for all but the smallest amplicon, and melt curve changes were observed for all of the larger amplicons. The larger amplicons were also affected by inhibitor concentration sooner than the small amplicon, and the amplicon with the lowest Tm appeared to be the least affected by inhibition (Figure 6). Additional Taq did not reduce inhibition by hematin, but additional magnesium increased the effects of inhibition in samples with hematin. Based on the fact that there is minimal shift in the template melt curve we believe hematin to be a Taq inhibitor.

Tannic Acid

Tannic acid is an agent found in leather, as well as in some types of plant material (20). It may be also be encountered in samples which have been exposed to leaf litter. No change in the melt curve was observed for samples inhibited with tannic acid for any of the primer sets (Figure 7). The smallest amplicon and lowest melting temperature primer set did not produce a Ct shift in the presence of tannic acid, however a Ct shift was observed for the larger amplicons. Some loss of product through limiting effects was observed for all primer sets but there was no significant change in reaction efficiency. Additional Taq and additional magnesium did relieve inhibition by tannic acid. Tannic acid thus appears to be a Taq inhibitor that also affects availability of the DNA template.

Indigo

Indigo is a dye used in certain types of fabrics, and this inhibitor may be encountered in DNA extracted from stains on denim or other dyed fabrics (19). Analysis of this inhibitor by qPCR proved to be problematic. Amplification could not be detected by the instrument due to interference by the dark blue color of the reaction mixture. It was decided that this was not a realistic representation of an inhibited sample, and the real time results indicated a loss of efficiency that was possibly related to the quenching of the dye.

Overall Results

The results of these experiments indicate that there are major differences in the mechanism by which different inhibitors affect the PCR reaction (Table 3). Some of the inhibitors, such as calcium and tannic acid, appear to be interacting with the polymerase. This is evidenced by the improvement in amplification with additional Taq enzyme, indicating a competitive inhibition reaction. Calcium, a divalent cation, is likely acting as a competitive inhibitor to magnesium, a cofactor for the polymerase enzyme. However, the addition of increased levels of magnesium to the reaction mixture does not relieve the inhibition. Tannic acid inhibition is reduced with both the addition of Taq and magnesium. Tannic acid contains a large number of electronegative groups, and could be chelating the magnesium which would render the Taq inoperable. The improvement of the reaction with an excess of magnesium supports this hypothesis. Humic acid produces both a shift in the Ct cycle and a melt curve change. For this substance both amplicon size and primer melting temperature affect the level of inhibition. This inhibitor is binding to the DNA and the effect is related to sequence and the strength of the hydrogen bonds in the amplicon.

Other inhibitors, such as hematin and melanin, appear to affect the processivity (rate of extension) of the DNA polymerase during primer extension. For these

compounds, the larger size amplicons are more sensitive to inhibition than smaller ones, indicating that the polymerase is being affected during primer extension, Since a change in the melt curve is also observed for these inhibitors, it is probable that inhibitor is binding to the DNA rather than the polymerase. While tannic acid also produces a Ct shift (and loss of available DNA template) for the larger amplicons, it does not affect the melting temperature (Figure 6). This indicates that the inhibitor is binding taq instead of the DNA.

Collagen appears to be binding to the DNA due to a melt curve shift, but the larger amplicons are less affected. In addition, the signal from the amplified samples decreases with the number of cycles, which indicates some sort of effect (quenching) of the reaction. A possible explanation for this is that the collagen is overwhelming the DNA and reducing the signal obtained from the intercalating dye. The smaller amplicons would be more likely to be overwhelmed due to the size of the collagen molecule in comparison to the size of the amplified DNA of the smaller amplicon.

Hematin and indigo, as well as the highest concentrations of tannic acid and melanin, had melt curves where incomplete melting was present (the signal never reaches baseline at low temperatures). This same phenomenon, as well as the lower maximum level of amplification associated with limiting effects, was observed for lower concentrations of SYBR Green in uninhibited samples (Figure 7). This suggests that these inhibitors function in such a way to limit the incorporation of the dye in the DNA strand, or have a quenching effect on the dye itself.

A summary of all results and effects is listed in Table 4.

Conclusions

A variety of inhibition mechanisms have been observed in the analysis of the inhibition of PCR by a variety of known inhibitors, and some inhibitors, such as tannic

acid, appear to affect the reaction in more than one manner. While smaller amplicon size does appear to be advantageous in the propensity of inhibition for some compounds, this is not a consistent rule for all inhibitors. Thus the hypothesis that reduced sized amplicons are more efficient in amplifying samples that are inhibited is not always correct.

For those amplicons with higher primer melting temperatures, the sequence of the amplicon as well as the primer is likely to determine the level of inhibition for those inhibitors which bind (intercalate) with the DNA. For those inhibitors which are interfering with the Taq, the addition of PCR components such as Taq or magnesium may alleviate the problem, but the extent to which this will help may vary. While an understanding of the mechanism of these inhibitors can help the analyst in attempts to alleviate the problem, an identification of the inhibitors present and their relative concentrations are necessary to effectively address the problem. Identification of possible inhibition can not always be made by visual inspection, but the qPCR data can indicate the presence of these inhibitors.

With the exception of calcium and collagen, additional BSA can often relieve inhibition when added to the PCR reaction (6). Sample dilution is also a useful technique but will further reduce template concentration. Other treatments, such as rinsing the sample with NaOH (1) or purification with silica based spin columns(2) or agarose (6) result in a loss of DNA template (21)

Overall, knowledge of the type of inhibitor present, especially melt curve data from SYBR green based qPCR data, should help the analyst select the best method to effectively remove inhibitors without compromising the amount of DNA or further compromising the PCR reaction. This knowledge will also help the analyst determine

the type of STR analysis to use, and if reduced sized amplicons will improve their results.

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Inhibition tables

Inhibitor	Units	1	2	3	4	5	6	7
Calcium	uM	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Humic Acid	ng/uL	0.5	1	1.5	2	2.5	3	3.5
Collagen	ng/uL	16	20	24	28	32	36	40
Melanin	ng/uL	1	1.5	2	2.5	3	3.5	4
Hematin	uM	1.5	1.75	2	2.25	2.5	2.75	3
Indigo	uM	100	150	200	250	300	350	400
Tannic Acid	ng/uL	1.5	2	2.5	3	3.5	4	4.5

Table 1 Final inhibitor concentrations for the 20 µL reaction mix

Size/ Tm	Primer	Sequence
100 bp (Tm 60)	Forward	5’-AAATAGGGGGCAAAATTCAAAG-3’
	Reverse	5’-CACAGGGAACACAGACTCCAT-3'
200 bp (Tm 60)	Forward	5’-ATTGGCCTGTTCCTCCCTTA-3’
	Reverse	5’-CAAGGTCCATAAATAAAAACCCATT-3’
300 bp (Tm 60)	Forward	5’-GCAAAATTCAAAGGGTATCTGG-3’
	Reverse	5’-GGAAATGACACTGCTACAACTCAC-3’
300 bp (Tm 58)	Forward	5'-ATAGGGGGCAAAATTCAAAG-3'
	Reverse	5'-CCTGTGTCCCTGAGAAGGTA- 3'
300 bp (Tm 62)	Forward	5’-AAATTCAAAGGGTATCTGGGCTCT-3’
	Reverse	5’-ACCTGGAAATGACACTGCTACAAC-3’

Table 2 Size (approximate), melting temperature, and sequences for the final five TH01 primers

Inhibitor	Melt	Efficiency	Limiting	Ct Shift	Other
Calcium		all	all		
Hematin	6	1,2,6,9	all	2,3,6,9	
Melanin	3,6		6	2,3,9	
Humic Acid	2,3,6,9		6	all	
Collagen	2,3,6,9	1,2,3	1,6	1,3,6,9	3,9~
Tannic Acid				2,3,9	
Indigo *					2*

~ loss of intensity in later cycles

* only one primer kit tested with indigo due to dye effect

Table 3: Summary of effects on qPCR for the five primer sets and seven inhibitors Primer sets: 1-100 bp Tm 60; 2 – 200 bp Tm 60; 3- 300 bp Tm 60; 6 – 300 bp Tm 58; 9 – 300 bp Tm 62.

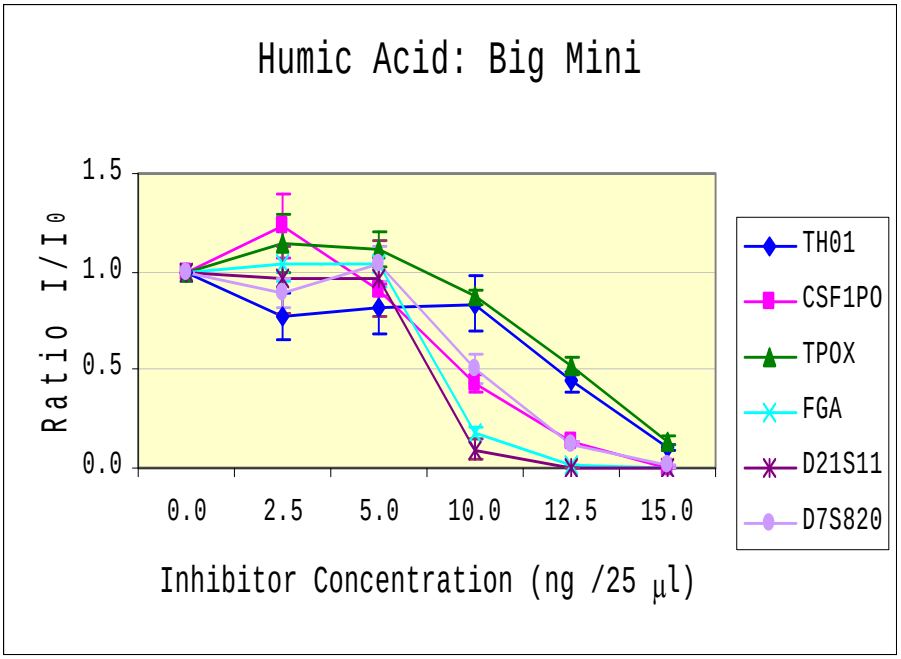

Figure 1: Inhibition by humic acid in amplification of various STR loci in the Big Miniplex STR kit. DNA samples were spiked with different concentrations of humic acid ranging from 0-15 ng/25 µL. I/I_0 is the ratio of signal with inhibitor in the sample to the signal without inhibitor in the sample. TH01 and TPOX are inhibited at a higher concentration than the other 4 loci. These two loci have the highest primer melting temperatures of the set. This suggests that the inhibitor is binding to the DNA and is displaced by the primers due to higher bond strength of the primers. (6)

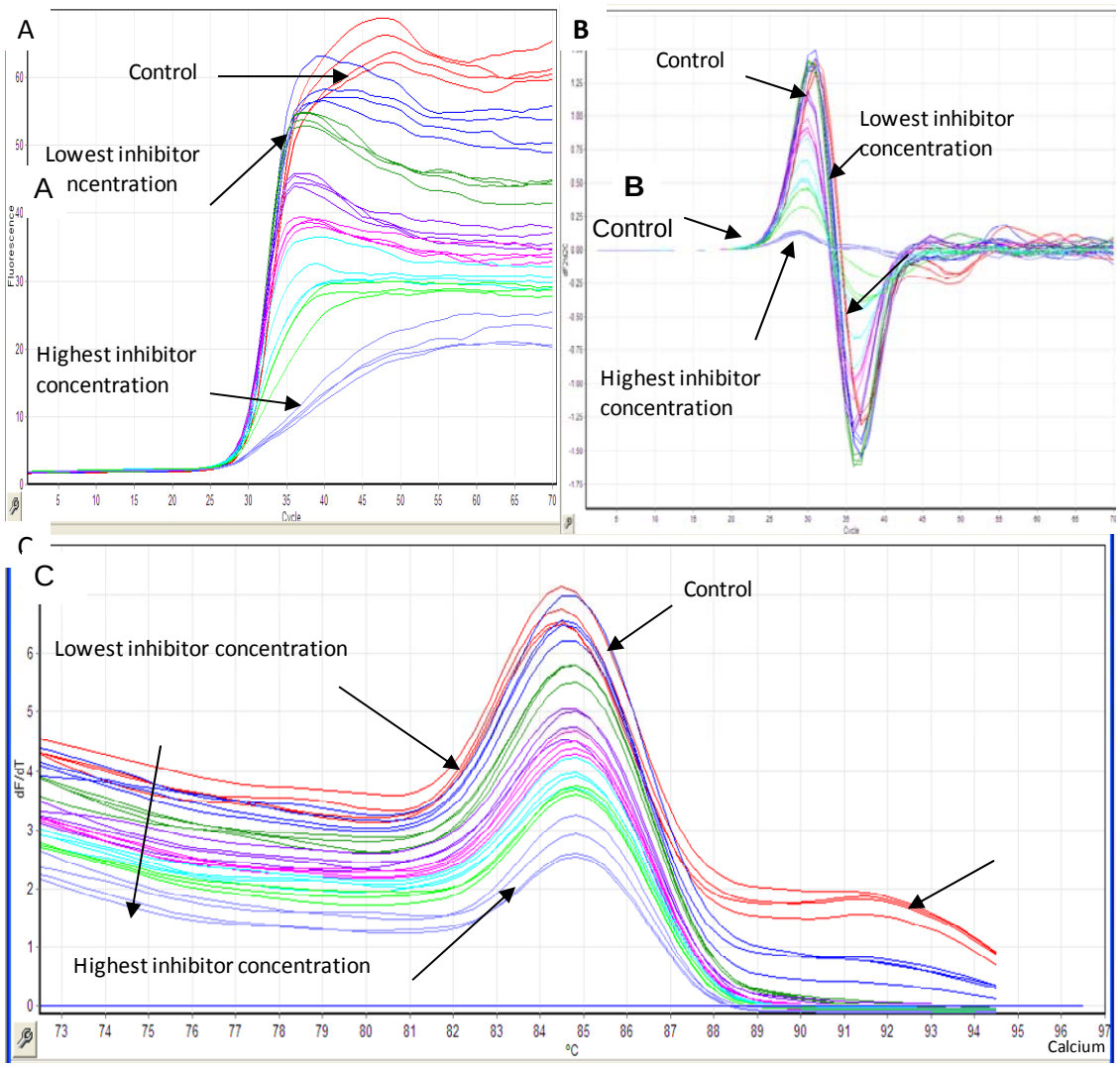

Figure 2: Real time data showing the effect of varying levels of calcium added to the 100 bp primer set (see Table 1 for concentrations). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there is little effect on the takeoff cycle (Ct), however the efficiency of reaction (slope of exponential amplification curve) changes greatly as does the final product concentration; In figure 2C, the DNA melt curve shows little if any effect with added calcium. These results are consistent with calcium's role as a Taq inhibitor

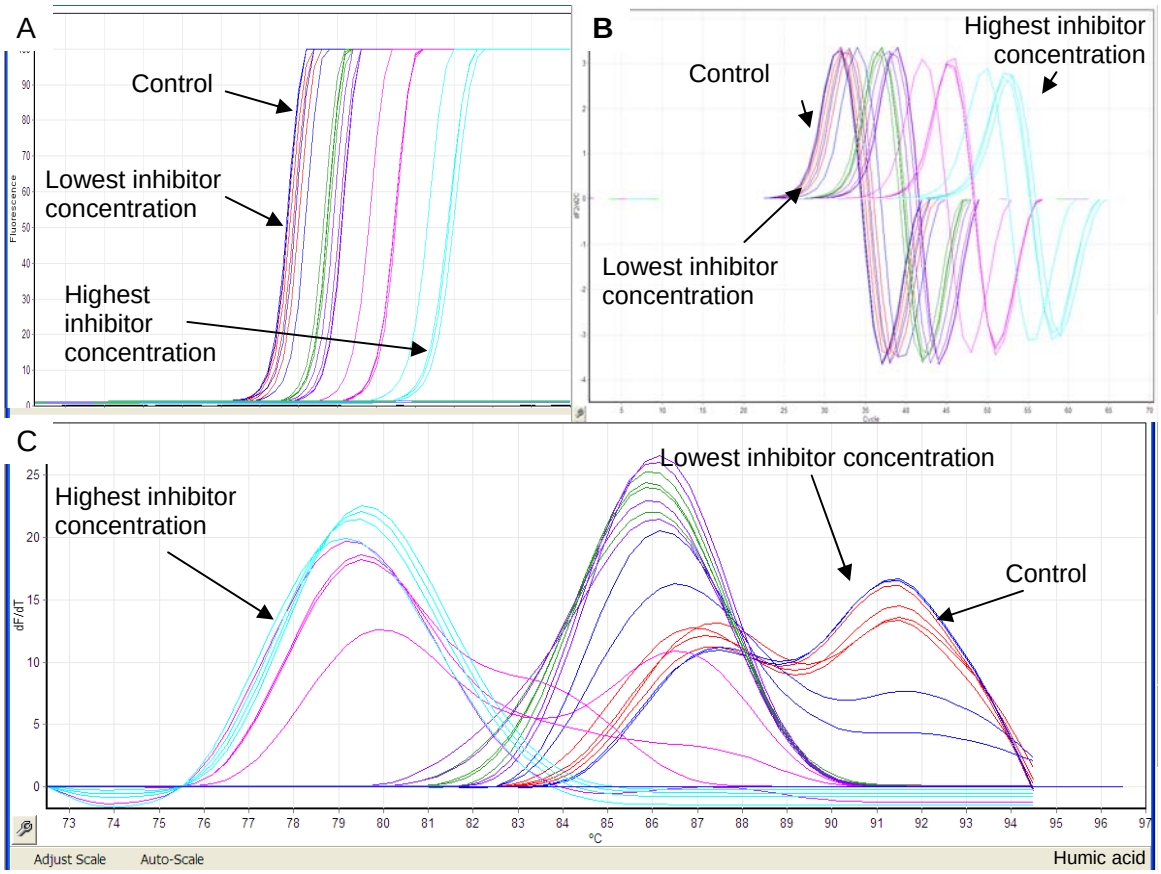

Figure 3: Real time data showing the effect of varying levels of humic acid added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there shift in the takeoff cycle (Ct), however the efficiency of reaction (slope of exponential amplification curve) does not change, nor is there any major loss in product; In figure 2C, the DNA melt curve shows extensive changes with inhibitor concentration. These results are consistent with humic acid inhibiting the PCR through binding the DNA and reducing the amount of available template.

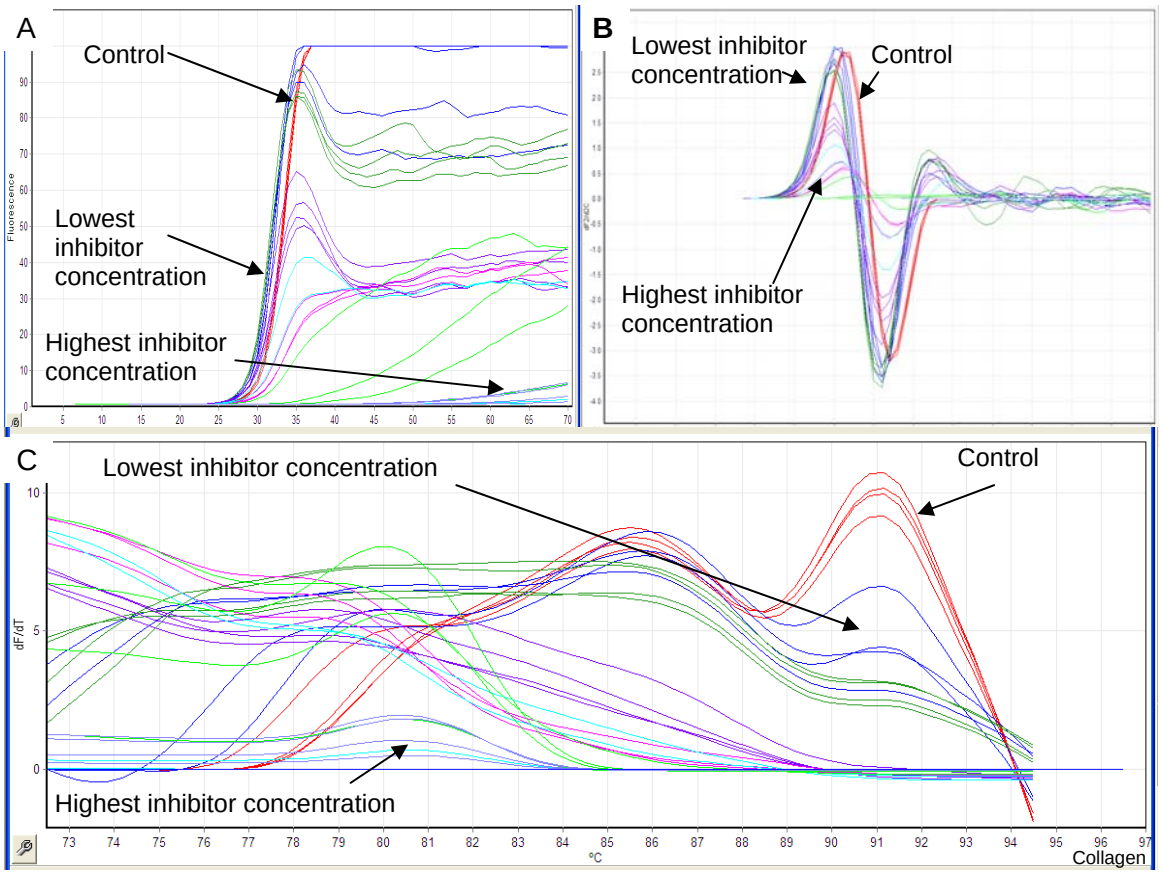

Figure 4: Real time data showing the effect of varying levels of collagen added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there in little effect on the takeoff cycle (Ct), however the efficiency of reaction (slope of exponential amplification curve) changes greatly as does the final product concentration. In addition, a drop off in fluorescence occurs over time. In figure 2C, the DNA melt curve changes at higher levels of inhibitor. These results are consistent with Taq inhibition, but unlike calcium, there is also some binding to the DNA template at later stages of the reaction and higher inhibitor concentrations.

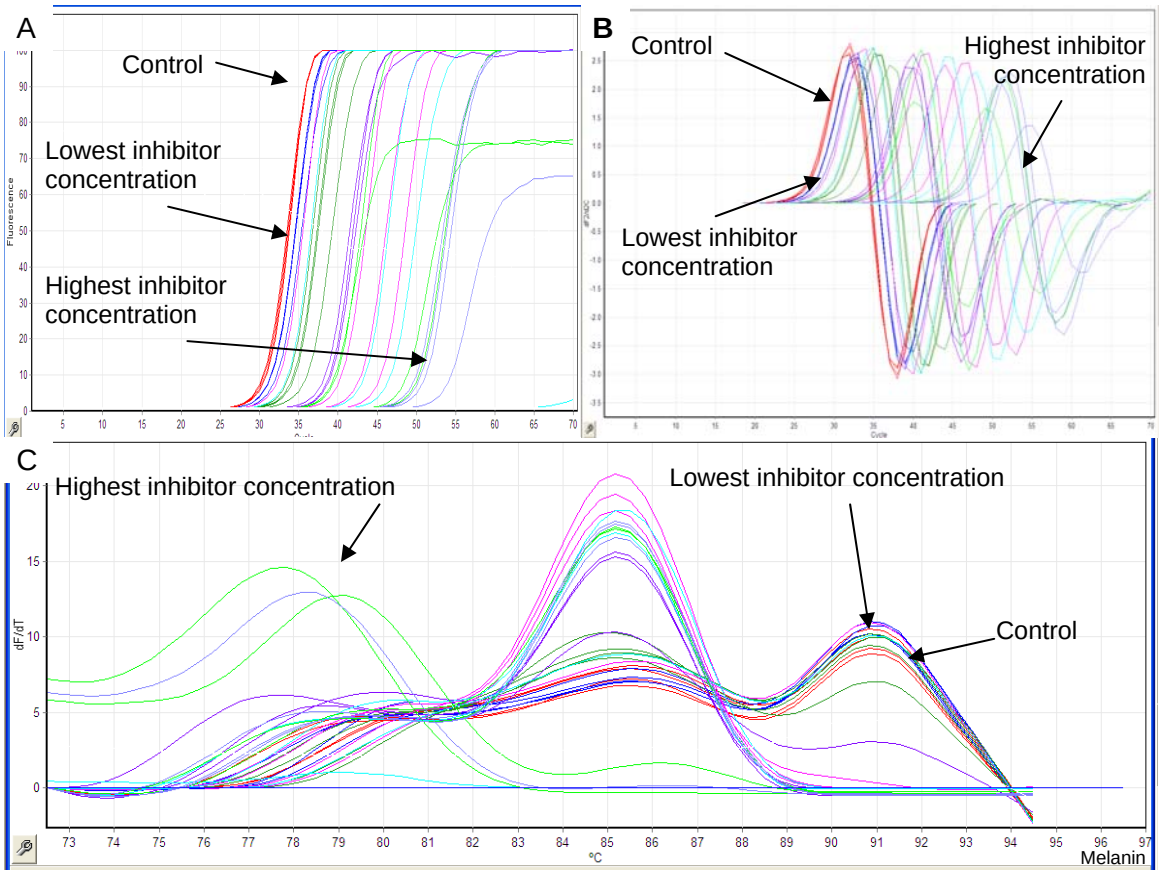

Figure 5: Real time data showing the effect of varying levels of melanin added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there is a strong effect on the takeoff cycle (Ct), however the efficiency of reaction (slope of exponential amplification curve) undergoes little change with [inhibitor]; In Figure 2C, the DNA melt curve shows three transitions as the [inhibitor] increases. These results are consistent with melanin inhibiting the PCR through binding the DNA and reducing the amount of available template.

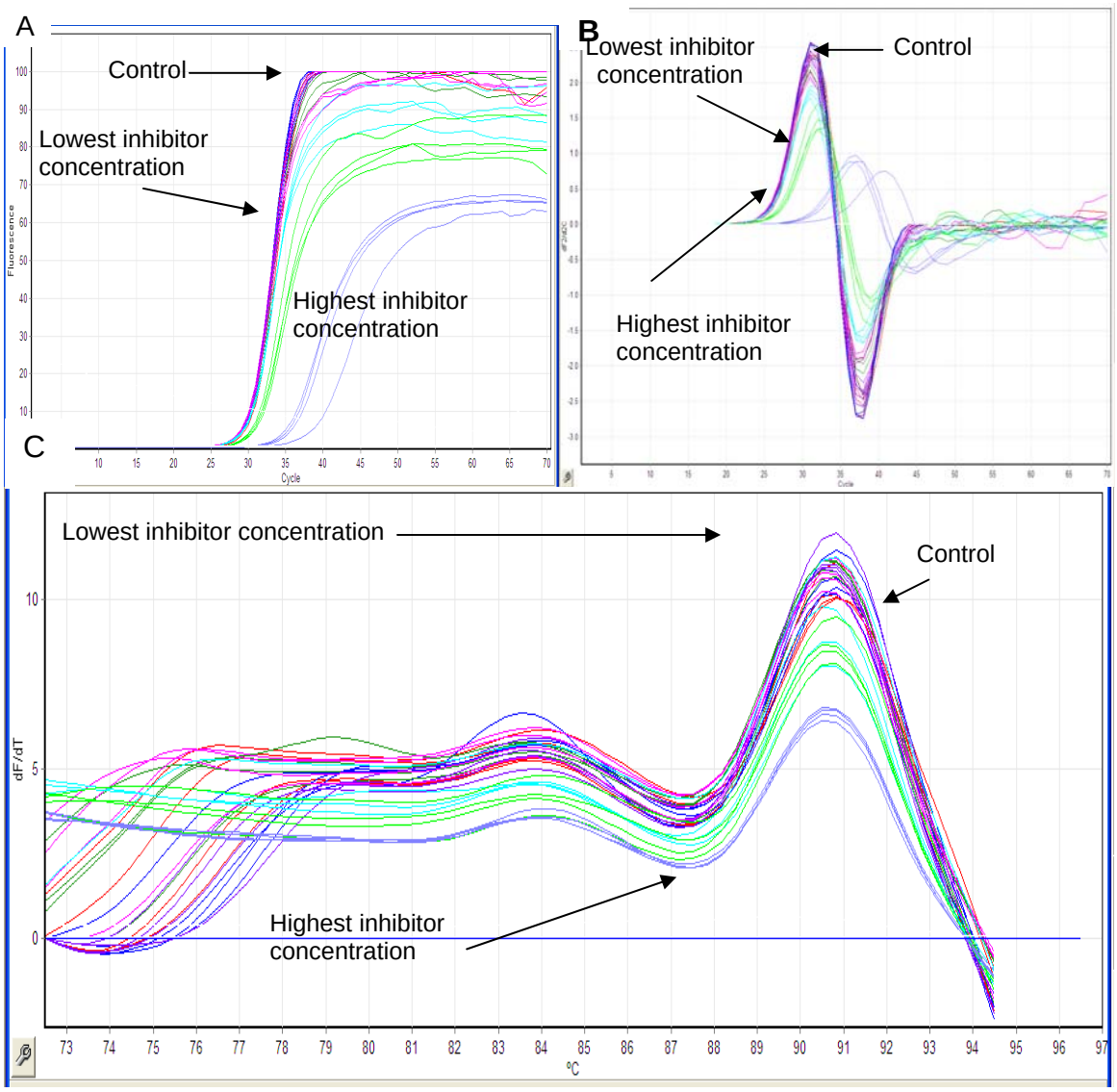

Figure 6: Real time data showing the effect of varying levels of hematin added to the 200 bp primer set (Set 2) (see Table 1 for concentrations). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there is an effect on the takeoff cycle at high inhibitor concentrations(Ct), as well as effects on the efficiency of reaction (slope of exponential amplification curve) and the production of PCR product; In Figure 2C, the DNA melt curve shows minimal effects with increasing [inhibitor]. These results are consistent with hematin as a Taq inhibitor and also show its ability to reduce PCR product formation.

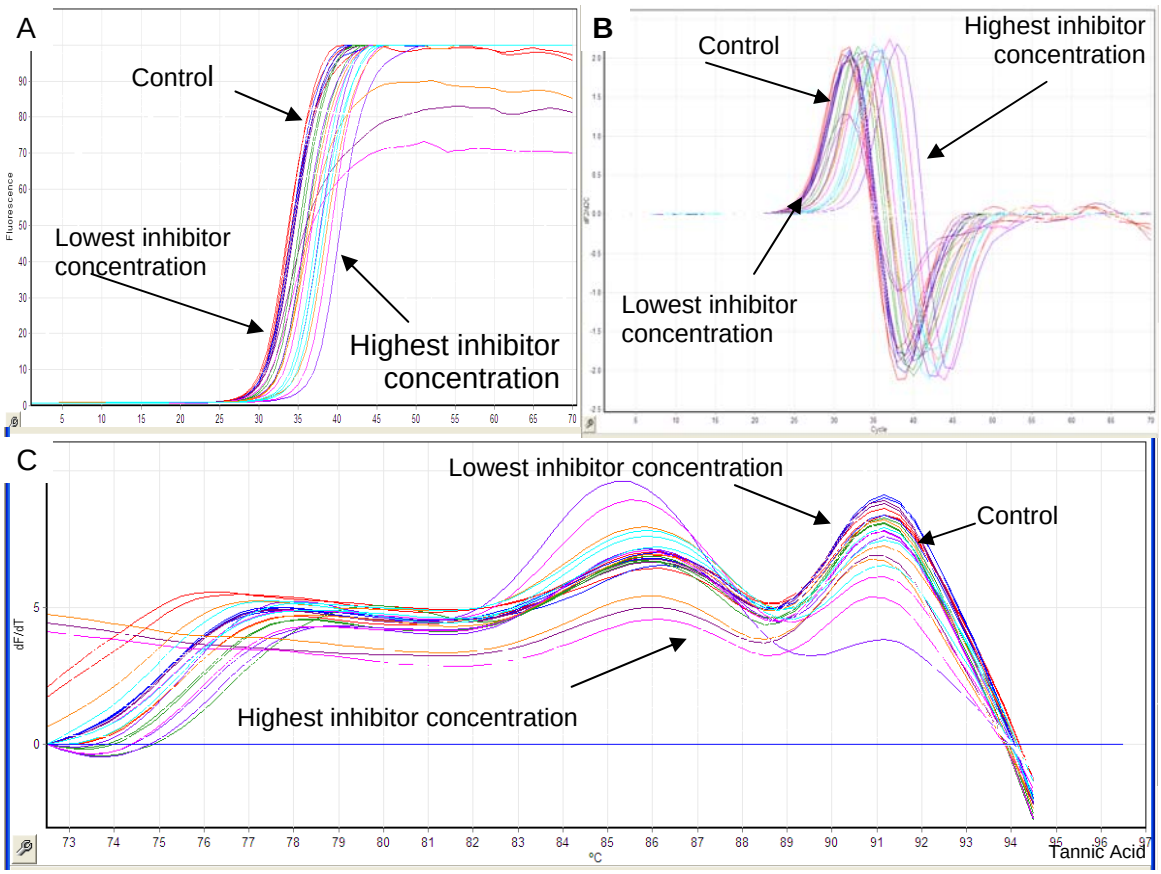

Figure 7: Real time data showing the effect of varying levels of tannic acid added to the 300 bp primer set (Set 3) (see Table 1 for concentrations). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there is an effect on the takeoff cycle (Ct), however the efficiency of reaction (slope of exponential amplification curve) does not change; In figure 2C, there are very minor DNA melt curve minor effects with added calcium. These results are consistent with tannic acid affecting the quantity of available DNA template.

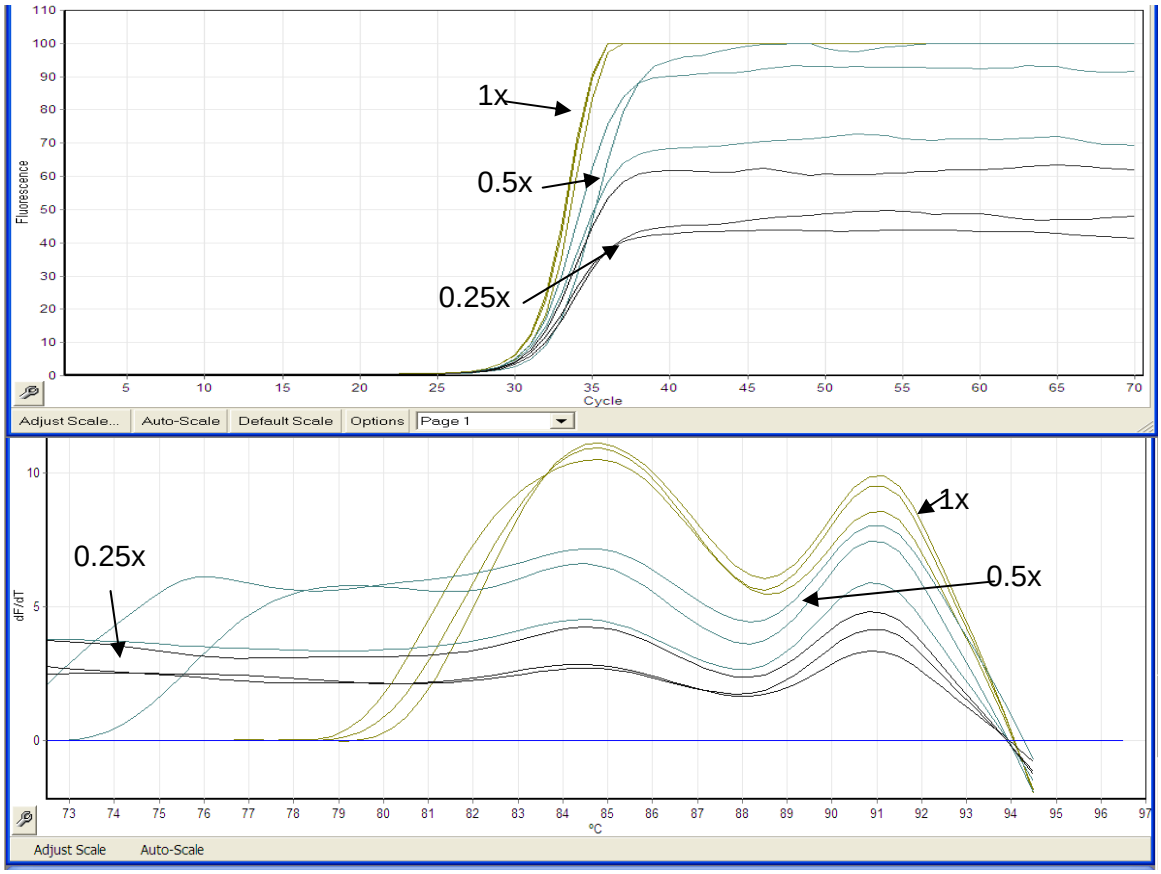

Figure 8: Real time data showing the effect of varying levels of SYBR Green added to the primer set 2 (200 bp). (A) Real time amplification curve (B) comparative quantitation (first derivative of A) (C) and product melting temperature analysis. As seen in plot A, there is little effect on the takeoff cycle (Ct), however the efficiency of reaction (slope of exponential amplification curve) changes as does the apparent final product concentration; In figure 2C, the DNA melt curve shows minor effects as the [SYBR Green] is dropped. As SYBR green is the visualizing agent for all reactions, these data indicate a potential effect that could occur if inhibitors block the interaction of SYBR green with product.

6. The effect of DNA degradation on STR profiles

A recurrent problem in forensic DNA analysis is the presence of DNA degradation in extracted samples. Electropherograms of degraded samples commonly show a ski slope effect, with loss of larger alleles and imbalance of smaller ones. A variety of different mechanisms have been suggested to account for DNA degradation, including the release of nucleases from putrefying cells, bacterial decomposition, and radiative crosslinking. Furthermore, oxidation, deamination, depurination and other hydrolytic processes can also lead to destabilization and breaks in DNA molecules (1). The major site of oxidative attack on the DNA bases is the C=C double bond of pyrimidines, and purines, leading to ring fragmentation and base modifications (2). Many of these oxidized base products will block replication, negatively impacting amplification with the standard Taq-DNA polymerases used in PCR (3).

DNA damage occurs in three primary ways: through hydrolytic cleavage, through oxidative damage to bases, and through radiative crosslinking of purines. While there have been a number of papers and reports suggesting potential mechanisms to repair damaged forensic DNA (3-5), there has been very little research on methods to detect the actual damage to degraded forensic DNA (6-9). In particular, there has been little work done examining oxidative damage in forensic samples, in spite of the fact that such damage is well documented in a number of disease processes (10).

Modified purine and pyrimidine bases constitute one of the major classes of oxidative DNA damage. Guanine nucleobases are frequently targeted by oxidants due to the fact that their oxidation potential is the lowest among the DNA bases. 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-OH-dG), is an adduct for which specific cellular repair enzymes exist and it has been shown to cause GC→TA transversions. Its presence in

DNA causes mutations resulting in mispairing and multiple amino acid substitutions (11). As such, the detection of this oxidative product provides a bellwether for the presence of oxidative DNA damage.

Various techniques exist for the detection of 8OHdG. The three most commonly used methods are: (i) high performance liquid chromatography coupled with electrochemical detection (HPLC-EC), (ii) gas chromatography coupled with mass spectrometry (GC-MS), and (iii) immunometric detection (12-16). Many laboratories studying DNA oxidation use enzymatic digestion of the DNA oligomer followed by HPLC-EC analysis of the individual bases. This technique is highly selective for 8OHdG since other, non-oxidized bases will not produce a signal. A further advantage of this technique is that it also permits quantitative analysis of the individual bases by HPLC/UV or mass spectrometry, facilitating the determination of additional oxidative lesions (17-19). Levels of 8OHdG in cells, tissues, and whole animals have been reported as an important biomarker for oxidative stress when evaluating pathological diseases. (2) Thus it is likely that this compound may provide insight into the relative amount of oxidative damage to target tissues used in forensic STR and mitochondrial analysis. The aim of this study was to evaluate the relative contribution of oxidative damage and hydrolytic damage to DNA by determining the 8OHdG concentration in DNA from both degraded and non-degraded biological samples, and comparing these data with amplification success using multiplexed STR typing.

Material and Methods

Chemical and Reagents

The following biological enzymes were used in the study: deoxyribonuclease I and nuclease phosphate Type IV phosphodiesterase I (Sigma Aldrich, St Louis, MO). alkaline phosphatase (AP) and phosphodiesterase II (Worthington, Lakewood, NJ)

proteinase K (USB, Cleveland, OH) nuclease P1 (Roche Diagnostics, Indianapolis, IN) phosphodiesterase I (Crotalus Adamenteus Venom - Worthington) ribonuclease A (RNase A), and ribonuclease T1 (Sigma-Aldrich St. Louis, MO). Chemical reagents included trisma base, EDTA, sodium dodecyl sulfate (SDS), 8-hydroxy-2'-deoxyguanosine (8OHdG), HPLC-grade methanol, absolute ethanol, chloroform–isoamyl alcohol, 24:1, 3% hydrogen peroxide, and $Fe(NH_4)_2(SO_4)_2.6H_2O$, all purchased from Sigma–Aldrich, St. Louis, MO, USA. Microcon devices (YM-10) were purchased from Millipore, Bedford, MA, USA. Sodium hypochlorite was prepared from commercial bleach at a concentration of 6% (w/v).

DNA Extraction

Human tissue samples were collected from 4 different individuals at the Forensic Anthropology Center of The University of Tennessee. These tissue samples were skin and muscle collected from the upper back from bodies placed on the surface under a natural canopy of trees. Following collection, samples were immediately frozen and stored for later extraction. Over the collection period of 0-4 weeks, from mid to late summer in Knoxville, TN, remains were exposed to a range of temperatures and were shaded part of the day. For DNA extraction, frozen human tissue (1 g) was thawed, and homogenized under liquid nitrogen using a 6750 freezer mill (Spex Certiprep, Inc., Meruchen, NJ). The milling cycle began with 10 min of pre-cooling followed by 3 cycles of 2 min grinding and 2 min resting. An impact frequency of 15 was used. After homogenization the mixture was digested using 4 mL of stain extraction buffer (10 mM Tris-Cl pH=8, 100 mM NaCl, 39 mM dithiothreitol, 10 mM EDTA, 2% SDS) 300 µL RNase A (1 mg/mL), 1 µL RNase T1 (500 U/µL) and proteinase K (50 µl of 20 mg/mL) and incubated overnight at 38 °C with agitation. DNA was extracted with 4mL of 24:1 chloroform:isoamyl alcohol. For each extraction step, vigorous shaking for 30 s was

followed by centrifugation at 13,000 rpm for 15 min to separate the phases. 350 μL of 3 M sodium acetate was added to the isolated aqueous phase. The solution was then precipitated by an equal volume of cold absolute ethanol and stored at -20 °C overnight. The DNA was pelleted by centrifugation at 13,000 rpm for 15 min. The pellet was washed with 70% ethanol (4 °C) to remove salt and diluted in 700 μL of distilled water.

Blood and buccal swab samples from living individuals were also collected and examined in this study. Organic extraction of DNA was performed as mentioned above.

Chemical Oxidation

To verify the ability of the HPLC-EC system to detect oxidative damage, a series of reactions were performed on DNA extracted from human blood and buccal swabs as well as from bovine tissue using either H_2O_2 or NaClO. The reactions were performed at 37 °C with 0.3% H_2O_2 using 100 μg of total DNA and incubated for 1 hour. To increase the rate of oxidation, DNA samples were also treated with 0.3% H_2O_2 in a solution containing 0.05 M $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.1 M HEPES for both 1 h and 3 h at 37 °C. All experiments were performed in triplicate. In order to obtain a tissue control, 500mg of bovine tissue was also treated with 1% H_2O_2 or 2% NaClO at 37 °C for 18 h. DNA from these samples was then extracted using the above organic extraction method. To verify that the chemical oxidation step also affected recovery of amplified STRs, replicate samples of 100 μg of DNA extracted from blood was incubated in 1% H_2O_2 or 0.6% NaClO under gentle shaking at 37 °C overnight. In order to remove excess chemical oxidants, all treated DNA samples were further purified by running them on an 0.8% agarose gel and excising the oxidized fragments. The purified samples were then quantified by real time PCR and amplified using the Powerplex® 16 STR kit (Promega).

Enzymatic Digestion

We examined and optimized two different hydrolysis reactions to obtain a complete DNA digestion prior to HPLC analysis. The initial hydrolysis protocol (20, 21) was performed using 100 μg of human DNA at a concentration of 0.5 μg/μL in 10 mM Tris-HCl (pH=7.4). The sample was denatured at 95 °C for 15 min before digestion. Samples were first treated with phosphodiesterase I type IV (0.5 U) and phosphodiesterase II (0.2U) at 50 °C for 1 h in a pH=7.4. The sample was then treated with 10 μL of 1 M Tris-HCl (pH=8) and further digested with 10 μL of alkaline phosphatase (1 U/μL) at 37 °C for 1 h. Finally, the reaction mixture was purified using a YM-10 microcon to remove enzymes prior to HPLC injection.

A second, alternative procedure was developed to improve the enzymatic digestion and increase the yield of the individual nucleotide bases (22). Extracted DNA samples at a concentration of 0.5 μg/μL were diluted in 10 mM Tris-HCl (pH=7.4), containing 100 mM NaCl and 10 mM $MgCl_2$, and treated with 40 U of DNaseI at 37°C for 30 min. The pH was then adjusted with 1 μL of 3 M sodium acetate (pH=5.2), and the fragmented DNA was further digested with 1 μL of Nuclease P1 (1U/1 μL) at 37°C for 1 h. Next 10 μL of 1 M Tris-HCl (pH=8) and 1 μL of alkaline phosphatase (1 U/μL) were added, followed by a 1 h incubation at 37°C. Finally, 1 μL of phosphodiesterase I type IV (0.05 U/μL) and 1 μL of phosphodiesterase II (0.02 U/μL), were added to the reaction mixture at 37°C for an additional 1 h to ensure the completeness of the DNA digestion. Following a total digestion time of 3.5 h, the reaction mixture was purified with a YM-10 microcon to remove enzymes prior to HPLC injection.

To compare and quantitate the individual bases obtained following the hydrolysis reactions, a set of standards was prepared through the hydrolysis of 200 μM of dATP,

dCTP, dGTP and dTTP. The hydrolysis reactions with the dNTPs were performed under the same conditions as the DNA samples, with the omission of DNaseI.

High Performance Liquid Chromatography

Fifty μL of hydrolyzed DNA was analyzed by HPLC coupled with dual UV and EC detectors. The HPLC system consisted of a pump (SP8800, Spectra Physics) autosampler (Model SP8880), and a programmable UV/VIS detector (Model 783 Programmable Absorbance Detector, Applied Biosystems). An electrochemical analyzer (Model 800B series, CH instruments) was linked to the system. The HPLC column used was an XBridge™ C18, 5 μm (Waters). The mobile phase consisted of 7.5% aqueous methanol containing 50 mM KH_2PO4 buffer (pH=5.5) and used a 1 mL/min flow rate. Normal nucleosides (dC, dT, dG, dA) were detected by the UV absorption at 260 nm. The electrochemical detection of 8OHdG was performed using an amperometric cell that was fitted with one glassy carbon working electrode, stainless steel auxiliary electrode and Ag/AgCl reference electrode. The detector was operated at a potential of 600 mV vs. Ag/AgCl. Overall, a linear relationship existed between detector response and concentration for 8OHdG (1 nM - 50 nM) by HPLC/ECC detection and for dG (20 μM - 200 μM) by HPLC/UV. The oxidative damage was expressed as a ratio 8OHdG/10^6 dG.

STR-PCR Amplification

A subset of the samples tested for the presence of 8OHdG was also examined to determine the effect of oxidative treatments on amplification success. Both oxidized and non oxidized DNA samples were examined. 200 pg of DNA was used in the STR-PCR analysis. Prior to amplification the DNA was quantified using a multicopy Alu-based real time PCR protocol with a RotorGene RG3000 cycler (Qiagen) with Sybr green detection (23). All samples were amplified using the Powerplex® 16 system following the parameters specified in the technical manual in a total reaction volume of 12.5 μL

with 0.5 μg nonacetylated BSA added to improve the detection of degraded/inhibited DNA (24). Reaction products were analyzed using an ABI PRISM 310 Genetic Analyzer and the Gene Scan ILS 600 size standard.

Results and Discussion

HPLC UV-EC

The goal of this paper was to develop a method to determine the relative effect of oxidation on a forensic DNA sample and to compare these results with natural degradation processes. In the DNA degradation process, large oligomers gradually break down into smaller and smaller pieces. The processes involved in this destruction are digestion via cellular and bacterial nucleases, oxidation, and hydrolysis. Oxidative damage is an alternative mechanism for DNA damage (25). Here DNA becomes unreadable due to the inability of the enzyme to read and copy the DNA sequence. Since oxidation is frequently mentioned as one of the processes leading to DNA degradation (26,27), we felt that it was important to develop a method to directly measure this process in forensic samples. Due to their structure, guanine bases will be the first to oxidize if this type of damage occurs, so 8OHdG was a logical target to detect DNA oxidation in such samples.

To perform this type of measurement, it is first necessary to completely digest the sample and then measure the relative amount of 8OHdG to dG by HPLC with electrochemical detection. While other techniques such as immunoassays can be used to determine the presence of 8OHdG, the advantage of the HPLC procedure is that it facilitates downstream analysis of other types of base damage via mass spectrometry or HPLC/UV (28). In our experiments we used HPLC/UV detection to provide feedback on the quality of the digestion. This test is not possible with immunoassay techniques.

Enzymatic digestion of DNA depends on the enzyme activity, the amount of DNA used, and factors such as time and temperature. In our study, two different protocols for different amounts and kind of enzymes were examined (29). The degree of DNA digestion was determined by examining the peak areas of the individual bases produced following the digestion using HPLC with UV detection, Figures 1,2. The elution times for the normal DNA nucleosides were as follows: dC, 2.9 min; dG, 5.3 min; dT, 6.9 min; dA, 12.5 min. In all cases, 2'-deoxyinosine (di) was also observed, eluting at 5.2 min. This compound is produced by deamination of 2'-deoxyadenosine by deaminases present in commercial alkaline phosphatase preparations (30).

Figure 1 shows the UV and EC separation profiles of normal nucleosides and 8OhdG, respectively, for the saliva and blood DNA samples that were digested with the first hydrolysis protocol. With this procedure, a full profile of all DNA nucleosides was not obtained due to an excessive concentration of alkaline phosphatase (10 U) and an insufficient concentration of hydrolytic enzymes. Using HPLC-UV detection, low levels of nucleosides (dC, dG, dT, dA) were identified in both blood and saliva but a high level of adenine was seen in the saliva samples, indicating that saliva degraded faster than blood samples. In addition HPLC-EC detection showed that only saliva produced levels of 8OHdG using this hydrolysis protocol. This unusual result indicated a potential problem with the protocol that may have been a result of amylases and other enzymes in saliva interfering with the sample digestion (31).

To correct for this problem, a second hydrolysis protocol was developed based on the work of Huang (22), Figure 2. Here the HPLC-UV profiles from untreated DNA is compared with DNA that was oxidized with 0.3% H_2O_2 + Fe^{+2}. The peak heights of the individual bases have increased 20 fold when compared to the previous digestion protocol. In addition, the untreated DNA samples showed no evidence of oxidation

when examined by HPLC-EC. However, a one hour treatment of the same sample with $H_2O_2 + Fe^{+2}$ produced a reduction in the concentration of nucleoside peaks, and the HPLC-EC result demonstrated the presence of 8OHdG. In comparing Figures 1 and 2, it is apparent that the addition of DNaseI and phosphodiesterases (PDE) I and II to the NP1 + AP system improved the DNA digestion by improving the release of normal nucleosides as well as 8OHdG. PDE I and II are exonucleases that attack the 3`- and 5`-terminal OH-groups, releasing 5`- and 3`-, respectively. We did not utilize saliva samples for the chemical oxidation experiments because we found 8OHdG in all untreated saliva samples.

To test the effect of chemical oxidation on tissue samples, additional experiments were performed on bovine tissue samples for 18 h with both H_2O_2 and bleach. These data were compared with naturally degraded human tissue samples obtained from skin and muscle tissue from bodies placed outdoors. Table 1 compares the background levels of chemical oxidation from DNA blood and bovine tissue samples for different oxidative treatments. Figure 3 shows the DNA profiles from naturally and chemically degraded tissue samples. The naturally degraded human tissue and all chemically oxidized samples had relatively low nucleoside peaks following digestion. 8OHdG was not detected in human tissue samples that were degraded for extended periods of time in the environment, although 8OHdG signals were seen in bovine tissue when H_2O_2 oxidation was performed (Table 1). Independent of the presence of 8OHdG, a very strong degradation profile was seen following STR amplification of the DNA from naturally degraded and chemically oxidized tissue samples when compared to the undegraded controls.

Blood DNA that was oxidized with $H_2O_2 + Fe^{+2}$ produced relatively high $8OHdG/10^6dG$ ratios of 117 ± 21.6. A comparison between DNA oxidized for 3 hours

by both H_2O_2 and bleach oxidation showed reduced oxidation with bleach treatments (15.8 ± 2.7 8OHdG/10^6dG) when compared with hydrogen peroxide (117 ± 21.6 8OHdG/10^6dG). It may be that the application of bleach (NaClO) to the DNA sample led to chlorination of cytosine and adenine to form 5-Cl cytosine and 8-Cl adenine, reducing the relative concentration of 8OHdG (32). Figure 4 shows an HPLC-EC chromatogram illustrating the separation of 8oxodG and 8OHdG in chemically oxidized samples. The limit of detection of for 8OHdG was 0.67 nM and the results were linear over a range from 1 nM to 50 nM.

STR-PCR AMPLIFICATION

All oxidized samples and untreated controls were amplified with the Powerplex® 16 kit (Promega). Figure 5 shows the comparison of an untreated DNA sample extracted from blood with the same sample oxidized with H_2O_2 and bleach. The results clearly demonstrate that the oxidative treatments induce degradation of the sample, particularly for the larger alleles. Relative levels of degradation were assessed by examining the percentage of alleles recovered. DNA that was oxidized with 0.6% bleach yielded 60 ± 1.0% of the total number of alleles present in the sample compared with 74.6 ± 0.6 % from blood DNA that was oxidized with 1.0% H_2O_2, as shown in table 2.

Aged human tissue samples obtained from the Forensic Anthropology Center of the University of Tennessee were also examined to determine the relative effects of oxidation and degradation. These tissue samples were obtained from bodies placed in mid to late summer at the Forensic Anthropology Center's facility. Allele dropout increased over the time of exposure, indicating gradually increasing degradation of the DNA in the tissue, Figure 6. The sample with 20 days of exposure time and three years of storage time resulted in the fewest alleles, producing only 34.6 ± 0.6% of the total alleles present in the sample. 8OHdG was not detected in these tissue samples,

indicating that oxidation is not the major reason for the relatively poor PCR amplification of these samples.

Conclusions

We have developed a method to test DNA extracts from blood, saliva, and tissue samples for oxidative damage using 8OHdG as a biomarker. The procedure comprises the digestion of extracted DNA into its constituent bases. Naturally degraded human tissue shows undetectable levels of this oxidized base; however, it is easily detected in blood, saliva, and bovine tissue samples that are chemically oxidized with bleach and hydrogen peroxide.

The examination of aged and buried tissue samples revealed no evidence of oxidation, but large amounts of degradation. These results indicate that while oxidative damage can clearly occur with chemical degradation, it is not a major factor in poor amplifications from degraded tissue.

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Table 1: Comparison of levels of 8OHdG/10^6 dG in human blood and bovine tissues samples for different treatments.

SAMPLE / TREATMENT	**Exposure time**	**8OHdG/10^6dG (mean ± SD)**
Saliva DNA control	-	18.6 ± 3.2
Human blood control *	-	-
Blood + 0.3% H_2O_2 *	1 hour	94 ± 13
Blood + 0.3% H_2O_2 + Fe^{+2} *	3 hours	117 ± 22
Blood + 0.6% NaClO *	1 hour	4.2 ± 1.3
Blood + 0.6% NaClO + Fe^{+2}*	3 hours	15.8 ± 2.7
Bovine Tissue control*	-	-
Bovine Tissue in 1% H_2O_2 *†	18 hours	59.2 ± 5.6
Bovine Tissue in 2% NaClO *†	18 hours	2.7 ± 0.6

* 100 μg DNA samples were digested with 40 U DNAseI for 0.5 h, followed by 1 U NP1 for 1 h, and 0.01 U PDE I and 0.02 U PDE II for 1 h, all digestions were performed at 37 °C in triplicate.
† After oxidation treatments, DNA samples were extracted from bovine tissue treated with 1% H_2O_2 and 2% NaClO, all digestions were perfomed at 37 °C with the optimized protocol.

Table 2: Percentage of Alleles recovered from DNA samples that were both naturally and chemically degraded, using STR Powerplex® 16 amplification.

Samples / Treatment	**Exposure Time∗**	**Storage Time†**	**% Alleles recovered (mean ± SD)**
Blood DNA control (untreated)	-	1 day	100
Blood NA + 1% H_2O_2	18 hours	1 day	74.6 ± 0.57
Blood DNA + 0.6% NaClO	18 hours	1 day	60 ± 1.0
Naturally degraded human tissue	2 days	2 months	81.2 ± 0.57
Naturally degraded human tissue	4 days	3 months	77.2 ± 1.15
Naturally degraded human tissue	5 days	3 months	65.2 ± 0.6
Naturally degraded human tissue	20 days	3 years	34.6 ± 0.57

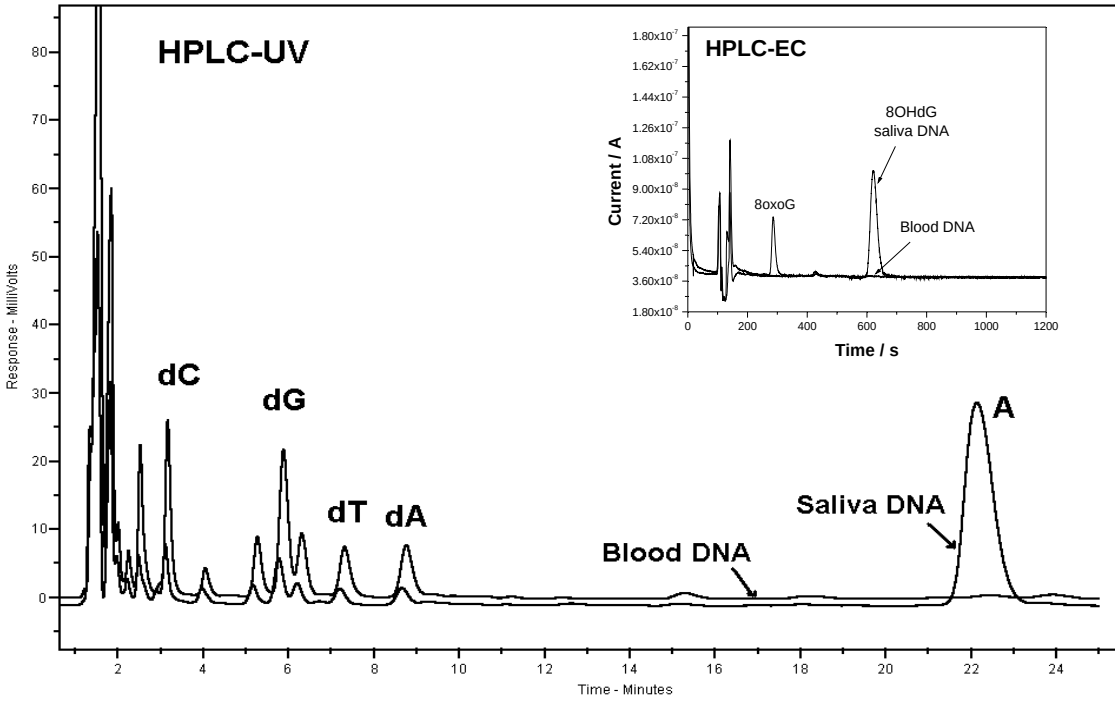

Figure 1: A chromatogram showing the results of the first of two hydrolysis protocols described in the paper. This protocol resulted in poor recovery of individual bases and excess formation of adenine. The figure shows a comparison between digested DNA from blood and from saliva. The insert shows the detection of the oxidation product 8OHdG. Samples were analyzed using HPLC with UV and EC detection using an eluent consisting of 92.5% 50mM KH_2PO_4, (pH=5.5) with 7.5 % Methanol. C18 column, flow: 1.0 mL/min, injection 50 µL, 260 nm. Insert shows amperometric detection at 600mV.

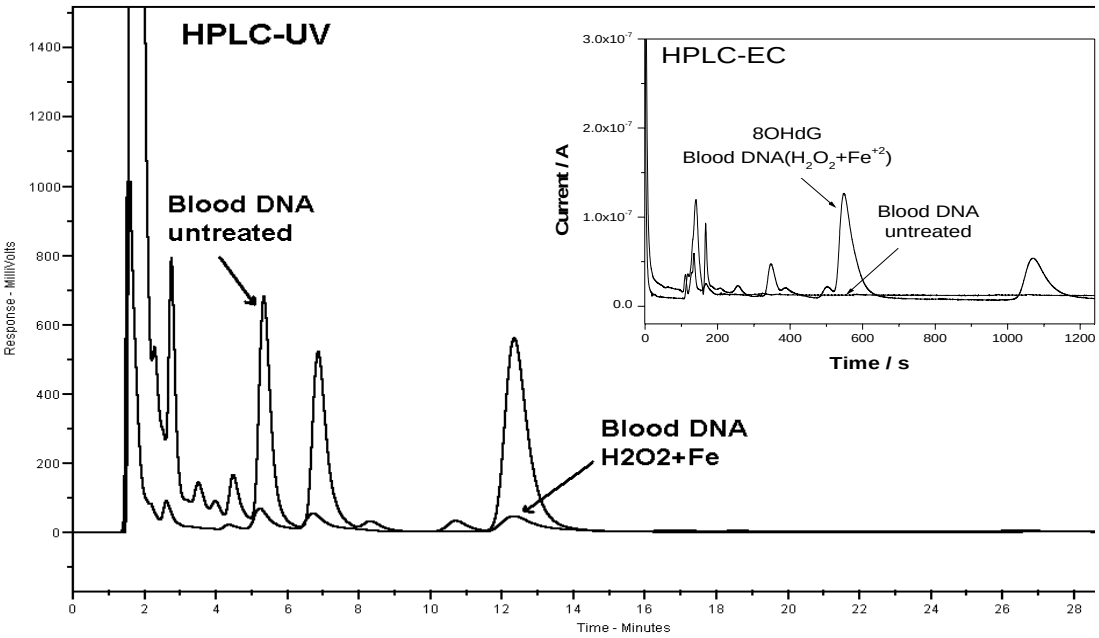

Figure 2: A chromatogram showing the results of the second of two hydrolysis protocols described in the paper. This protocol resulted in improved recovery of individual bases. The figure shows a comparison between untreated and oxidized DNA extracted from a blood sample. The insert shows the detection of the oxidation product 8OHdG. Samples were analyzed using the same conditions as in Figure 1.

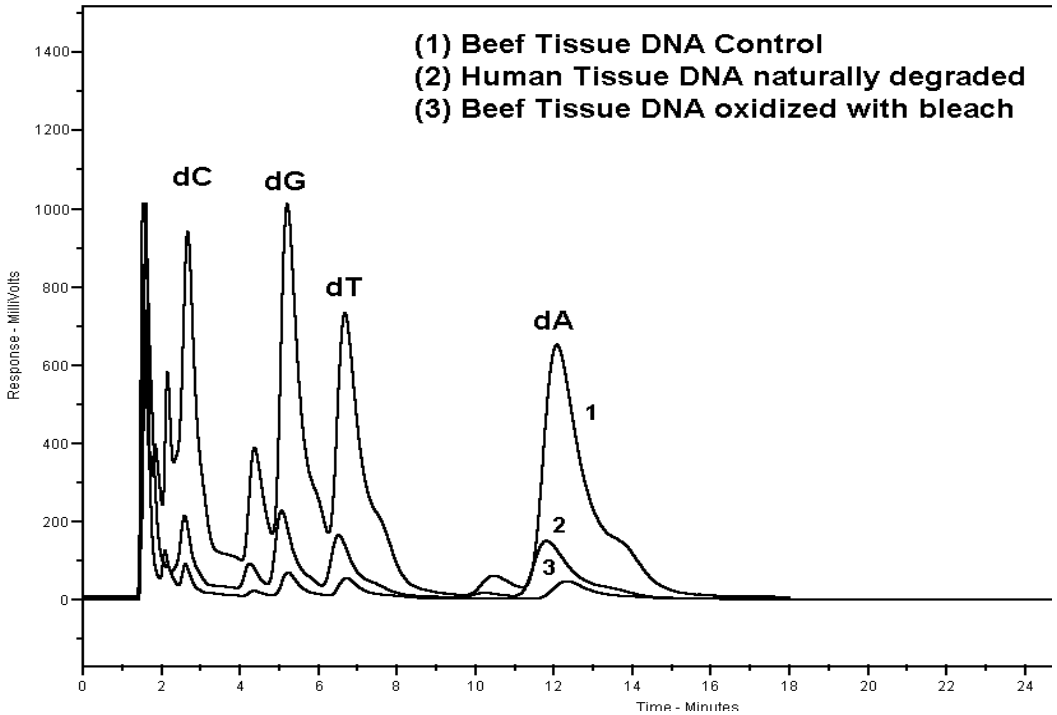

Figure 3: A chromatogram showing a comparison of beef and human tissue digested using the second protocol. One of the bovine DNA samples is treated with peroxide to illustrate the effect of this treatment on the recovery of intact DNA bases.

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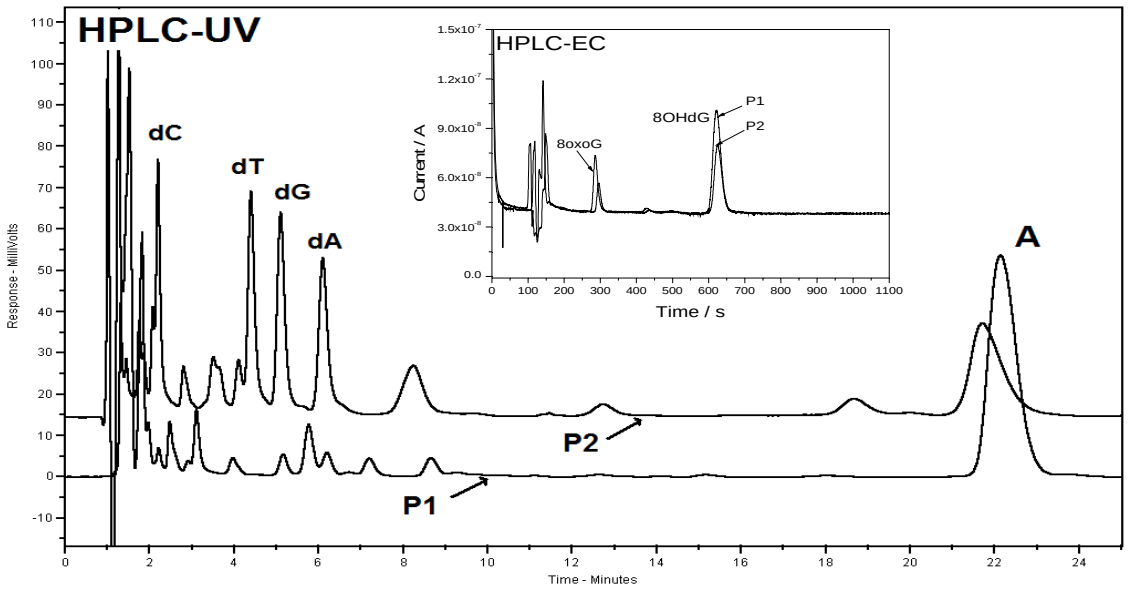

Figure 4: A chromatogram showing formation of 8OHdG and 8oxodG following the treatment of DNA with various oxidants. Samples were exposed to the oxidants for 3 hours and then digested using the 2nd protocol described in the paper. HPLC conditions were the same as described in Figure 1.

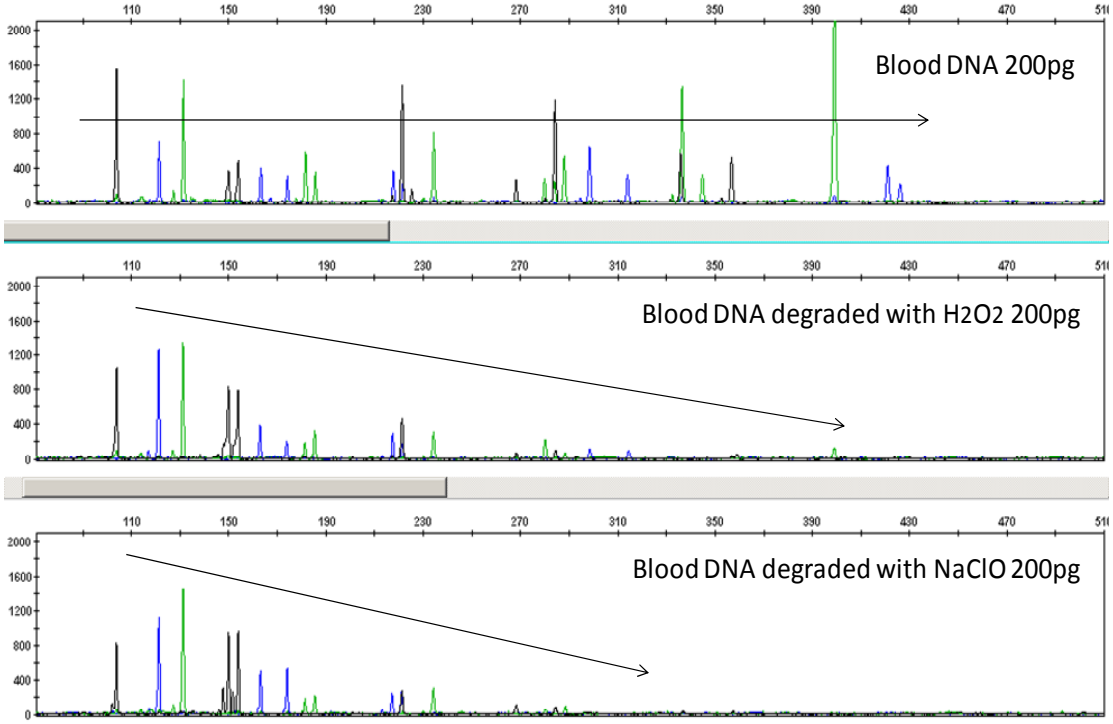

Figure 5: A comparison of the amplification of a DNA sample extracted from blood with the Powerplex ® 16 STR multiplex kit with that same sample treated with bleach and peroxide. The concentration of the DNA template was 200pg. PCR amplification and genotyping were performed using manufacturer's suggested protocols using an ABI310.

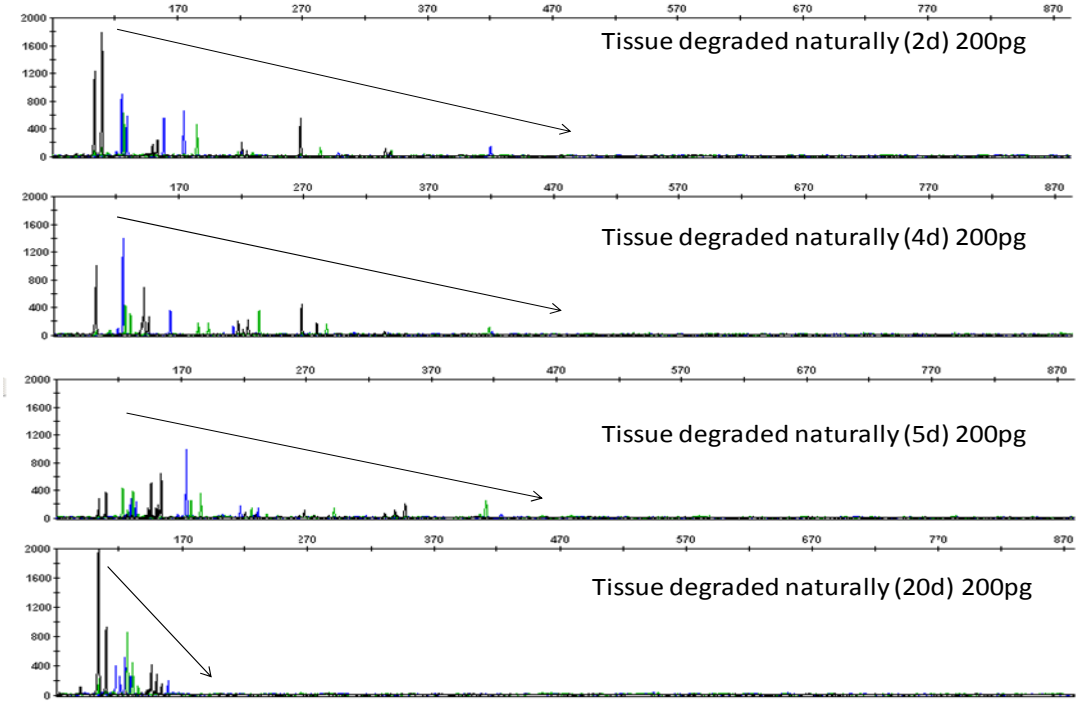

Figure 6: The effects of time on the ability to recover DNA from tissue samples recovered from bodies buried in shallow graves. DNA recovered using organic extraction techniques and amplified using the Powerplex 16 kit.